Conformational Transition of κ-Carrageenan in Aqueous Solution

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ABSTRACT: The interaction of Cs^+ ions with κ-carrageenan in aqueous solution was studied by using spectroscopic (^{133}Cs NMR, optical rotation, and turbidimetry) and thermodynamic (isothermal microcalorimetry and dilatometry) techniques. All data collected seem to confirm the previous evidence of the occurrence of a conformational transition, induced by temperature and/or ionic strength. From NMR and calorimetry data the relative amount of species in equilibrium (disaccharide repeat units present as disordered coil, single helices, and dimers of single helices) is computed. Comparison of calorimetric and dilatometric data with theoretical predictions allows one to discriminate between overall and purely electrostatic contributions to the binding of Cs^+ ions by κ-carrageenan.

Introduction

Whereas X-ray fiber diffraction analysis has revealed a bunch of ordered arrays of polysaccharide conformations in the condensed phase,[1] progress in understanding the conformational properties of polysaccharides in the solution and gel phases has been comparatively slow. Current ideas about polysaccharide conformations in solution derive mainly from molecular model building[2–6] and extrapolations from the condensed phase;[7] both of these ignore the roles of the solvent and cosolutes, and the latter disregards also the modifying influence of crystal field forces.

We have been interested for many years in the thermodynamic properties of polymers in solution[8–13] and considered that the ability of microcalorimetric and dilatometric measurements to contribute to the understanding of conformational processes of polysaccharides could be exploited more. It is evident that these thermodynamic measurements alone cannot lead directly to conformations or mechanisms; they can be fitted following some theoretical model, which is then tested for its ability to predict other measurable quantities.

One theory that has an excellent track record in accounting for the electrostatic properties of ionic polymers is the counterion condensation theory developed by Manning.[14–16] It led us to suspect, from measurements of the enthalpy of dissociation and dilution, that pectic acid undergoes a pH-induced conformational transition, as confirmed by potentiometric, viscosimetric, and chiroptical measurements.[12] We have also used Manning's theory to explore molecular models for the mechanism of gelation of κ-carrageenan,[17] a sulfated galactan extracted from certain seaweeds. It is composed of linear chains of alternating 1,3-linked β-D-galactopyranose residues, most of which are sulfated on position 4, and 1,4-linked α-D-galactopyranose residues, most of which are present as 3,6-anhydride.[18] The results of this analysis were in favor of a process that envisages a two-step process:[19,20] (i) a unimolecular conformational transition from random coil (favored by high temperature and low ionic strength) to an ordered conformation (favored by low temperature and high ionic strength), which is presumed, from model building, to be a single helix; (ii) side-by-side dimerization of the conformationally ordered chains by cation-selective salt-bridge formation.

In this paper, we present new microcalorimetric, dilatometric, and chiroptical data from the Trieste laboratory and new NMR data from the Trondheim laboratory and show how the Manning theory can bring them into agreement upon the assumption of the above model. Some new equations for the volume changes in polyelectrolyte solutions are also presented, after a brief summary of the relevant parts of the Manning theory.

Theory

The theory of linear polyelectrolytes developed by Manning is based on the concept of counterion condensation, which is postulated to take place as long as the linear charge density of the polymer exceeds a critical value. When the real chain is represented as an infinite, linear array of charges with an average spacing, b, the linear charge density parameter, ξ, is given by

$$\xi = e_c^2/(Dk_BTb) \tag{1}$$

where e_c is the value of the elementary charge, D the dielectric constant of the bulk medium, k_B the Boltzmann constant, and T the absolute temperature. Assuming monovalent charges on both counterions and fixed groups, the critical condition occurs when $\xi = 1$, which for water at 283.15 K corresponds to $b_{crit} = 7.034$ Å. For values of $b < b_{crit}$, the theory predicts that a fraction r ($r = 1 - \xi^{-1}$) of counterions will "condense" into a limited volume around the polyion, to reduce its effective charge density to unity. Taking as the reference state that of an ionized solution of simple salt containing an equivalent number of charges and treating the mobile ions by the Debye–Hückel approximation, the theory provides the following expressions for the ionic free energy of a system containing n_{e_c} charges:

For $\xi < 1$

$$g^{ion} = g^{el} = \frac{G^{el}}{n_e\mathbf{R}T} = -\xi \ln [1 - \exp(-kb)] \tag{2}$$

where $\mathbf{R}$ is the gas constant, I the ionic strength of the solution, and k the Debye screening parameter

$$k = [(8\pi e_c^2/Dk_BT)I]^{1/2} \tag{3}$$

For $\xi > 1$

$$g^{ion} = g^{el} + g^{mix}$$

$$g^{el} = -\xi(1 - r)^2 \ln [1 - \exp(-kb)] = -\xi^{-1} \ln [1 - \exp(-kb)] \tag{4}$$

g^{mix} contains all the terms for counterions, co-ions, and

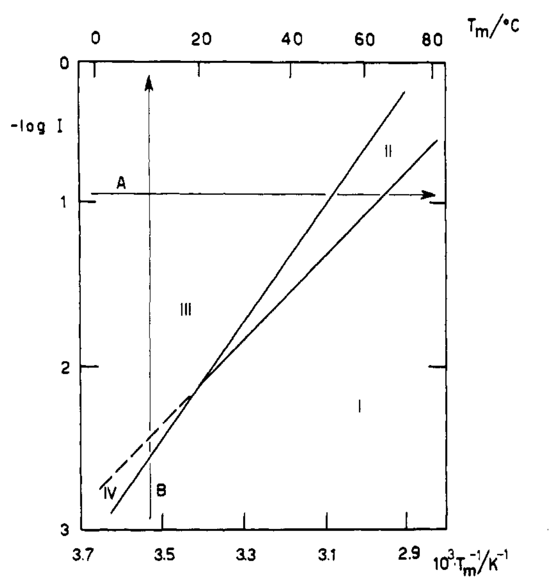

Figure 1. Phase diagram for the K^+ and Cs^+ salt forms of κ-carrageenan in aqueous solution. The conformations prevailing in regions I–IV are described in the text.

solvent, which are ideally supposed to be of purely entropic origin

$$g^{\mathrm{mix}} = (1 - \xi^{-1}) \ln \frac{1 - \xi^{-1}}{V_p C_p (R + 1)} + (R + \xi^{-1}) \ln \frac{R + \xi^{-1}}{R + 1} - (2R + \xi^{-1}) \ln (1 - V_p C_p) + 2 - \xi^{-1} \quad (5)$$

R is the molar ratio of the simple salt to charged groups on the polymer, S/C_p. S and C_p are the molar concentration of simple salt and the number of equivalents of disaccharide repeat units per unit volume, respectively. V_p is the volume of the condensed phase per mole of charge. For the limiting case of $kb \ll 1$ and $R \rightarrow \infty$, the term ln $[1 - \exp(-kb)]$ will take the form $^1/_2 \ln I + \text{cont} = {}^1/_2 \ln S + \text{const}$, and

$$V_p = 8\pi e N(\xi - 1)b^3 \quad (6)$$

where b is expressed in cm, N is Avogadro's number, e is the base of natural logarithms, and V_p is given in L mol^{-1}.

