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Effect of calcium ions on the organization of iota-carrageenan helices: an X-ray investigation

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Abstract

X-ray fiber diffraction analysis confirms that calcium ι-carrageenan forms a threefold, right-handed, half-staggered, parallel, double helix of pitch 26.42 Å stabilized by interchain hydrogen bonds. According to the detailed structural results, three helices are packed in a trigonal unit cell ($a = 23.61$ and $c = 13.21$ Å). Strong interactions between the sulfate groups of neighboring helices, mediated by calcium ions and water molecules, are responsible for stabilizing the three-dimensional structure.

Keywords: Iota-carrageenan; X-ray diffraction; Three-dimensional structure; Junction zone

1. Introduction

A plethora of literature is available on bacterial and algal polysaccharides, which have been utilized in the food industry as gelling agents, thickeners, syneresis inhibitors, and binders. In particular, carrageenans usage has increased dramatically in the dairy industry as they can complex with other hydrocolloids and proteins. Carrageenans are water-soluble sulfated galactans of the marine red algae *Rhodophyceae* with alternating α-(1→3)- and β-(1→4)-linked galactopyranosyl residues. The α-galactopyranosyl residue is converted to accommodate a 3,6-anhydro ring either by enzymatic reaction or chemical treatment. In general, carrageenans are distinguished by the amount of 3,6-anhydro group, sulfation, and position of the sulfate group, and so far seven different carrageenans have been identified.[1,2] These carrageenans have medical applications as well. For example, with the highest degree of sulfation, three per disaccharide repeat, λ-carrageenan is the most potent anticoagulant followed by the other two.[3,4] However, their clinical usage is currently limited due to the high immunogenicity and gel formation tendency.

The chemical repeat of ι-carrageenan may be written as →3)-*A*-(1→4)-*B*-(1→ where *A* and *B* represent β-D-Gal-4-SO_3^- and 3,6-anhydro-α-D-Gal-2-SO_3^-, respectively. κ-Carrageenan also has the same sequence except that its anhydride residue does not possess a sulfate moiety. In the case of λ-carrageenan, the sulfate group of β-D-Gal in *A* shifts to the second position and *B* becomes 2,6-di-*O*-sulfato-α-D-galactopyranosyl unit. These chemical differences are evident clearly in their distinct physical properties. For example, κ-carrageenan produces the strongest gels within the family; the gels are rigid and elastic in the presence of potassium, whereas calcium develops stiff and brittle gels that are susceptible to syneresis. In contrast, ι-carrageenan gels are syneresis free and they have the unique ability of thixotropic flow and good freezing stability. Both κ- and ι-carrageenans form thermally reversible gels upon heating and cooling of aqueous solution. λ-Carrageenan does not form gels at any concentration.

As cations affect the gelling properties to varying extents, knowledge of the three-dimensional structures of different types of carrageenans will be useful in understanding the junction zone details and gelling mechanism at the molecular level. Our recent X-ray study on sodium ι-carrageenan[5] has revealed that its

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double helices are organized in a trigonal net and stabilized by either SO_3^-–Na–SO_3^- or SO_3^-–Na–water–Na–SO_3^- interactions. Since then, we have obtained polycrystalline and oriented fibers from calcium ι-carrageenan and determined its detailed three-dimensional structure. The calcium-mediated 'helix–helix' interactions are found to be stronger than those in the monovalent form.

2. Experimental

Fiber preparation.—A pure and homogeneous sample of ι-carrageenan in the sodium form was kindly provided by the FMC Corporation, USA. About 15 mg of the polysaccharide was dissolved in 1 mL of distilled water, adequate for preparing ten or more fibers. A few drops of this solution were placed in between the beaded ends of two glass rods in a fiber puller maintaining 75% relative humidity in its chamber. As the solution dried slowly and reached a semi-solid state after few hours, it was stretched at regular intervals of time so that it formed an oriented fiber, about 3–5 mm long.[6] In order to convert into the calcium form, several such fibers were immersed in a beaker containing 1:9 0.05 M calcium chloride aq-isopropanol solution at rt for nearly a month. Subsequent energy dispersive spectra recorded on a Jeol 35 CF scanning electron microscope, from different regions, clearly indicated the presence of calcium but not sodium in the doped fibers. The quantitative estimate of the relative amounts of elements sulfur and calcium was in the 2:1 molar ratio.

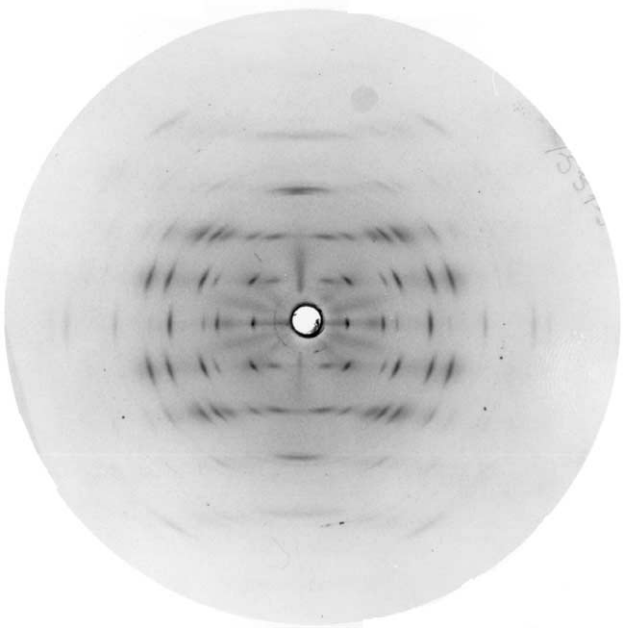


Fig. 1. X-ray diffraction pattern from a well oriented and polycrystalline fiber of calcium ι-carrageenan.

This confirmed the complete elimination of sodium in exchange for calcium as desired for conducting the X-ray analysis. The fiber density was measured by the flotation method using a mixture of bromoform and carbon tetrachloride.

