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Ultrastructural evidence for intramolecular double stranding in iota-carrageenan

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ABSTRACT

Kinetic studies of primary processes of conformational ordering in gel-forming biopolymers have suggested that a change in mechanism from intermolecular to intramolecular multistrand formation occurs on lowering the concentration of biopolymer. We report here ultrastructural observations consistent with intramolecular double stranding in a carbohydrate polymer, iota-carrageenan, by arresting this process of primary conformational ordering by an ultra-rapid freeze fixation technique. High-resolution transmission electron microscopy (TEM) revealed isolated iota-carrageenan chains showing a range of morphologies (linear, circular, and hairpin) consistent with intramolecular stranding. Control experiments in which iota-carrageenan was frozen in the disordered form revealed longer and thinner strands.

INTRODUCTION

Iota-carrageenan consists principally of an alternating copolymer of 1,3-linked β-D-galactose 4-sulphate and 1,4-linked 3,6-anhydro-α-D-galactose 2-sulphate (Fig. 1). X-ray fibre diffraction studies of calcium iota-carrageenan have revealed a three-fold right-handed double helix with parallel strands and a helix pitch of 2.66 nm[1]. In solution, iota-carrageenan undergoes a reversible disorder–order transition, with high ionic strength and low temperature favouring the ordered state. Intermolecular double-helix formation should produce a doubling of the observed molecular weight on going through the order–disorder transition. This has been observed using membrane osmometry and light-scattering in some studies[2–5] but not in others[6]. Some of the most conclusive evidence for dimer formation in solution has come from kinetic studies using polarimetric stopped-flow[2,7]. Reactions on a seconds-to-minutes timescale, initiated by mixing iota-carrageenan and

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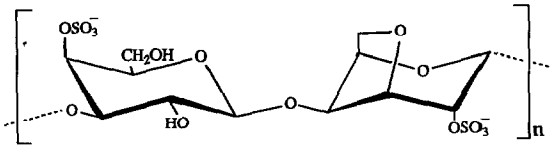

Fig. 1. Idealised iota-carrageenan structure showing the repeating disaccharide residue.

salt solutions [NaCl, KCl, $(CH_3)_4NCl$], fit to a kinetic model with a second-order forward reaction and first-order back reaction, indicating a reversible double-helix-forming process,

$$2C \underset{k_1}{\overset{k_2}{\rightleftharpoons}} H_2 \qquad (1)$$

with random-coil residues, C, and double-helix residue pairs, H_2. A secondary process follows on a minutes-to-hours timescale and is assumed to involve polymer annealing and aggregation[8]. Recent studies of the closely related anionic polysaccharide kappa-carrageenan showed that the rate of primary ordering over the concentration range 0.15–3 mg mL^{-1} in 0.02 M KCl solution was independent of polymer concentration, although the reaction progress curves were still those of a second-order forward reaction[9]. It was suggested that the mechanism of double stranding changes from inter- to intra-molecular[10] as the polymer concentration is decreased from above to below the critical overlap concentration, c^* (refs 9 and 11).

In the present study, the Group II cation Ca^{2+}, which has been shown to be particularly effective in promoting formation of the ordered state[12,13], was used to induce conformational ordering of iota-carrageenan at low polymer concentration. There have been no previous TEM investigations of iota-carrageenan, although work by Hermansson[14] on kappa-carrageenan in 0.01–0.2 M KCl has demonstrated the power of TEM for visualising ordered and aggregated structures. We have used TEM to observe directly the products of reaction *1* prior to significant progress in the second phase.

EXPERIMENTAL

Reaction *1* was carried out by mixing 2.5 mM $CaCl_2$ and iota-carrageenan (X6955/CECA SA France) concentrations suitable for TEM visualisation of non-overlapped molecules (0.5 or 5.0 μg mL^{-1}) at 35°C. After 4 min, an aliquot of the mixture (0.25 or 1.0 μL) was spread on a 5 × 5 mm square of freshly cleaved mica and rapidly frozen in a mixture of liquid propane in isopentane by a countercurrent plunge-freezing technique[15,16]. The frozen sample on mica was subsequently freeze-dried at −65°C or less and rotary-shadowed with 0.4 nm of platinum at an angle of 7° and 6.0 nm of carbon at 90°. The cleaned replicas were examined by TEM. In a control experiment, iota-carrageenan and $(CH_3)_4NCl$

were mixed at a concentration of 5.0 μg mL^{-1} and 2.5 mM, and rapidly frozen after 4 min at 35°C.