Phase Diagram. For a polyelectrolyte that, by a change in the ionic strength, temperature, or both, undergoes a cooperative transition between two conformations differing in average linear charge density, the theory[17,21] predicts a linear relationship between the logarithm of the ionic strength and the inverse of the transition temperature (T_m). Depending on the value of the initial and final linear charge density parameters, ξ_i and ξ_f ($\xi_i > \xi_f$), respectively, the equations given by the theory are

$$-\Delta H^m/[\mathrm{d} \log I/\mathrm{d}(1/T_m)] = 2.288(\xi_i - \xi_f) \quad (7)$$

$$-\Delta H^m/[\mathrm{d} \log I/\mathrm{d}(1/T_m)] = 2.288(2 - \xi_f - \xi_i^{-1}) \quad (8)$$

$$-\Delta H^m/[\mathrm{d} \log I/\mathrm{d}(1/T_m)] = 2.288(\xi_f^{-1} - \xi_i^{-1}) \quad (9)$$

The validity of eq 7–9 is subject to some conditions: (i) $kb \ll 1$; (ii) the dependence on I of the whole nonpolyelectrolytic contribution to the free energy of transition is much smaller than the purely polyelectrolytic term; (iii) the dependence on T of both the enthalpy, ΔH^m, and of the entropy, ΔS^m, of transition is negligible; (iv) ΔH^m is largely independent of I. Such conditions, which are largely met in most cases of interest, have been already discussed in detail.[17] Many biopolymers, including κ-carrageenan,[22-27] give linear plots in the log I vs. $1/T_m$ diagrams. The results for the K^+ and Cs^+ salt forms of κ-carrageenan are schematically shown in Figure 1. The two lines whose slopes are −3500 and −2600 K are found by different techniques. The figure can be regarded as a "phase diagram" of the relative stability of carrageenan conformations prevailing in the various regions (I–IV) of the diagram in which the following conformations have been proposed to predominate:[17] region I, disordered chains, rc (b = 10.3 Å); region II, side-by-side dimers of single helices, 1h.1h (b = 4.1 Å) + rc; region III, single helices, 1h (b = 8.2 Å) + 1h.1h; region IV, 1h + rc.

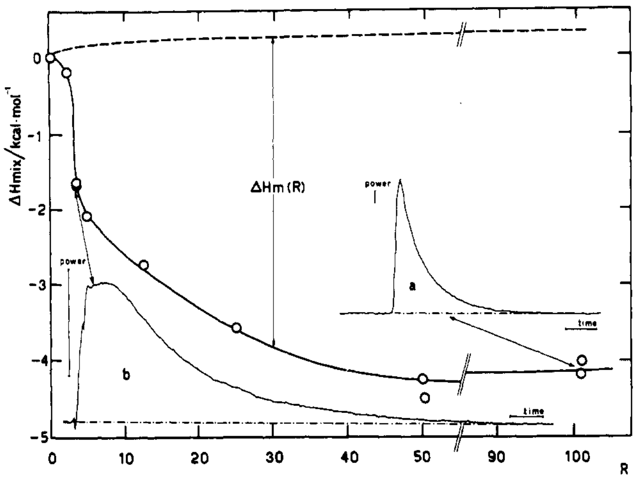

Figure 2. Microcalorimetric data of mixing CsCl with tetramethylammonium κ-carrageenate solutions at 283.2 K. Polymer concentration after mixing was 10^{-3} M. R is the molar salt to polymer ratio. The broken line is the theoretical electrostatic curve of mixing, calculated by use of eq 10. In the inserts two typical microcalorimetric peaks are shown: a, fast reaction; b, slow reaction. Time and power scales in both (a) and (b) are 2 min and 5×10^{-5} W, respectively.

Most experimental approaches have been centered on the temperature dependence of some physical property of the carrageenan solutions. However, owing to sensitivity limits, nearly all experiments had to be performed at relatively high I values, in which the stability of the aggregated structures, 1h.1h, was greater than that of the single-chain ordered conformation (e.g., along line A in Figure 1). Therefore, it is particularly important to look for conditions in which the order of stabilities is modified, at least partially. Line B of Figure 1 corresponds to an ion-induced disorder–order isothermal transition (at T = 283.15 K) in which the stability of 1h within a "window", from about $I = 10^{-3}$ to 10^{-2} M, is expected to be higher than that of 1h.1h.

Enthalpy and Volume Changes of Electrostatic Origin in Polyelectrolyte Solution. A detailed derivation of the enthalpy changes of electrostatic origin accompanying the mixing of a polyelectrolyte solution with a solution of a simple salt is reported elsewhere.[28] The net heat effects, ΔH^{el}, corrected for the dilution effects of both polymer and salt vs. solvent, are given by the following expression:

$$\Delta H^{\mathrm{el}} = -{}^1\!/_2 RT[1 + (\mathrm{d} \ln D)/(\mathrm{d} \ln T)]\xi^r\{2 \ln ([1 - \exp(-k_f b)]/[1 - \exp(-k_i b)]) + k_f b/[\exp(k_f b) - 1] - k_i b/[\exp(k_i b) - 1]\} \quad (10)$$

where $r = 1$ for $\xi < 1$, r = −1 for $\xi > 1$, $k_i = (2\lambda I_i)^{1/2}$, $k_f = (2\lambda I_f)^{1/2}$, $\lambda = 4\pi e_c{}^2/Dk_B T$, $I_{i,f} = C_p(R_{i,f} + {}^1/_2\xi^{-n})$ ($n = 0$ for $\xi < 1$, $n = -1$ for $\xi > 1$), and $R_{i,f}$ is the initial (i) and final (f) molar ratio of simple salt to polymer, respectively. All other symbols have been previously defined.