Diffraction patterns.—Fiber-diffraction patterns were recorded on flat photographic film in a pinhole camera using Ni-filtered Cu K_α X-rays (wavelength 1.5418 Å). The specimen chamber was flushed with a steady stream of helium gas bubbled through a saturated salt solution to minimize air scatter and maintain the fiber at constant humidity. The fiber was dusted with calcite powder (characteristic spacing 3.035 Å) as an internal calibrant for determining the unit cell dimensions. One of the diffraction patterns chosen for structure analysis is shown in Fig. 1. It contains Bragg reflections up to the fourth layer line extending out to 2.8 Å resolution. The spots are sharp and small near the center, but they become arcs of increasing length towards the edge of the pattern. This means that large crystallites have formed in the fiber, but unlike in the case of sodium ι-carrageenan,[5] they are a bit short of perfect alignment parallel to the fiber axis. To the extent of the Bragg reflections, the pattern in Fig. 1 resembles those of calcium and strontium ι-carrageenan[7] that are overlaid with continuous intensity on layer lines due to statistical disorder in the fibers.

Unit cell dimensions and intensity data.—The meridional reflection on the third layer line (Fig. 1) indicates that calcium ι-carrageenan forms a threefold helix. All the 33 observed reflections could be indexed on a trigonal unit cell, $a = 23.61(2)$ and c (fiber axis) = 13.21(1) Å. By comparing with the reported smaller trigonal cell ($a = 13.73$, $c = 13.28$ Å) for the same polymer,[7] we find that there is a good match in the axial repeat. This suggests that the molecular structures could be similar in the two cases, but the packing arrangements are not. On the other hand, the present calcium form, showing approximately 2% lateral contraction as well as axial expansion, is isomorphous with that of sodium ($a = 24.02$ and $c = 12.93$ Å).[5]

The radial trace of the optical density of each reflection in the pattern was measured using a Joyce Loebl Mark III microdensitomer. Baseline for the radial scan was drawn before measuring the trace area that is proportional to the intensity of the reflection. The intensities were averaged over all the quadrants in the pattern. A set of 30 reflections is too weak to be seen on the pattern and the lowest measured intensity was assigned as the threshold value to each of these unobserved reflections. The intensities were then converted into observed structure amplitudes (F_o) after applying Lorentz and polarization corrections. While all the observed reflections were used in the structure refinement, an unobserved reflection was included as datum only if its threshold F_o were less than the calculated

structure amplitude (F_c) and omitted otherwise implying that it had been fitted well by the model.

3. Structure analysis

Molecular model and packing arrangement.—The threefold, right-handed, half-staggered, double helix of sodium ι-carrageenan,[5] incorporating $^4C_1$ and $^1C_4$ sugar-ring conformations for *A* and *B*, respectively, was used as the starting model. The main chain conformation angles (ϕ_1, ψ_1) and (ϕ_2, ψ_2), as well as those of the side groups, were refined so as to generate a stereochemically optimized calcium carrageenan helix of pitch 26.42 Å ($=2c$) using the linked-atom least-squares (LALS) method.[8]

The fiber density (1.69 g/mL) suggests that the unit cell has room for nine disaccharide units, corresponding to half-a-pitch of three double helices, along with nine calcium ions and 108 water molecules. In other words, the crystallographic asymmetric unit should account for six galactosyl sulfate groups, three calcium ions and 36 water molecules. Analogous to the packing scheme of sodium ι-carrageenan,[5] three helices (I, II, III) were positioned in the unit cell such that their fractional coordinates (u, v) were (2/3, 1/3), (1/3, 2/3) and (0, 0), respectively. Two distinct models were considered with regard to the polarities of the helices. In model P, all the helices had the same polarity, but in model A, helix II was aligned antiparallel to I and III. For each helix, the orientation (μ) relative to a^*-axis and translation (w) relative to *ab*-plane had to be determined. By setting the relative translation of helix I to zero, this arrangement corresponds to the space group $P3_2$ and five packing parameters (μ_1, μ_2, μ_3, w_2 and w_3) had to be determined. Details of the LALS structure refinement are the same as those reported in our previous publications[5,9,10] and the *R*-values computed were based on water-smeared scattering factors.[11]

Structure refinement and guest molecules.—The three helices in the unit cell are 13.6 Å apart from each other, a value close to the helix diameter. However, due to lack of intimate interactions between them at any orientation and translation for each helix, a conventional (μ, w) survey of helix–helix contacts was not helpful in choosing the preferred packing arrangement. Using the X-ray data, the five packing parameters, μ_1–w_3, were then stepped at small intervals (5° for μ and 0.05 for w) and the corresponding *R*-values examined in search of a possible solution. Several minima having $R \approx 0.32$ and 0.25 were found for models A and P, respectively, and tested in a series of difference Fourier maps for locating guest molecules. In many cases, we could identify only a few of them but not progress further due to lack of improvement in the X-ray fit.

In the case of model P, although the X-ray fit was improving down to $R = 0.15$, the main difficulty was that the guest molecules shown by the difference maps were so scattered that they were unable to form hydrogen bonds either amongst themselves or with the polysaccharide chains. Such a loose packing arrangement was deemed unstable and unsatisfactory and not considered further. The same behavior was experienced in the structure analysis of sodium ι-carrageenan also.

However, the set up $\mu_1 = 40°$, $\mu_2 = -45°$, $\mu_3 = 80°$, $w_2 = -0.25$ and $w_3 = -0.30$ for model A, somewhat similar to that in sodium ι-carrageenan, 45°, −29°, 101°, −0.10 and −0.09 in the same sequence,[5] led us to the correct solution. The first difference Fourier map showed many positive-density regions between the helices. Initially, 12 prominent peaks were chosen, named as water, and included one after another in the refinement. The reduction in *R* was monitored with the criterion that the guest molecules must be less than 3.2 Å from at least two of the carbohydrate oxygen atoms. Three peaks, each able to connect the sulfate oxygen atoms of adjacent helices, were then re-labeled as calcium ions. When the positions of these three ions and nine water molecules were refined, they were found to be stable and *R* dropped to 0.21. The second difference Fourier map using the improved phases of the augmented structure was cleaner and it yielded three more water molecules and their inclusion reduced *R* marginally to 0.20. Refinement of the main- and side-chain conformation angles of the carrageenan helix along with the positions of guest molecules slightly improved the X-ray fit. The convergence was smooth, shifts in variables were very small, all the non-bonded contacts were satisfactory, and *R* was 0.185 at the end of the refinement. This X-ray fit includes all the 33 observed and 15 out of 30 unobserved reflections. The atomic coordinates of this model are given in Table 1. The observed and calculated structure amplitudes are listed in Table 2.