RESULTS AND DISCUSSION

At low concentrations of $(CH_3)_4NCl$, iota-carrageenan exists in the disordered form[2]. Fig. 2A shows an electron micrograph prepared from the control disordered sample (5.0 μg mL^{-1}) in 2.5 mM $(CH_3)_4NCl$ rapidly frozen from 35°C. The long fine fibre seen in Fig. 2A (apparent width, 2 nm with the replicated platinum) is typical of the fibres observed in this preparation and is identified as a single-stranded random coil. Studies of iota-carrageenan in 2.5 mM $CaCl_2$, using optical rotation, have shown that 35°C is just below the melting temperature of the polymer, and an equilibrium distribution contains both disordered and ordered residues[17]. Conductometric stopped-flow studies have showed that, on mixing $CaCl_2$ and iota-carrageenan, the primary ordering process *1* is effectively complete after 4 min[17]. Figs. 2B and 2C show iota-carrageenan molecules at 5.0 μg mL^{-1} rapidly frozen 4 min after mixing in 2.5 mM $CaCl_2$. Numerous circular polymers of a range of diameters (Fig. 2C) and simple loop and complex multilooped structures

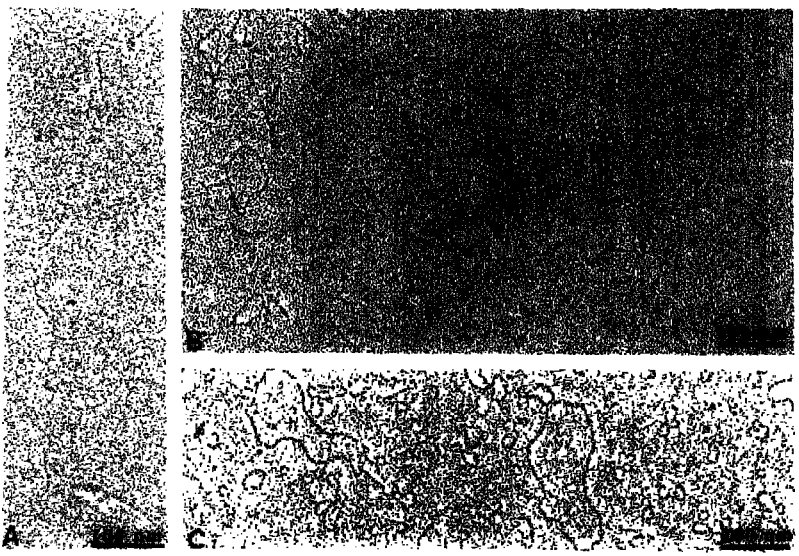

Fig. 2. Electron micrographs of platinum-replicated samples of iota-carrageenan: single-stranded random coil (A); ordered double-stranded forms showing (B) a variety of morphologies and (C) cyclic structures. Solutions of iota-carrageenan (5.0 μg mL^{-1}) in 2.5 mM $(CH_3)_4NCl$ (A) and 2.5 mM $CaCl_2$ (B and C) were sampled 4 min after mixing. Aliquots (A, 0.25 μL; B and C, 1.0 μL) spread on 5×5 mm mica were rapidly frozen in propane–isopentane, stored in liquid nitrogen, freeze-dried, and then rotary-shadowed with platinum at an angle of 7° and carbon at 90°. Replicas were collected onto uncoated 400-mesh copper grids and examined in a transmission electron microscope (Jeol 1200 EX) at 80 kV.

(Fig. 2B) are visible. Figs. 3A–3E focus on individual iota-carrageenan molecules at 0.5 μg mL^{-1} ordered with 2.5 mM $CaCl_2$. Fig. 3A shows both ordered and disordered molecules alongside each other. Fig. 3B can be interpreted as a single-stranded hairpin loop closing into a double-stranded region. A double-helical twist can be almost discerned between the arrows. In Fig. 3C, a closed double-stranded loop is seen with two regions of uncoiled single strands. This coexistence of disordered and ordered structures is expected in a sample frozen close to its melting temperature. Fig. 3D is that of a fully closed double-stranded loop. Fig. 3E suggests regions with secondary folding (supercoiling). The numerous circular polymers and the loop structures can be interpreted as evidence for intramolecular stranding. The distribution of chain length in Figs. 2 and 3 accords