The curve calculated for b = 10.3 Å and T = 283.15 K is shown as a broken line in Figure 2. The heat effect is

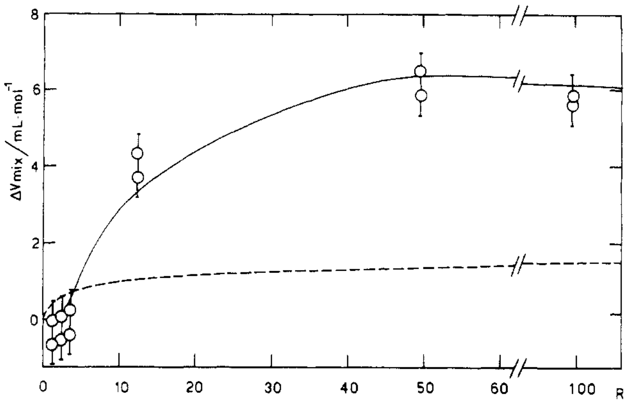

Figure 3. Dilatometric data of mixing CsCl with tetramethylammonium κ-carrageenate solutions at 283.2 K. Polymer concentration after mixing was 10^{-3} M. R is the molar salt to polymer ratio. The broken line is the theoretical electrostatic curve of mixing, calculated by use of eq 11.

endothermic and small, and in the absence of conformational changes it is expected to be very slightly dependent on the value of ξ. A wide variety of polyelectrolytes, including ionic polysaccharides, have been shown to conform quite well to the theoretical behavior.[28-33] Deviations from endothermicity have always been recorded in systems for which it was independently possible to suggest the onset of a conformational transition.[30-33]

The expression for the electrostatic volume change for the isothermal mixing of a polyelectrolyte with a simple salt, ΔV^{el}, can be derived from eq 4 by taking the proper pressure derivative, dG^{el}/dP. Details of the derivation and a discussion of the assumptions, similar to those used in the case of enthalpy,[28] will be reported elsewhere. The final expression of ΔV^{el}, corrected for the volume effects upon dilution of polymer and salt, is

$$\Delta V^{el} = -\tfrac{1}{2}(RT/P)[(\mathrm{d}\ln D)/(\mathrm{d}\ln P)]\xi^{r}\{2\ln[1 - \exp(k_f b)]/[1 - \exp(k_i b)] + [k_f b/\exp(k_f b)^{-1}] - [k_i b/\exp(k_i b)^{-1}]\} \quad (11)$$

where all symbols have been previously defined. For the particular case of $k_{i,f} \ll 1$ and $R_{i,f} = 0$, eq 11 coincides with eq 13 by Škerjanc,[34] derived from the Manning theory for the isothermal dilution with water of pure polyelectrolyte.

The curve calculated from eq 11 for $b = 10.3$ Å and $T = 283.15$ K is reported as a broken line in Figure 3, using $R/P = 82.1$ mL mol^{-1} K^{-1} and $(\mathrm{d}\ln D)/(\mathrm{d}\ln P) = 4.71 \times 10^{-5}$.[34]

The predicted volume effect is positive, of the order of a few mL mol^{-1}. As in the case of the enthalpy changes, the deviations from the expected behavior will be taken as an effect of conformational changes.

Experimental Section

Materials. The sample of κ-carrageenan used was an extract of *Euchema cottonii* (CECA SA, France), the same as that already described[35] and found by ^{13}C NMR analysis to conform well with the theoretical monomer composition. It was purified by repeated precipitation with aqueous KCl. The tetramethylammonium (TMA$^+$) salt form of the polymer was obtained by percolating a solution of κ-carrageenan through a Dowex cation exchanger. The equivalent weight of the repeating unit was taken to be 495.

All reagents were of analytical grade. Freshly triply distilled water was constantly used.

Methods. Optical activity data were recorded at $\lambda = 313$ nm with a Perkin-Elmer spectropolarimeter Model 141 M using a mercury lamp and a quartz cell of 10-cm path length. The results are expressed as molar optical activity, $[\alpha]$ ($[\alpha] = \alpha l^{-1}C_p^{-1}$, where α is the observed rotation in degrees, l is the cell pathlength in dm, and C_p is the carrageenan equivalent concentration).

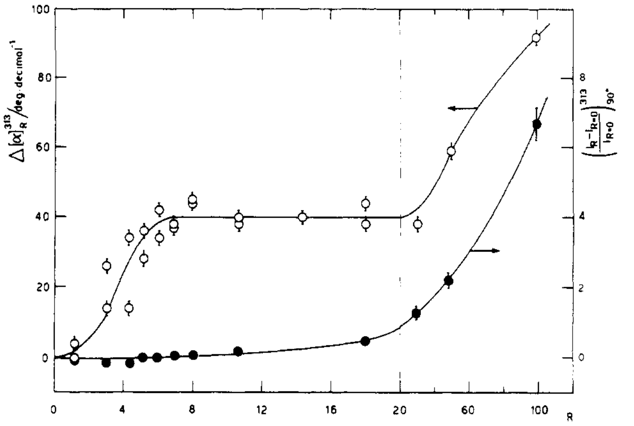

Figure 4. Increment in optical rotation (open symbols) and specific increase of the intensity of the scattered light at 90° (filled symbols) of solutions of tetramethylammonium κ-carrageenate vs. the molar ratio of CsCl to polymer, R, at 313 nm and 283 K.

The intensity of the light scattered at 90° was recorded with a Perkin-Elmer Model MFP-3L spectrofluorimeter, equipped with a xenon lamp. The two monochromators were fixed at 313 nm with a slit width of 2 nm; a standard 1-cm square-section quartz cell was used. Both polymer and CsCl solutions were filtered prior to mixing.

ΔH^{mix} data were obtained with an LKB isothermal batch-type microcalorimeter Model 10700-2, equipped with twin gold cells, with a time constant of about 100 s. Tetramethylammonium κ-carrageenan solution (2 mL, initial concentration 2×10^{-3} M) was mixed with 2 mL of CsCl solution of variable concentration. The two compartments of the reference cell contained equal amounts of solvent and of CsCl solutions, which afforded a simultaneous correction for the dilution effect of the salt. The ΔH of dilution of the polymer was measured separately without CsCl in the solutions. Treatment of the calorimetric data followed the already described procedure.[12]

In all the above experiments the temperature was kept at 283.2 K.