4. Results

Molecular morphology.—Two mutually perpendicular views of the ι-carrageenan double helix are shown in Fig. 2. The sulfate groups are on the exterior of the sinuous helix. The major conformation angles are listed in Table 3. There are no intrachain hydrogen bonds but two interchain hydrogen bonds, O-2H···O-5 and O-6H···O2, are formed per disaccharide repeat between the galactosyl units to provide helix stability. These are denoted as ***1*** and ***2*** in Table 4 that lists the details of all the predominant attractive interactions (2.1–3.1 Å between functional groups) in the structure. The hydrogen bond donors O-2 and O-6 are also linked to a water molecule (*W*-5 in I, *W*-6 in II and *W*-4 in III as seen

Table 1
Cartesian and cylindrical polar atomic coordinates of a repeating unit of the ι-carrageenan helix, and those of calcium ions and water molecules in an asymmetric unit

Group	Atom	x (Å)	y (Å)	z (Å)	r (Å)	ϕ (°)
Gal-4-SO_3^- (*A*)	C-1	−0.169	1.777	5.262	1.785	95.45
	C-2	−0.076	2.146	6.740	2.147	92.04
	C-3	−1.372	1.801	7.459	2.265	127.30
	C-4	−2.560	2.427	6.740	3.528	136.52
	C-5	−2.532	2.061	5.261	3.265	140.86
	C-6	−3.625	2.746	4.469	4.548	142.85
	O-1	0.976	2.231	4.623	2.435	66.38
	O-2	1.026	1.465	7.325	1.789	54.98
	O-3	−1.317	2.281	8.807	2.633	120.00
	O-4	−2.519	3.848	6.857	4.599	123.21
	O-5	−1.282	2.455	4.674	2.769	117.57
	O-6	−3.507	2.459	3.075	4.283	144.96
	S	−3.892	4.558	7.271	5.993	130.49
	O-S1	−4.687	3.645	8.069	5.937	142.13
	O-S2	−3.606	5.752	8.043	6.788	122.08
	O-S3	−4.628	4.920	6.075	6.755	133.24
	H-1	−0.310	0.690	5.166	0.757	114.17
	H-2	0.119	3.224	6.837	3.226	87.89
	H-3	−1.513	0.710	7.464	1.671	154.85
	H-4	−3.495	2.063	7.190	4.059	149.45
	H-5	−2.629	0.971	5.152	2.803	159.73
	H-61	−3.504	3.837	4.544	5.196	132.40
	H-62	−4.604	2.500	4.906	5.239	151.49
3,6-Anhydro-Gal-2-SO_3^- (*B*)	C-1	2.624	1.055	0.879	2.828	21.90
	C-2	3.330	0.604	2.165	3.384	10.27
	C-3	2.923	1.541	3.310	3.304	27.79
	C-4	1.401	1.495	3.480	2.048	46.86
	C-5	1.031	2.292	2.233	2.513	65.77
	C-6	2.029	3.436	2.283	3.990	59.43
	O-1	2.633	0.000	0.000	2.633	0.00
	O-2	2.948	−0.724	2.542	3.036	−13.80
	O-3	3.209	2.886	2.917	4.316	41.96
	O-4	0.976	2.231	4.623	2.435	66.38
	O-5	1.238	1.417	1.096	1.881	48.85
	S	3.945	−1.893	2.096	4.376	−25.64
	O-S1	5.229	−1.708	2.745	5.501	−18.09
	O-S2	3.390	−3.176	2.483	4.645	−43.13
	O-S3	4.121	−1.857	0.658	4.520	−24.25
	H-1	3.163	1.911	0.446	3.695	31.13
	H-2	4.419	0.651	2.022	4.467	8.38
	H-3	3.407	1.213	4.242	3.617	19.60
	H-4	0.720	0.646	3.317	0.967	41.89
	H-5	0.011	2.689	2.339	2.689	89.76
	H-61	2.274	3.755	1.259	4.390	58.80
	H-62	1.625	4.248	2.906	4.548	69.06
Calcium	Ca-1	4.205	5.541	−1.741	6.956	52.80
	Ca-2	−0.409	12.559	0.161	12.566	91.86
	Ca-3	7.284	−0.378	10.517	7.293	−2.97
Water	*W*-1	−1.044	9.053	−1.381	9.113	96.58
	W-2	6.176	2.234	−2.374	6.568	19.89
	W-3	7.822	−5.037	0.669	9.304	−32.78
	W-4	−0.351	−4.373	0.303	4.387	−94.58
	W-5	10.759	−3.150	4.279	11.211	−16.32

Table 1 (*Continued*)

Group	Atom	x (Å)	y (Å)	z (Å)	r (Å)	ϕ (°)
	W-6	3.971	−8.445	−7.353	9.333	−64.82
	W-7	2.963	6.404	0.742	7.056	65.17
	W-8	0.565	8.027	0.680	8.047	−5.97
	W-9	−6.349	−3.071	−6.416	7.053	−154.19
	W-10	11.598	−5.754	1.852	12.947	−26.39
	W-11	7.937	−1.630	1.051	8.102	−11.60
	W-12	10.079	−6.257	−0.421	11.863	−31.83

The cylindrical coordinates of the next two disaccharides in one chain are (r, $\phi+120°$, $z+2c/3$) and (r, $\phi-120°$, $z+4c/3$). The second chain of the double helix is generated from the first by adding c to their z coordinates. For a down-pointing double helix, the coordinates are (r, $-\phi$, $-z$). The packing parameters are:

Molecule	Sense	μ (°)	*u*	*v*	*w*
Helix I	up	46.4	2/3	1/3	0.0
Helix II	down	-48.7	1/3	2/3	-0.2380
Helix III	up	84.3	0.0	0.0	-0.3064
Ca and Water		0.0	0.0	0.0	0.0

from ***6***, ***7***, and ***8***, respectively, in Table 4) that is already connected to two of the oxygen atoms of a 2-sulfate group in the same helix. In all these respects, the helix shape is exceptionally similar to that of the sodium form[5] suggesting that the carrageenan helix has a robust geometry. This can be verified by comparing the conformation angles for the two salt forms in Table 3. While the backbone angles match within 10°, the largest change (18°) occurs in the 4-sulfate group. Consequently, superposition of the two helices suggests that the root mean-square deviation for atoms in the backbone is only 0.2 Å, the same as that in the 2-sulfate group, but it is 0.4 Å in the 4-sulfate group.