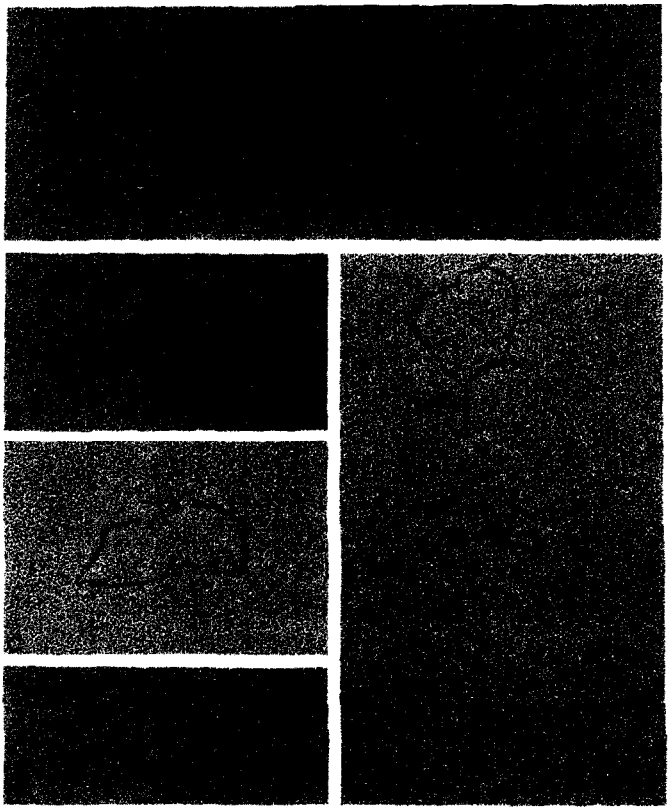

Fig. 3. Electron micrographs of partially ordered iota-carrageenan. Sample preparation as in Figs. 2B and C, except with 0.5 μg mL^{-1} iota-carrageenan. In A, a structure on the right is considerably longer and thinner than that on the left, suggesting the coexistence of single- and double-stranded forms; B, a single-stranded loop of a hairpin structure closing into a double-stranded region (arrows); C, an incompletely folded cyclic structure with potential double-strand separation (arrows); D, a fully ordered cyclic structure; and E, an example of a thicker folded (double helical) form folding back again and twisting to form a secondary fold (supercoiling) in regions. Arrows point to those secondary foldings, while arrow heads point to regions of unfolded random coil.

with the nonhomogeneity of the original iota-carrageenan sample. Although this precludes the making of quantitative estimates, in general, the structures formed in $CaCl_2$ appear to be shorter than those in $(CH_3)_4NCl$, again suggesting intramolecular folding. Furthermore, the apparent width (3–4 nm) of the polymer in $CaCl_2$ is approximately double that in $(CH_3)_4NCl$. All observations in $CaCl_2$ are consistent with double-stranded species formed by intramolecular ordering.

Whilst circular double-stranded DNA is well documented[18], circular forms of polysaccharides have only recently been discovered in TEM studies of triple-helical schizophyllan and lentinan[19,20]. Our observation of cyclised forms of double-helical iota-carrageenan suggests that this morphology may be a common feature in biopolymers ordered at low concentration. In intramolecular double-helix formation, cyclised forms can result only from parallel strand nucleation whereas hairpins result from antiparallel strands (Fig. 4). There may be some energetic advantages to the former in iota-carrageenan, since X-ray fibre diffraction shows parallel strands in the solid-state double helix[1]. Supercoiling in a polysaccharide was also only recently first observed in the circular triple-helical structures of schizophyllan and lentinan[20]. The possible functional and structural significance of circular and supercoiled polysaccharides was discussed by Stokke et al.[19,20], but left unresolved. As with DNA, we believe that this intramolecular stranding is simply a common feature in general in flexible biopolymers at low concentrations.

The ultra-rapid freeze fixation technique used here has enormous potential for time-resolved studies, both because of the low dead time for freezing (< 1 ms) and the ability to use solutions which are complementary to those used in kinetic and equilibrium studies, in that they are free of such chemicals as glycerol that have traditionally been added to TEM preparations of this type[21]. Stokke et al.[20] were cautious in their interpretation of supercoiling in that they could not exclude the possibility of introduction of structural artefacts caused by sample preparation for TEM. Addition of glycerol, spray deposition of sample, and vacuum drying at 20°C can introduce numerous structural artefacts[21]. Exclusion of any additive, gentle deposition of sample, ultra-rapid freeze fixation, and freeze-drying in our preparation eliminate the possibility of preparation artefacts in that we can confidently say

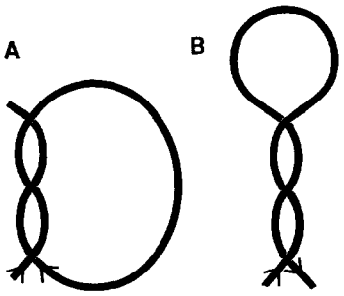

Fig. 4. Schematic diagrams for intramolecular nucleation of A, cyclised forms from parallel stranding; and B, loop forms from antiparallel stranding: arrows indicate the direction along the chain in Fig. 1.