Volume changes of mixing were obtained with Carlsberg dilatometers, according to the method described previously.[36] The treatment of the dilatometric data was already described.[9]

The time scale of the mixing experiments both in microcalorimetry and in dilatometry is about half an hour, but constancy of the readings was checked also after some hours, ensuring completeness of the reaction.

^{133}Cs NMR spectra were recorded at 283 ± 1 K with a JEOL FX-100 NMR spectrometer equipped with a multinuclear probe. The resonance frequency was 13 MHz. Chemical shifts in polymer-containing solutions are given in hertz with respect to a reference CsCl solution at the same concentration in a coaxial tube. All details about experimental setup and discussion of the features of the observed resonance signal have been described.[37]

Results

Measurements of Optical Activity. Figure 4 shows the optical activity at 313 nm and 283 K of a 0.5 g L^{-1} (10^{-3} M) solution of κ-carrageenan (TMA$^+$ salt) as a function of the molar ratio, R, of added CsCl per carrageenan repeating unit. The data are reported as the difference, $\Delta[\alpha]$, in molar optical activity between the solution at R, $[\alpha]_R$, and the salt-free aqueous solution of the TMA$^+$ salt, $[\alpha]_{R=0}$

$$\Delta[\alpha]_R = [\alpha]_R - [\alpha]_{R=0} \quad (12)$$

Despite the scattering of the points due to the very low polymer concentration ($C_p = 10^{-3}$ M), there is a sigmoidal increase in $\Delta[\alpha]$ with increasing R, centered around $R = 4$, which eventually attains a constant value from $R > 7$ to $R = 20$. This behavior adds to various experimental findings[19,22,24,38] in which a temperature-induced or ionic strength induced change in the optical activity of carrageenan solutions has been attributed to the occurrence of a conformational transition.

For R values larger than 20, a marked increase in the readings was noticed together with a severe loss in reproducibility and the appearance of visible amounts of microgels. The presence of anisotropic microaggregates is known to prevent the interpretation of optical activity data in terms of molecular parameters.[39]

To verify whether macromolecular aggregation also occurred at $R < 7$, the intensity of the scattered light at a fixed angle (90°) was measured at 283 K on the same solutions and at the same wavelength employed in the optical activity measurements ($\lambda = 313$ nm). The scattering results were expressed on a relative scale as the specific increase in the intensity of the scattered light, $(H_R)_{90°}$

$$(H_R)_{90°} = [(I_R - I_{R=0})]/I_{R=0}]_{90°} \qquad (13)$$

where I_R and $I_{R=0}$ are the observed scattering intensity values, in arbitrary units, of the polymer solution at R and of the salt-free solution, respectively. The results are reported in Figure 4 as a function of R. The main features of the curve are as follows: (i) there is no increase in $(H_R)_{90°}$ in the interval of R in which there is full development of the increase in optical activity ($0 < R < 7$); (ii) for $R > 20$ there is a very strong increase in $(H_R)_{90°}$ with R, the intensity of the scattered light reaching a value several times larger than that of the salt-free solution. The interpretation of the anomalous increase in $\Delta[\alpha]$ at high R values ($R > 20$) as an experimental artifact due to extensive formation of (possibly birefringent) microgels is therefore fully supported by the scattering experiment. The $\Delta[\alpha]$ points at $R > 20$ will not be further considered in the discussion of the chirooptical properties of the molecularly disperse system.

Microcalorimetric Measurements. The experimental results of mixing a salt-free κ-carrageenan (TMA$^+$ form) solution at 283 K with CsCl in a batch microcalorimeter are reported in Figure 2. The heat effects were corrected for the dilution effects of both the salt-free polymer solution and CsCl and were expressed per mole of disaccharide repeating unit. The curve shows a very clear sigmoidal behavior, starting from almost athermic values at the lowest accessible R value and rapidly changing to exothermic values, with the steepest change at $3 < R < 4$. The $\Delta H^{\mathrm{mix}}(R)$ curve decreases further with increasing R but with a more gentle slope. It reaches a minimum value of about -4.3 kcal mol^{-1} at around $R = 50$ and then slightly increases to -4.1 kcal mol^{-1} at $R = 100$.

An important aspect of the calorimetric curve is that the shape of the experimental (power vs. time) response was dependent upon R (Figure 2, inserts). In particular, both at very low and at very high R values the signal was typical of chemical reactions that undergo complete transformation in a time much shorter than the response time of the calorimeter (i.e., about 100 s).[40] On the contrary, the signal at intermediate values of R ($5 < R < 20$) qualitatively showed at least two components: the first still corresponded to a fast process, but the second indicated that another process was taking place on a time scale longer than ca. 100 s. On increasing the value of the ionic strength (i.e., on increasing R), the overall shape of the calorimetric signal progressively developed again toward the familiar shape for fast reactions. Another important point is that microgel formation at high R values did not at all impair the accuracy or reproducibility of the calorimetric measurements, as already found for other systems containing ionic polysaccharides.[28,32]

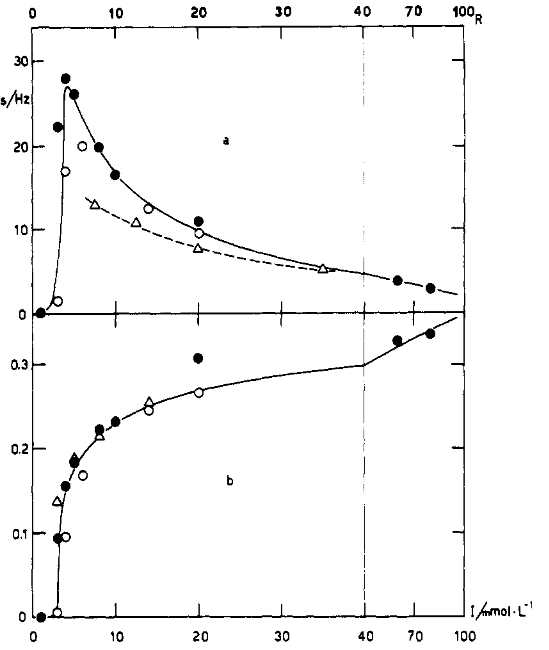

Figure 5. (a) Dependence of the observed ^{133}Cs NMR shift of Cs$^+$ ions in the presence of κ-carrageenan on the molar ratio of CsCl to polymer, R, with respect to a dilute solution of CsCl in water. (b) Dependence of the fraction of Cs$^+$ ions bound to specific sites of κ-carrageenan in water, f_b, on the ionic strength, I. f_b is calculated by use of eq 14. Triangles indicate polymer concentration, C_p, 4×10^{-4} M and circles indicate $C_p = 10^{-3}$ M. For the particular case of $C_p = 10^{-3}$ M, the numerical values of the scales R and I (mM) practically coincide except for the lowest R values. Open circles and filled circles refer to two different series of measurements.