Calcium ions and water molecules.—Direct 'helix–helix' hydrogen bonds do not exist in either sodium ι-carrageenan[5] or this structure. Instead, ions and water molecules are able to hold the helices, two 'up' and one 'down', together as shown in Fig. 3. A calcium ion and four out of 12 water molecules have been located near a disaccharide repeat in each helix. The remaining eight water molecules are probably disordered. The ions span the helices in a concerted fashion. Whereas the 2-sulfate groups of II and III are linked by Ca-1 (***3***), and likewise the 4-sulfate groups of I and III by Ca-3 (***5***), the 2-sulfate of I is linked to the 4-sulfate of II by Ca-2 (***4***). These features are shown in Fig. 4 in which each panel highlights that the ion is anchored securely by at least four oxygen atoms in the carbohydrate chains in addition to one or two water molecules similar to the six or more ligands often observed for calcium in anionic polysaccharide structures.[10,12] The Ca···O distances (2.1–3.0 Å) agree with reported values in related structures.[10,13,14]

The 12 water molecules in the asymmetric unit, *W*-1 through *W*-12, fall under two sets depending on the extent of their involvement in stabilizing the structure. In the first set of five water molecules, each is linked to only one helix. Three of them, *W*-4 through *W*-6, as already mentioned as participants in ***6***–***8*** sequentially, reinforce the interchain hydrogen bonds ***1*** and ***2***, enhance double-helix stability and thus become an integral part of the helix itself. The same feature is also observed in the sodium ι-carrageenan structure.[5] The other two, *W*-8 and *W*-12 are both confined only to helix II (***13***, ***16***).

The remaining seven water molecules, in the second set, are able to interact either directly or via other guest molecules with the functional groups in one or two helices simultaneously as listed in ***9*** through ***16*** and thus are responsible for the ordered aggregation of helices. Combining the data in Table 4 with the details in Fig. 4 reveals that water-mediated bridges tether the helices that are already held tight by calcium ions at their sulfate groups. Denoting 2-S as the surrogate for an oxygen atom in 2-sulfate and 4-S for 4-sulfate, the major tertiary interactions are the following: I and II are connected by the two long bridges 2-S–Ca–*W*–*W*–O-6, and 2-S–*W*–*W*–*W*–2-S (Fig. 4(a)); III and II by one short 2-S–*W*–2-S bridge, and three medium 2-S–*W*–*W*–4-S, 4-S–*W*–*W*–4-S, and 4-S–*W*–*W*–O-6 bridges (Fig. 4(b)); and I and III are glued by one short 4-S–*W*–2-S bridge, one medium 2-S–*W*–*W*–2-S bridge, and a long 2-S–*W*–*W*–*W*–2-S bridge (Fig. 4(c)). These features underscore the importance of both 2- and 4-sulfate groups for the aggregation properties of ι-carrageenan helices.

Table 2
Observed and calculated structure amplitudes for calcium ι-carrageenan

h k	l				
	0	1	2	3	4
0 0	M [*1391*]	N [*0*]	N [*0*]	M [*305*]	N [*0*]
1 0	(35) (*66*)	141 *418*	219 *210*	(79) (*84*)	[53] [148]
1 1 2 0	278 *284*	293 *307*	258 *265*	(158) (*180*)	(187) (*278*)
2 1	108 *188*	403 *500*	599 *436*	311 *302*	238 *325*
3 0	379 *366*				
2 2 3 1	251 *260*	537 *470*	560 *458*	202 *272*	241 *316*
4 0	(143) (*149*)	149 *237*	165 *228*	(187) (*212*)	[214] [*127*]
3 2 4 1	645 *555*	682 *627*	456 *475*	244 *310*	578 *425*
5 0	[183] [*118*]	[188] [*103*]	[203] [*187*]	[226] [*94*]	[255] [*83*]
3 3 4 2	199 *296*	776 *560*	251 *278*	333 *281*	
5 1	[207] [*194*]	(212) (*332*)	(227) (*237*)	(250) (*261*)	
6 0 4 3	(322) (*359*)	(329) (*332*)	[350] [*200*]	323 *372*	
5 2	518 *463*	[242] [*194*]	289 *321*		
6 1	[251] [*109*]	(256) (*292*)	[271] [*186*]	[295] [*207*]	
4 4 7 0	[270] [*131*]	278 *211*			
6 2	(283) (*305*)	(288) (*294*)			
7 1	606 *507*				
5 4	(314) (*341*)				
8 0	364 *379*				
7 2	[334] [*264*]				

In each reflection box, the observed amplitude is given in the first line and that calculated (*italic*) in the second. The curved and rectangular brackets refer to below threshold reflections included in, and rejected from, the least-squares refinement, respectively. M and N denote meridional and systematically absent reflections, respectively. The calculated structure amplitudes include a temperature factor with $B = 7.5$ Å^2.

5. Discussion

The main aim of our systematic study on the three-dimensional structures of carrageenans is three-fold. The first is to visualize the preferred interactions among the polysaccharide chains, cations, and solvent molecules, and hence the junction zone details at the molecular level that could be correlated with the observed rheological properties. The second is to examine the same carrageenan type in different salt forms in order to learn the molecular basis of the differences in their physical properties. The third is to unravel the distinct morphologies of all the carrageenan types under the influence of cations and water molecules, if any.

We have shown that ι-carrageenan forms a threefold, right-handed, parallel, half-staggered, double helix in the presence of sodium[5] and calcium ions (this study). In both cases, the three helices in the trigonal unit cell are not related by any crystallographic symmetry and are packed in the space group $P3_2$. This situation offers three possibilities for consideration with regard to helix shape. The first is to have distinct conformations for the helices in the main chain as well as in the sulfate groups. The second is to retain the same main-chain geometry for all the helices, but allow their sulfate groups adopt alternate orientations if accessible. The third is to utilize conservatively, identical geometry for all the helices. The entropy of these systems will decrease in the same order. The first choice is quite complex in terms of modeling because of the large number of additional degrees of freedom. The second is an intermediate state, perhaps a compromise between the other two, yet tedious to compute and analyze. The third choice is more conservative and practical as it preserves the same low-energy conformation for the biopolymer helices when they are organized in the unit cell. Bearing in mind that the first two approaches would only lead to minor variations on the same basic theme, we selected the parsimonious and simple solution as the most probable representation of the carrageenan crystal structure.