that the circular and supercoiled morphologies of iota-carrageenan are real and therefore the similar structures in schizophyllan and lentinan are also probably real. Our interpretation that the ordered structures are intramolecular double strands is based on the apparent doubling of the chain thickness, the shorter chain lengths, and the circular and looped structures of ordered iota-carrageenan compared to the disordered form. Observation of single-stranded random coils in kappa-carrageenan is believed to be impossible[22]. In our investigations, the visualisation of the random coil was clearly possible in the presence of $(CH_3)_4Cl$ (Fig. 2A) but was very difficult in the absence of the salt (results not shown). Our ability to visualise the iota-carrageenan random coil in this investigation is therefore probably due to the good structural preservation by the ultra-rapid freeze fixation and the very fine film of platinum (0.4 nm) deposited for replica preparation, as well as to a possible amplification of the coil thickness by interacting $(CH_3)_4Cl$ ions. Future experiments, using this technique and iota-carrageenan concentrations above and below c^* and with short-chain segments, may give further insight into the nature of the ordered structures of this biopolymer.

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REFERENCES

1 S. Arnott, W.E. Scott, D.A. Rees, and C.G.A. McNab, *J. Mol. Biol.*, 90 (1974) 253–267.
2 K.R.J. Austen, D.M. Goodall, and I.T. Norton, *Carbohydr. Res.*, 140 (1985) 251–261.
3 R.A. Jones, E.J. Staples, and A. Penman, *J. Chem. Soc., Perkin Trans. 2*, (1973) 1608–1612.
4 E.R. Morris, D.A. Rees, and G. Robinson, *J. Mol. Biol.*, 138 (1980) 349–362.
5 I.T. Norton, D.M. Goodall, E.R. Morris, and D.A. Rees, *J. Chem. Soc., Faraday Trans. 1*, 79 (1983) 2489–2500.
6 O. Smidsrød, I.L. Anderson, H. Grasdalen, B. Larsen, and T. Painter, *Carbohydr. Res.*, 80 (1980) c11–c16.
7 I.T. Norton, D.M. Goodall, E.R. Morris, and D.A. Rees, *J. Chem. Soc., Faraday Trans. 1*, 79 (1983) 2501–2515.
8 K.R.J. Austen, D.M. Goodall, and I.T. Norton, *Biopolymers*, 27 (1988) 139–151.
9 D.M. Goodall, in W. Knoche and R. Schomäcker (Eds.), *Reactions in Compartmentalized Liquids*, Springer-Verlag, Berlin, 1989, pp 153–162.
10 W.F. Harrington and N.V. Rao, *Biochemistry*, 9 (1970) 3714–3724.
11 E.R. Morris, A.N. Cutler, S.B. Ross-Murphy, D.A. Rees, and J. Price, *Carbohydr. Polym.*, 1 (1981) 5–21.
12 S. Nilsson and L. Piculell, *Macromolecules*, 22 (1989) 3011–3017.
13 I.T. Norton, in G.O. Phillips, P.A. Williams, and D.J. Wedlock (Eds.), *Gums and Stabilisers for the Food Industry*, Vol. 5, IRL Press, Oxford, 1990, pp 511–520.
14 A.-M. Hermansson, *Carbohydr. Polym.*, 10 (1989) 163–181.
15 P.W. Le, R. Murray, A.W. Robards, and P.R. Waites, *J. Microsc.*, 156 (1988) 173–182.
16 A.W. Robards, in J.L. Hall and C. Haws (Eds.), *Electron Microscopy of Plant Cells*, Academic Press, London, 1991, pp 257–312.
17 E.T. Bergström, D. Phil. Thesis, University of York, 1991.

18 J.C. Wang, in J.A. Semlyen (Ed.), *Cyclic Polymers,* Elsevier Applied Science, London, 1986, pp 225–260.
19 B.T. Stokke, A. Elgsaeter, D.A. Brant, T. Kuge, and S. Kitamura, *Biopolymers*, 30 (1991) 1161–1182.
20 B.T. Stokke, A. Elgsaeter, D.A. Brant, and S. Kitamura, *Macromolecules,* 24 (1991) 6349–6351.
21 A.W. Robards and R.M. Abeysekara, in L. Megias-Megias, M.L. Rodrigues-Gracia, A. Rios, and J.M. Arias (Eds.), *Electron Microscopy 1992*, Vol. 3, Secretariado de Publicaciones de la Universidad le Granada, 1992, pp 77–78.
22 A.-M. Hermansson, in W. Burchard and S.B. Ross-Murphy (Eds.), *Physical Networks. Polymers and Gels,* Elsevier Applied Science, London, 1990, pp 271–282.