Dilatometric Measurements. The volume changes on mixing CsCl solutions of increasing concentration with a salt-free solution of tetramethylammonium κ-carrageenate at 283 K are reported in Figure 3. The values have been corrected for the volume changes of dilution of both the polymer and the added salt. Despite the error affecting the final result (±0.5 mL mol^{-1}) due to the extremely low polymer concentration employed, a clearly sigmodal curve comes out as a function of R. It starts from practically zero up to about $R = 4$ and then increases to a positive value of about 6.0 mL mol^{-1} at about $R = 50$. Thereafter, the curve is practically constant up to $R = 100$. Similarly to what was pointed out about the calorimetric measurements, no difference could be noted in the quality of the dilatometric measurements (e.g., reproducibility, time effects, etc.) on moving from the optically featureless solutions to those containing microgel.

^{133}Cs NMR Measurements. ^{133}Cs NMR measurements, carried out as a function of R on a 10^{-3} M solution of κ-carrageenan in H$_2$O at 283 K, revealed the characterstic line shape and line shift already reported for a 3% gel of cesium κ-carrageenate in D$_2$O.[37] Such spectral features indicate the presence of specific binding sites on the ordered, aggregated polysaccharide, which gives rise to a quadrupole relaxation of the $^7/_2$ nucleus of Cs$^+$, involved in a two-site exchange.[37] Moreover, the line shift is a sensitive probe of the thermally induced or ion-induced conformational transition/aggregation, and we therefore use this parameter to follow the specific interaction of Cs$^+$ with κ-carrageenan. The observed line shift data, δ_{obsd}, relative to a dilute solution of CsCl in water, are reported in Figure 5a as a function of R (upper abscissa) both for 10^{-3} M (circles) and for 0.4×10^{-3} M (triangles) tetramethylammonium κ-carrageenate solutions. The curve drawn through the circles shows a sharp maximum at around $R = 4.5$ and a slow decrease on increasing R.

According to the two-site model,[41] in the presence of the rapid averaging shown by the spectra of cesium κ-carrageenate the observed shift is the weighted average of the contributions of the "bound" ions through an "intrinsic shift", δ°, and of the "free" ions, whose resonance frequency is equal to that of CsCl and is taken as the arbitrary zero

$$\delta_{\mathrm{obsd}}(R) = \frac{[\mathrm{Cs}^+]_{\mathrm{bound}}}{[\mathrm{Cs}^+]_{\mathrm{tot}}}\delta^\circ + \frac{[\mathrm{Cs}^+]_{\mathrm{free}}}{[\mathrm{Cs}^+]_{\mathrm{tot}}}\delta_{\mathrm{CsCl}} = \frac{[\mathrm{Cs}^+]_{\mathrm{bound}}}{RC_{\mathrm{p}}}\delta^\circ = \frac{\delta^\circ}{R}f_{\mathrm{b}} \quad (14)$$

To evaluate the fraction of bound cesium ions per disaccharide unit, f_{b}, one needs to know the value of δ° for the particular system, which is not available on a priori grounds. At first we induced a high conformational order (i.e., large number of "binding" sites) on the polysaccharide by addition of large amounts of TMACl in the presence of small amounts of Cs^+ ions (the classical condition of excess ligand). Surprisingly, at high $\mathrm{TMA}^+/\mathrm{Cs}^+$ ratios, competition of TMA^+ with Cs^+ gave rise to barely detectable chemical shifts despite the known, high polymer selectivity for Cs^+. We then resorted to preparing different solutions (or gels) or κ-carrageenan by mixing different ratios of the pure TMA^+ and pure Cs^+ salt forms, at the same equivalent concentration and without the addition of any simple salt. Rather concentrated solutions were employed (the total polymer concentration was in most cases 3% w/w, and in some cases 6% w/w) to reach high values of I through the contribution of the polyelectrolyte's own counterions.

Starting from the pure Cs^+ form and moving toward the TMA^+ one, the observed shift increased to about 600 Hz (a gel was observed in all cases). This suggsts that only a fraction of the disaccharide units involved in the aggregated structure give rise to specific sites for Cs^+. For a molar fraction of the Cs^+ form (X_{Cs^+}) less than 0.06, a sharp decrease in shift paralleled the passage from a true gel to a very viscous solution. It probably indicates that, at the given polymer concentration, TMA^+ ions by themselves are unable to give rise to a coherent structure and to binding sites. A plot of the reciprocal of δ_{obsd} as a function of X_{Cs^+} for all the X_{Cs^+} values larger than 0.06 gave a good linear correlation, with an intercept and slope of $(1.40 \pm 0.08) \times 10^{-3}\ \mathrm{Hz}^{-1}$ and $(4.38 \pm 0.14) \times 10^{-3}\ \mathrm{Hz}^{-1}$, respectively. The reciprocal of the intercept can be taken as a measure of the intrinsic shift, δ°; it is equal to 715 ± 41 Hz. The intrinsic shift for bound cesium (55 ppm) compares quite well with those reported for cryptate complexes of Cs^+ with various macrocyclic ligands.[42]

It was possible to evaluate the fraction of bound Cs^+ ions in the former experiments by rearranging eq 14; the results are reported in Figure 5b as $\delta_{\mathrm{obsd}}R/\delta^\circ$ vs. I for both the solutions investigated. The $f_{\mathrm{b}}(R)$ curve shows a rapid increase at around $I = 4$ mM; above $I = 7$ mM it grows more slowly. The similarity with the calorimetric and dilatometric curves is quite strong and suggests a common origin for the thermodynamic and spectroscopic phenomena. The plot is independent of C_{p} provided that the abscissa is the total ionic strength. This is to be expected if the "binding" phenomenon is not governed by chemical equilibrium dependent on the reactant concentrations but, rather, by the long-range electrostatic interactions typical of polyelectrolyte solutions.

Discussion

Solution. All the experimental results were obtained under conditions ranging from true solutions of independent chains to microgels. In particular, the scattering data of Figure 4 show that, up to about $R = 20$, the solution can be considered as molecularly disperse and devoid of possible artifacts due to situations of thermodynamic nonequilibrium.