Table 3
Major conformation angles (and e.s.d.) in degrees in the calcium ι-carrageenan helix (*a*) this study (*b*) Arnott et al.[7] compared with those in (*c*) the sodium form[5]

Parameter	Location	*a*	*b*	*c*
ϕ_1(O-5–C-1–O-4–C-4)	β-(1→4)	−91(1)	−87	−89
ψ_1(C-1–O-4–C-4–C-5)	β-(1→4)	104(1)	94	109
ϕ_2(O-5–C-1–O-3–C-3)	α-(1→3)	70(1)	75	60
ψ_2(C-1–O-3–C-3–C-4)	α-(1→3)	76(1)	79	77
χ(C-4–C-5–C-6–O-6)	hydroxy-methyl	176(6)	176	−172
θ_1(C-3–C-4–O-4–S)	4-sulfate	−133(3)	−124	−115
θ_2(C-4–O-4–S–O-S1)	4-sulfate	29(4)	81	32
θ_3(C-1–C-2–O-2–S)	2-sulfate	97(2)	101	99
θ_4(C-2–O-2–S–O-S1)	2-sulfate	63(3)	75	63

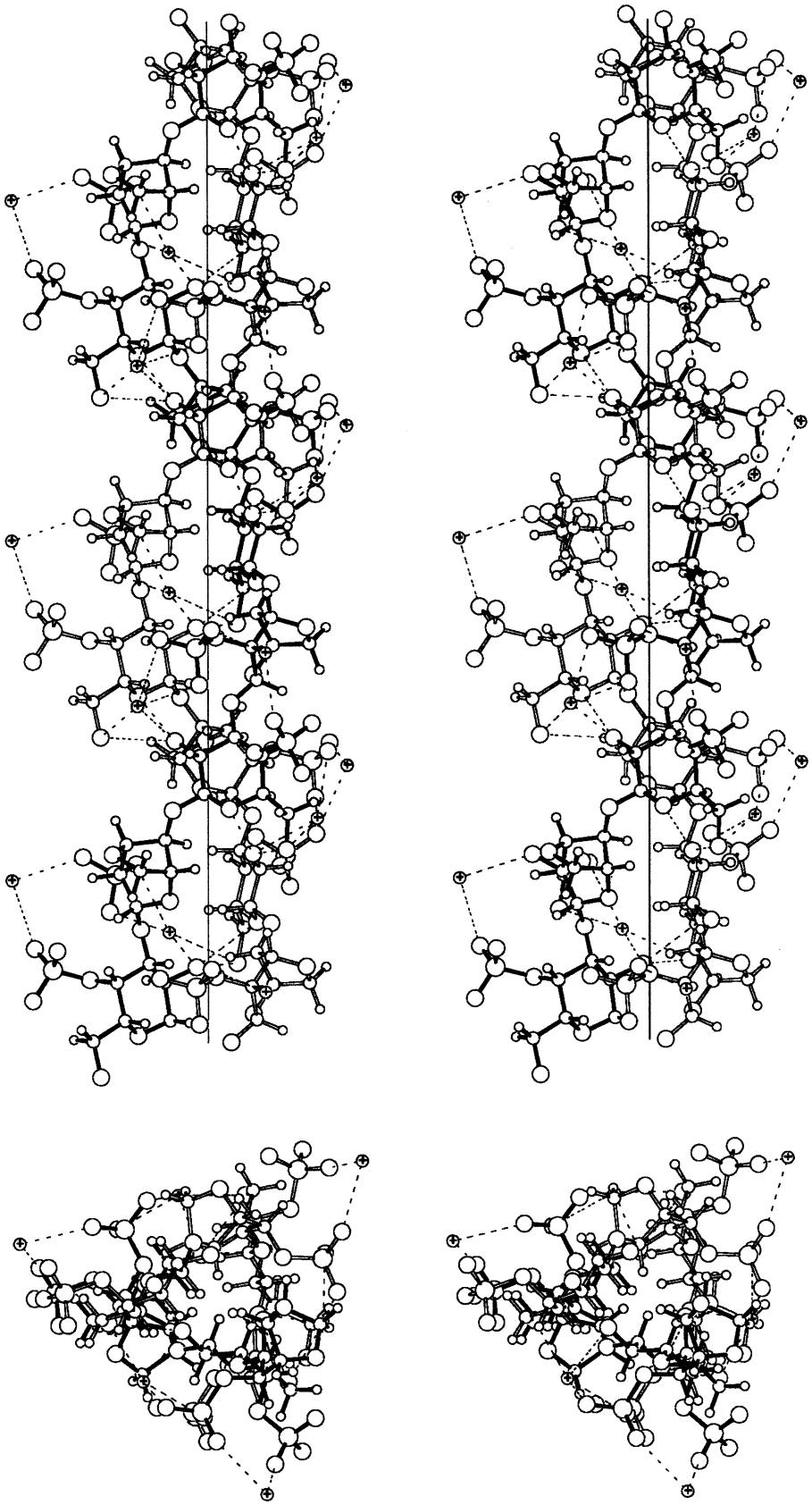


Fig. 2. Stereo drawing of two mutually perpendicular views of the ι-carrageenan double helix stabilized by interchain hydrogen bonds (dashed line). Water molecules (crossed circle) enhance structural stability by connecting sulfate groups to neighboring hydroxymethyl groups. All the hydrogen atoms are included only in this drawing. The vertical line (top) is the helix axis.