Calorimetric Results: Comparison with Theoretical Predictions and DSC Data. Following the procedure described in Theory, the calculated $\Delta H^{\mathrm{el}}\ (R)$ function is reported as a dashed line in Figure 2. It corresponds to the electrostatic heat effect that ensues from mixing a solution of a polyelectrolyte with b corresponding to that of a random coil form of κ-carrageenan ($b = 10.3$ Å) with an increasing amount of salt, corrected for the dilution of pure polymer and of salt in water.[28] Recent calorimetric data of mixing κ-carrageenan with TMACl in the absence of conformational effects[33] are in quantitative agreement with the theoretical predictions reported in Figure 2. According to the theory, in the case of ion-induced conformational transitions of polyelectrolytes, the deviations of the experimental data from the calculated curve can be interpreted as a measure of the progress of such transitions as a function of R. Therefore, for the particular equilibria that are considered to take place in the present case, one can write for the average enthalpy of order formation, $\Delta H^{\mathrm{m}}(R)$

$$\Delta H^{\mathrm{m}}(R) = \Delta H^{\mathrm{mix}}(R) - \Delta H^{\mathrm{el}}(R) = X_{1\mathrm{h}}\Delta H_{1\mathrm{h}} + X_{1\mathrm{h.1h}}\Delta H_{1\mathrm{h.1h}} \quad (15)$$

X_{rc}, $X_{1\mathrm{h}}$, and $X_{1\mathrm{h.1h}}$ are the mole fractions of dimeric repeat units present as random coil, single helix, and dimer of single helices, respectively. $\Delta H_{1\mathrm{h}}$ and $\Delta H_{1\mathrm{h.1h}}$ are the enthalpy changes per mole of charge for the reactions

$$\mathrm{rc} \rightarrow 1\mathrm{h} \quad \text{(a)}$$

and

$$\mathrm{rc} \rightarrow 1\mathrm{h.1h} \quad \text{(b)}$$

respectively.

The first important result of an analysis of the $\Delta H^{\mathrm{mix}}(R)$ data of Figure 2 is that, for R values corresponding to complete transformation to the ordered form (see later), $\Delta H^{\mathrm{m}}(R)$ is not constant but ranges from about -2.5 kcal mol^{-1} at $R = 7$ to about -4.6 kcal mol^{-1} at $R = 50$, slowly decreasing in value to about -4.45 kcal mol^{-1} at $R = 100$. For complete transformation, by using the experimental slopes derived from the phase diagram of κ-carrageenan (Figure 1) one predicts for ΔH^{m} the limiting values of -1.4 ± 0.1 and -4.35 ± 0.3 kcal mol^{-1} for the reactions (a) and (b), respectively. The agreement between the range of experimental ΔH^{m} of Figure 2 and that predicted from the assumed single chain/dimer model is very good. This seems to indicate that both processes take place to a relative extent that depends on R. Moreover, the ΔH^{m} values are in good agreement with the reported DSC data, which range from -1.3 ± 0.2 to -4.3 ± 0.1 kcal mol^{-1}, depending on the ionic strength.[22,25,27] With respect to the DSC results, we stress the importance of the present isothermal microcalorimetric data for two reasons: (i) they have been obtained under conditions of polymer concentration that are lower by at least one order of magnitude (this ensures both a better comparison with theoretical predictions (derived from the limiting laws) and a higher molecular dispersity); (ii) they have been collected as a function of the variable R, thus providing a picture of the whole course of the conformational/aggregational transformation induced by the ionic strength.

The novelty of the method is increased by the finding that the profile of the $\Delta H(R)$ curve is not altered upon passing from the true molecularly disperse solution to the microgel. The isothermal microcalorimetric method ap-

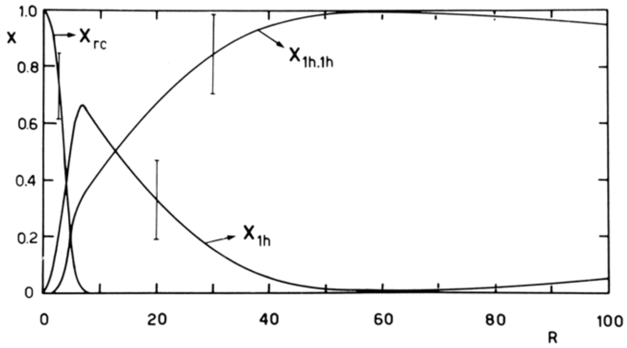

Figure 6. Distribution of mole fractions of κ-carrageenan disaccharidic units in solution at 283 K as a function of the molar CsCl to polymer ratio, R, computed from eq 16 and 17 and the calorimetric data.

pears therefore to be free of the limitations of more classical techniques (in particular of optical and chirooptical techniques) to follow the evolution of molecular processes to a supramolecular level.

Moreover, by using the equations

$$X_{1h} + X_{1h.1h} = \Delta[\alpha](R)/\Delta[\alpha]_{max} \qquad (16)$$

$$X_{1h} + X_{1h.1h} + X_{rc} = 1 \qquad (17)$$

in addition to eq 15, one can evaluate the mole fractions of the species in equilibrium. $\Delta[\alpha]$ is the value of the excess optical activity of solutions at $7 < R < 20$ (with respect to that at $R = 0$) for which the constancy of $\Delta[\alpha]$ is interpreted, as usual, as indicating full development of the ordered conformation.[43,44] From the experimental results, the values $\Delta H_{1h} = -1.4$ kcal mol^{-1} and $\Delta H_{1h.1h} = -4.6$ kcal mol^{-1} are used in eq 15–17 to calculate X_{rc}, X_{1h}, and $X_{1h.1h}$, respectively. The results are reported in Figure 6.

The evolution of the different conformations of κ-carrageenan upon increasing the ionic strength shows (i) a quite rapid sigmoidal decrease of the disordered form, which might indicate a strong cooperativity, and (ii) a similarly rapid increase of the single helix and of the dimer forms up to about $R = 7$, followed by a very slow transformation of the former into the latter. The number of carrageenan repeating units in the 1h conformation prevails over that in the 1h.1h form up to about $R = 10$. Hence, the conditions described in Theory as the "window" in the phase diagram, allowing one to detect the isolated fundamental ordered conformation of carrageenan, 1h, have been met, at least to some extent.

Another point to be remarked is the parallel increase of the calculated fraction of dimeric repeat units present as side-by-side dimers of helices, 1h.1h, and of aggregate formation, as monitored by the specific increase in the scattering intensity (Figure 4). The present results strongly support the idea that the molecular basis of gel formation of carrageenans resides in the tendency of segments of two ordered chains to pair off, as already proposed for alginate- and pectate–Ca^{2+} systems.[20] The cross-linking in the gel would then be given by several different chains, each forming a double-stranded aggregate of varying length with a particular chain, although, small bundles of chains of higher strandedness, as proposed for agarose,[45] cannot be excluded at high R values on the basis of the calorimetric data alone.

Interactions of Cs^+ Ions with Different Chain Conformations. Superimposed on the nonspecific effect of the ionic strength, a specific role of alkali metal ions of high atomic number toward the conformational transition of κ-carrageenan has been recognized.[19,20,46,47] Cs^+ ions give rise to new NMR phenomena (both chemical shift and quadrupole relaxation) in the presence of gelling κ-carrageenan.[37] The interpretation of the observed chemical shift of ^{133}Cs under such conditions in terms of the currently accepted two-state model for relaxation has been already dealt with in Results. In the present discussion, we aim at a correlation between the observed NMR effect of Cs^+ ions and the molecular interpretation of the calorimetric data.

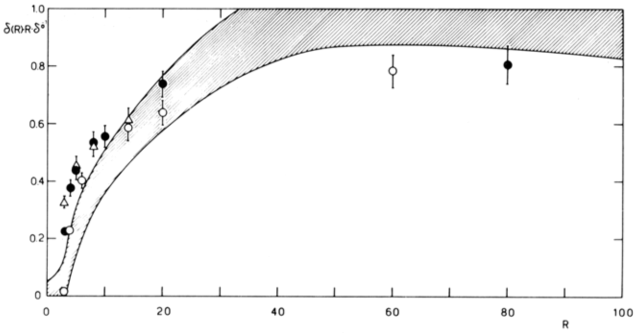

Figure 7. Dependence on R, the molar CsCl to κ-carrageenan polymer ratio, of the mole fraction of disaccharide units in the conformation 1h.1h in aqueous solution at 283 K. The data points were obtained from NMR measurements and use of eq 18 (see text). For explanation of symbols, see legend to the Figure 5. The dashed ribbon represents the $X_{1h.1h}$ vs. R curve derived from calorimetry with the range of estimated uncertainty.

According to the two-state model, the changes in $\delta_{obsd}(R)R/\delta°$ as a function of R (Figure 5b) represent the dependence on R of the fraction of "site-bound" Cs^+ ions per mole of disaccharide. The same ^{133}Cs NMR effects were reported to be uniquely dependent upon the existence of contact zones between carrageenan molecules, as manifested by macroscopic aggregates or a gel.[37] A notable feature of the curve of Figure 5b is the sharp sigmoidal increase at very low R values, followed by a smoother increase at high R values. This curve resembles that of the mole fraction of disaccharide units in the side-by-side dimer, 1h.1h, calculated from the calorimetric data (Figure 6). The simplest hypothesis is that the fraction of "site-bound" cesium ions, which give rise to a specific quadrupole relaxation, can be identified with that of the condensed couterions, f_c. This fraction is required by the condensation theory since the ξ value of the 1h.1h structure is larger than 1. Accordingly, the fraction f_c [$f_c = 1 - (1/\xi_{1h.1h}) = 0.419$, $\xi_{1h.1h}$ being 1.74] of monovalent cesium counterions should represent the *maximum* fraction of "site-bound" ions per disaccharide unit that can give rise to chemical shift and quadrupole relaxation. The ratio $\delta(R)R/(\delta° f_c)$ is then taken as equal to the mole fraction of disaccharide residues in the 1h.1h conformation, under the assumption

$$f_b(R) = f_c X_{1h.1h}(R) \qquad (18)$$

The results of the computations are reported in Figure 7 together with the calculated values of $X_{1h.1h}$ derived from the calorimetric curve. The agreement between the two sets of estimates is fairly good, despite the different hypotheses involved. Moreover, no adjustable parameter had to be introduced in either of the approaches. The success of the agreement cannot disguise the crudeness of the assumptions. For example, the population of condensed counterions in the 1h.1h conformation might be composed of different subpopulations, not all of which display equal NMR behavior. In addition, minor contributions to the chemical shift (although not to line shape) from sites on 1h cannot be ruled out a priori. The interpretation of the ^{133}Cs shift data would then imply the necessity of describing all the separate contributions. More experimental data in a wider range of conditions need to be collected before drawing detailed conclusions at the molecular level.

Solvation Effects in Conformational Changes. In the case of the dilatometric data of Figure 3, it is possible to write an equation similar to eq 14

$$\Delta V^{\mathrm{m}}(R) = \Delta V^{\mathrm{mix}}(R) - \Delta V^{\mathrm{el}}(R) = X_{1\mathrm{h}}\Delta V_{1\mathrm{h}} + X_{1\mathrm{h}.1\mathrm{h}}\Delta V_{1\mathrm{h}.1\mathrm{h}} \quad (19)$$

$\Delta V^{\mathrm{mix}}(R)$ stands for the observed volume change on mixing; $\Delta V^{\mathrm{el}}(R)$ is the calculated electrostatic volume change of mixing the disordered conformation with salt, calculated from eq 11. $\Delta V_{1\mathrm{h}}$ and $\Delta V_{1\mathrm{h}.1\mathrm{h}}$ stand for the molar volume changes of conformational transition, of both ionic (polyelectrolytic) and nonionic origin, pertaining to the aforementioned reactions (a) and (b), respectively. The theoretical curve of $\Delta V^{\mathrm{el}}(R)$ for the disordered chains is shown in Figure 3 (dashed curve). The calculated volume effect is always positive, with a trend resembling that of $\Delta H^{\mathrm{el}}(R)$. However, at variance with the latter one, the electrostatic contribution to $\Delta V^{\mathrm{mix}}(R)$ is an important fraction of the total observed effect.