Table 4
Attractive interactions among iota-carrageenan helices, calcium ions and water molecules

Interaction	Atom *X*	Atom *Y*	*X*···*Y* (Å)	Precursor P	*P*–*X*···*Y* (°)	Bridge
1	O-2*A*(1)	O-5*A*(2)	2.93	C-2*A*	165	
2	O-6*A*(2)	O-2*A*(1)	2.88	C-6*A*	105	
3	O-S2*B*(II,2)	Ca-1	2.54	S-*B*	148	2-S–Ca–2-S
	O-S1*B*(III,1)	Ca-1	2.10	S-*B*	95	
	O-S2*B*(III,1)	Ca-1	2.59	S-*B*	76	
	O-S3*B*(III,1)	Ca-1	3.02	S-*B*	61	
	W-7	Ca-1	2.91			
4	O-S2*B*(I,1,−10)	Ca-2	2.96	S-*B*	83	2-S–Ca–4-S
	O-S3*B*(I,1,−10)	Ca-2	2.46	S-*B*	103	
	O-S1*A*(II,2,10)	Ca-2	2.33	S-*A*	104	
	O-S2*A*(II,2,10)	Ca-2	2.67	S-*A*	89	
	W-5	Ca-2	2.81			
	W-10	Ca-2	2.77			
5	O-S1*A*(I,1)	Ca-3	2.54	S-*A*	111	4-S–Ca–4-S
	O-S2*A*(I,1)	Ca-3	3.04	S-*A*	89	
	O-S1*A*(III,1)	Ca-3	2.95	S-*A*	88	
	O-S3*A*(III,1)	Ca-3	2.46	S-*A*	109	
	W-2	Ca-3	2.86			
6	O-2*A*(III,2)	*W*-4	2.98	C-2*A*	96	2-S–*W*–(O-2,O-6)
	O-S2*B*(III,2)	*W*-4	2.84	S-*B*	95	
	O-S3*B*(III,2)	*W*-4	2.75	S-*B*	99	
	O-6*A*(III,1)	*W*-4	2.98	C-6*A*	74	
7	O-2*A*(I,2)	*W*-5	2.84	C-2*A*	95	2-S–*W*–(O-2,O-6)
	O-S2*B*(I,2)	*W*-5	2.90	S-*B*	93	
	O-S3*B*(I,2)	*W*-5	2.73	S-*B*	101	
	O-6*A*(I,1)	*W*-5	2.92	C-6*A*	76	
8	O-2*A*(II,2,0−1)	*W*-6	2.93	C-2*A*	95	2-S–*W*–(O-2,O-6)
	O-S2*B*(II,2,0−1)	*W*-6	2.96	S-*B*	95	
	O-S3*B*(II,2,0−1)	*W*-6	2.79	S-*B*	102	
	O-6*A*(II,1,0−1)	*W*-6	2.92	C-6*A*	77	
9	O-6*A*(I,2,−10)	*W*-1	2.98	C-6*A*	91	O-6–*W*–2-S
	O-S1*B*(II,2,10)	*W*-1	3.07	S-*B*	151	
	W-3	*W*-1	2.80			
	W-5	*W*-1	2.75			
	W-8	*W*-1	2.81			
	W-9	*W*-1	2.63			
	W-11	*W*-1	2.84			
10	O-S2*A*(I,2)	*W*-2	3.04	S-*A*	145	4-S–*W*–2-S
	O-S1*B*(I,2)	*W*-2	2.94	S-*B*	136	
	O-S2*B*(III,1)	*W*-2	2.92	S-*B*	146	
	W-4	*W*-2	2.83			
11	O-S2*A*(III,2)	*W*-3	2.74	S-*A*	136	4-S–*W*–2-S
	O-S1*B*(II,1,0−1)	*W*-3	2.75	S-*B*	151	
	W-9	*W*-3	2.84			
	W-12	*W*-3	2.79			
12	O-S2*B*(II,2)	*W*-7	2.80	S-*B*	93	2-S–*W*–2-S
	O-S3*B*(II,2)	*W*-7	2.78	S-*B*	93	
	O-S1*B*(III,1)	*W*-7	2.57	S-*B*	145	
	W-6	*W*-7	2.77			
	W-8	*W*-7	2.90			
13	O-S2*A*(II,2)	*W*-8	3.04	S-*A*	141	4-S–*W*–2-S
	O-S1*B*(II,2)	*W*-8	2.82	S-*B*	98	
	O-S3*B*(II,2)	*W*-8	2.82	S-*B*	98	
	W-9	*W*-8	2.87			

Table 4
Attractive interactions among iota-carrageenan helices, calcium ions and water molecules

Interaction	Atom *X*	Atom *Y*	*X*···*Y* (Å)	Precursor P	*P*–*X*···*Y* (°)	Bridge
14	O-S2*A*(III,2)	*W*-9	2.81	S-*A*	127	4-S–*W*–2-S
	O-S1*B*(III,2)	*W*-9	2.72	S-*B*	124	
	O-S1*B*(II,1,−1−1)	*W*-9	2.61	S-*B*	130	
15	O-6*A*(I,1)	*W*-11	2.64	C-6*A*	131	O-6–*W*–4-S
	O-S*A*1(III,2)	*W*-11	2.61	S-*A*	116	
16	O-6*A*(II,2)	*W*-12	2.83	C-6*A*	107	
	W-10	*W*-12	2.78			

I, II and III, or 1 and 2, in parentheses after the atom name refer to the three helices in the unit cell, or the two chains in the helix; the third and fourth digits, if any, refer to *a*- and *b*-translations. In the last column, 2-S and 4-S indicate 2- and 4-sulfate groups, respectively.

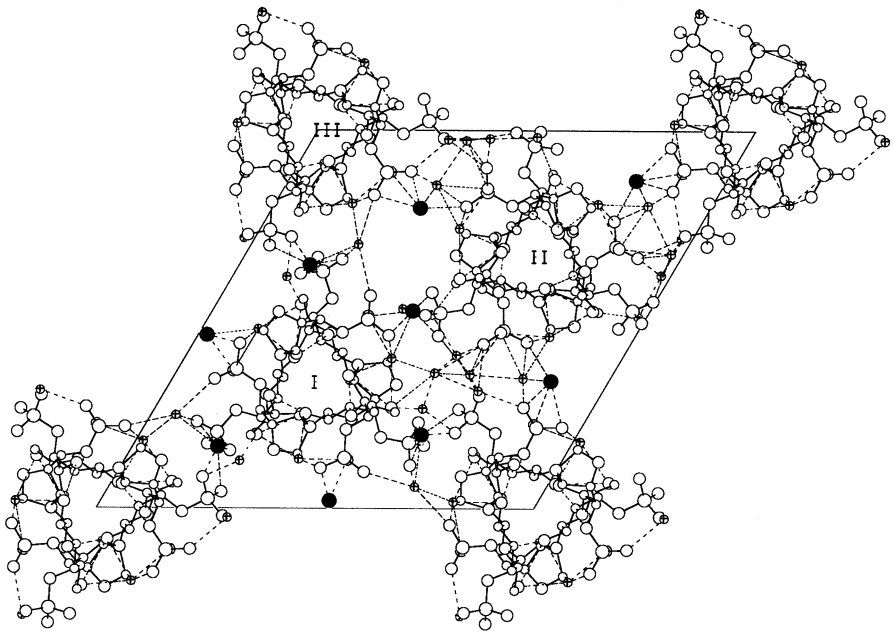
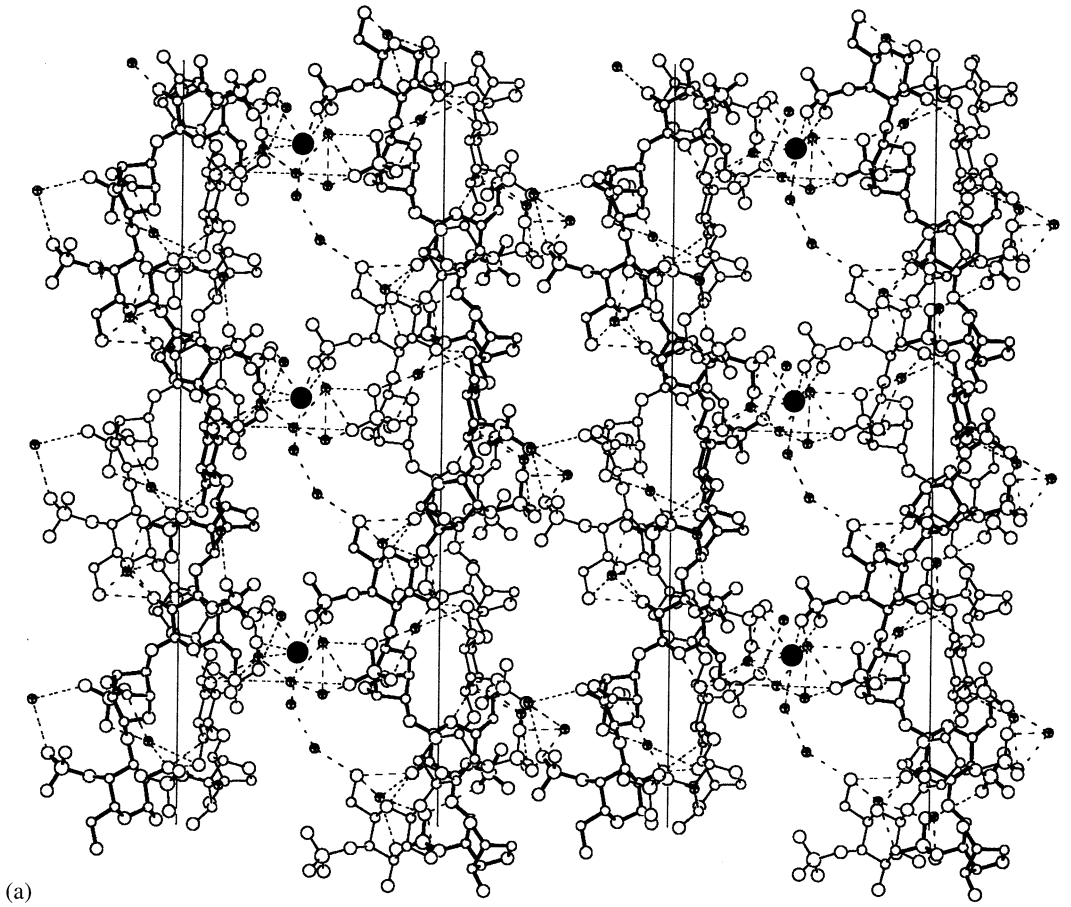


Fig. 3. A projection of the trigonal unit-cell down the *c*-axis. Helices I, II, and III are, respectively pointing up, down and up. Calcium ions (filled circle) and water molecules (crossed circle), connected to peripheral sulfate groups, are holding the helices tight.

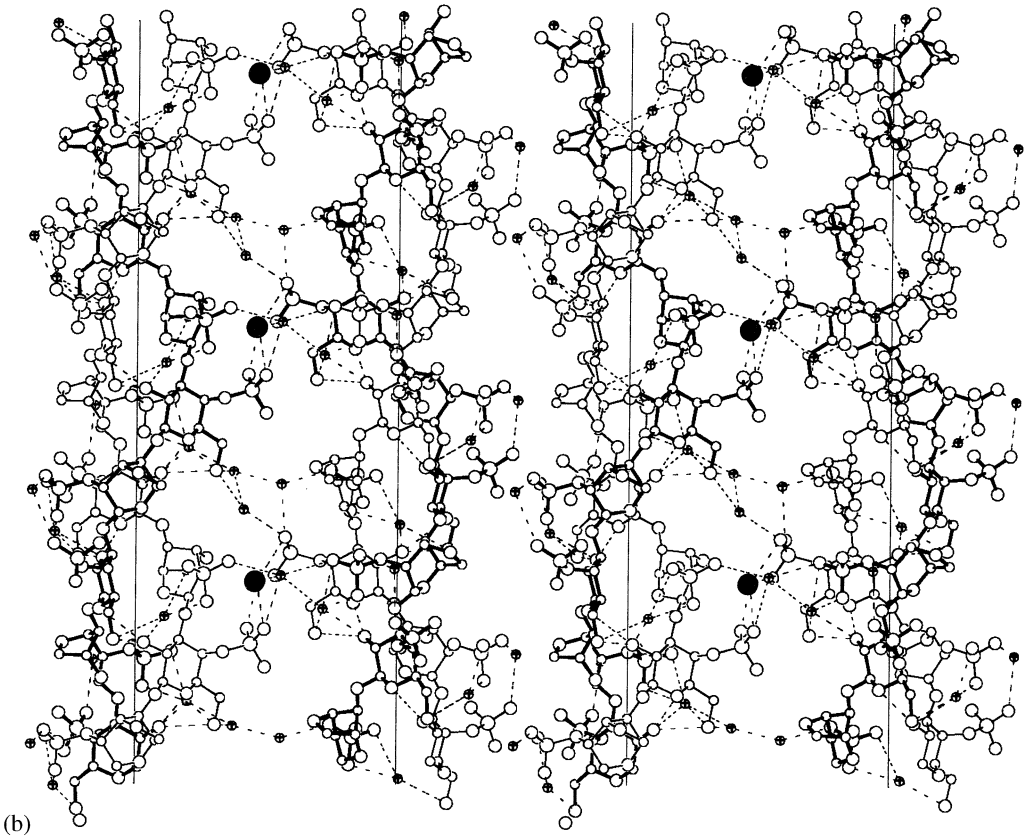
Bayley reported first the fiber-diffraction patterns of κ- and λ-carrageenans[15] and identified correctly the pitch of κ-carrageenan helix to be 25.2 Å. With mainly continuous intensity, these patterns were then not amenable for further investigation. Later, based on their good patterns from potassium κ- and ι-carrageenans, Anderson et al.[16] stated that three double helices of pitch 26 Å pass through a trigonal net of side 22.6 Å. Arnott et al.[7] subsequently described the morphology of calcium ι-carrageenan compatible with a statistical packing arrangement since their diffraction pattern was a composite of continuous and Bragg intensities. Our studies on the sodium[5] and calcium forms have yielded the precise structural details.