The number of data points and the experimental error (±0.5 mL mol^{-1}) did not allow us to use even the simplest two-parameter model, using $\Delta V_{1\mathrm{h}}$ and $\Delta V_{1\mathrm{h}.1\mathrm{h}}$ as fitting parameters independent of the ionic strength and the values of the mole fractions of single helix and dimer taken from the data of Figure 6. One can only suggest that $\Delta V_{1\mathrm{h}}$ is very small and negative, likely between −0.5 and −1 mL mol^{-1}. It compares favorably with the value reported for the intramolecular conformational transition of poly(α-D-galacturonate), which was found to be zero within the experimental accuracy.[12] On the other hand, one can more safely estimate, from the high R range, that $\Delta H_{1\mathrm{h}.1\mathrm{h}} = 4.5 \pm 0.5$ mL mol^{-1}. The positive value adds to those already found for other ionic polysaccharide systems undergoing an ion-induced chain aggregation.[48] The present, directly determined, value of $\Delta V_{1\mathrm{h}.1\mathrm{h}}$ is in very good agreement with that (about 4 mL mol^{-1}) reported by Rinaudo et al.[49] from the relative increase in the ultrasonic absorption of the K^+ form of κ-carrageenan with respect to that of the TMA^+ form in solution.

The volume change accompanying the change in conformation/aggregation of κ-carrageenan is very small compared with those of typical polyuronate/divalent ions systems, even more so if normalized per mole of pyranose unit. In the latter instances, the volume effects are larger by factors from 4 to almost 10.[50] Large and positive ΔV values are usually interpreted in terms of strong desolvation of the interacting species, which implies a favorable entropic contribution to aggregation. The carboxylate groups are well-known to be easily dehydrated, as well as the interacting Ca^{2+} or Cu^{2+} ions. On the other hand, both the Cs^+ ion and the $\mathrm{OSO_3}^-$ group of carrageenan give rise to small volume effects. The former is known to be little hydrated and the latter is currently believed to be surrounded by electrostricted water molecules too tightly bound to be easily displaceable. Whether the observed volume change stems from a small ionic desolvation or from a partial overlap of the hydration layers of two facing polysaccharide chains cannot be assessed at present. The second hypothesis could be the molecular counterpart of the observed macroscopic phenomenon of syneresis, which accompanies the phase separation of κ-carrageenan as well as of the related nonionic polymer agarose.[45]

Conclusion

The combined use of microcalorimetric measurements and polyelectroyte theory has proved to be quite successful in providing a self-consistent description of the ion-induced conformational/aggregational change of κ-carrageenan. Although the calorimetric measurements suffer from the intrinsic limitations of all classical thermodynamic methods, their use to derive one-dimensional structural informations is strongly justified by the molecular nature of the polyelectrolyte theory employed for their interpretation. Moreover, isothermal microcalorimetric measurements turned out to be very accurate in conditions that extend from the solution to the gel phase. In this respect, they are more reliable than other commonly employed techniques.

The molecular model of gel formation coming from the present results simply adds to the assumed model of intramolecular transition/dimerization[19,20] the evidence of a high cooperativity in the formation of the fundamental, ordered form; the final stages of the formation of gel network seem more to result from a slow process of parallel slipping of ordered chains. The specific interaction of Cs^+ ions with the interchain structure does not appear to be the dominant contribution to the driving force of the process. Cs^+ ions seem rather to be an almost positive "structural filler" than to actively link chains together.

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Conformation Characterization of *Bombyx mori* Silk Fibroin in the Solid State by High-Frequency ^{13}C Cross Polarization–Magic Angle Spinning NMR, X-ray Diffraction, and Infrared Spectroscopy

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ABSTRACT: We recorded X-ray diffraction, ^{13}C CP–MAS NMR, and infrared spectra of silk fibroin samples from *Bombyx mori* in the solid state, prepared under different conditions, in order to compare conformational features obtained by the respective methods. These three methods provided consistent data as to the presence of the most stable silk II form. Silk I and random-coil forms were distinguishable only by the extent of line broadening in ^{13}C NMR peaks, although the chemical shifts are the same. The most obvious advantage of the ^{13}C CP–MAS NMR approach is that this method is intrinsically nondestructive and very convenient for evaluation of the relative proportion of silk I and II forms in the same sample. We found that powder formation even at liquid-nitrogen temperature caused a change from silk I to random-coil form, although the amount of silk II form remains unchanged during this procedure. In view of the sample preparation conditions as well as the similarity of the ^{13}C chemical shifts and infrared spectra between the silk I and random-coil forms, it is plausible that the random-coil form is a mixture of silk I type forms whose long-range crystalline packing is distorted to the extent to give halo X-ray diffraction.

Introduction

Two kinds of crystalline modifications, silk I and silk II, as well as the random-coil form, exist as dimorphs of silk fibroin from *Bombyx mori* in the solid state, depending on the sample preparation conditions.[1] On the basis of X-ray diffraction studies, the conformation of silk II has been established as the antiparallel β-sheet form by Marsh et al.[2] and later by Fraser et al.[3] The conformation of silk I,[4-9] however, appears to be not well-defined, as compared with that of silk II. The translations along the molecular axis calculated from the fiber-repeat distance were reported as 2.27–2.4 Å, which is longer than the corresponding value for the α-helix (1.5 Å) and shorter than the value for silk II type (3.5 Å). Lotz and Keith proposed a crankshaft model on the basis of the similarity of the diffraction pattern with that of $(\text{Ala-Gly})_n$II.[9] There appears to be a conflict as to the classification of the random-coil form as viewed from the infrared absorption frequencies: Hayakawa et al.[10] proposed the silk I form for samples whose infrared frequencies are very similar to those of the random-coil form proposed by Miyazawa[11,12] and Magoshi et al.[8] We previously demonstrated that examination of the conformation-dependent ^{13}C chemical shifts as determined by high-frequency (75.46 MHz) cross polarization–magic angle spinning (CP–MAS) provides conformational characterization of the backbone moieties of peptides and polypeptides.[13-20] A similar approach has been applied to conformational analysis of polypeptides by Kricheldorf et al.[21-24] In particular, we showed that three forms of silk fibroins, silk I, silk II, and α-helix forms, are easily distinguished by the sizable displacement of the ^{13}C chemical shifts of the major amino acid residues: Ala, Gly, and Ser.[15,18]. However, we pointed out that new model building is required for the silk I form, because the Ala residue in the silk I form *does not* take torsion angles corresponding with the β-sheet form as proposed by the crankshaft model.

Our aim in this paper is to record and compare three sets of data, X-ray diffraction, ^{13}C CP–MAS NMR, and infrared spectra, of carefully prepared *B. mori* fibroin samples of silk I, silk II, and random-coil form. Our objective of this study is threefold. First, we clarify major advantages and disadvantages of these three methods and characteristic features inherent to the individual method

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