Interestingly, guest molecules (mono or divalent ions, and water molecules) induce polymorphism in certain anionic polysaccharides. For example, a twofold helix of chondroitin 4-sulfate with calcium ions[12] changes to a threefold helix in the presence of monovalent ions.[17] Three different allomorphs are possible for the sodium salt of dermatan sulfate.[18] As many as three distinct molecular structures in seven different packing arrangements exist for hyaluronan.[19] In contrast, the ι-carageenan double helix is conserved in both the sodium and calcium forms. With only small perturbations in the conformation angles (Table 3), the helix is not vulnerable to the surroundings. However, the cations and water molecules have specific roles (Table 4) in the aggregation of helices that in turn affect the bulk properties.

Rheological studies on ι-carrageenan gels suggest that calcium is more effective than monovalent ions



(a)



(b)

Fig. 4.

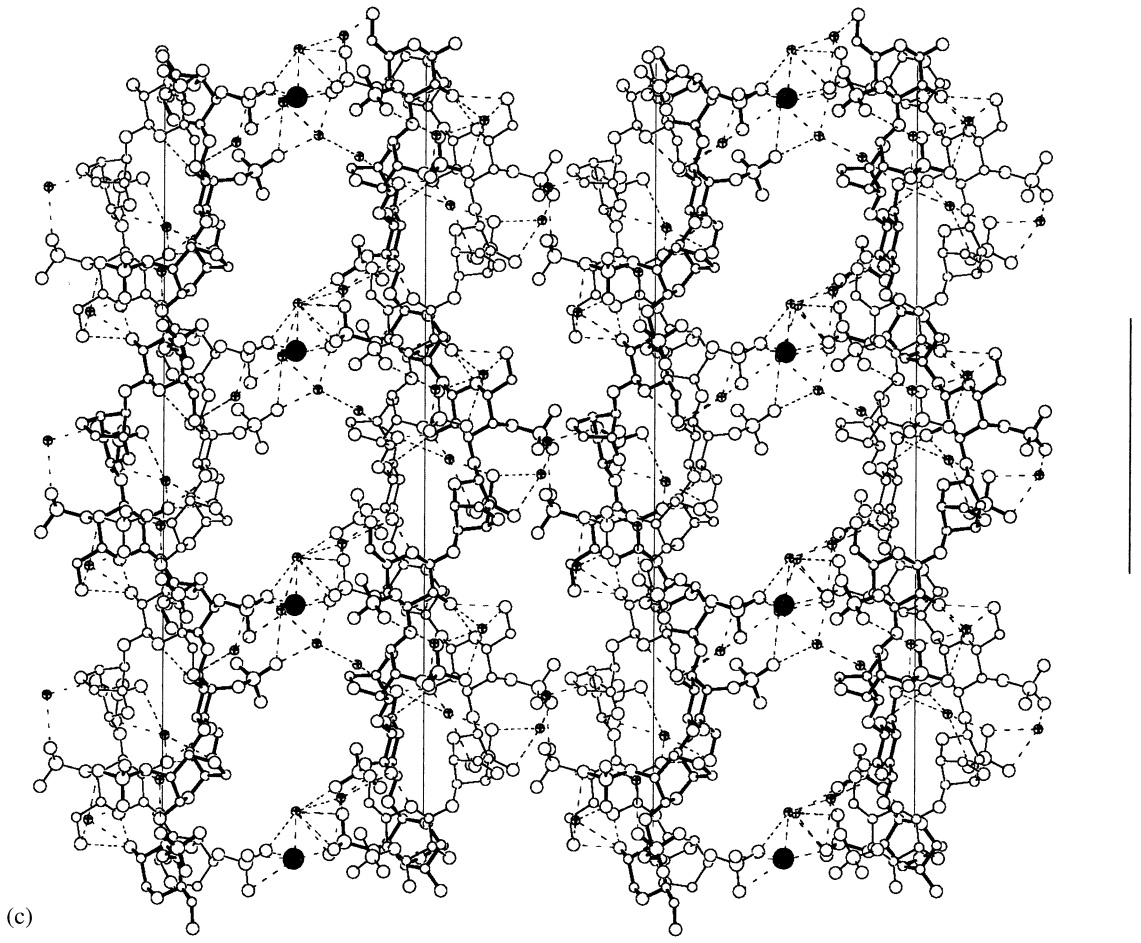


Fig. 4. Stereo drawings showing pairwise interactions between ι-carrageenan helices in the unit cell viewed normal to helix–helix separation (13.6 Å). Hydrogen bonds and coordination bonds for calcium ions are in dashed lines: (a) I (up) and II (down); (b) III (up) and II (down); and (c) I (up) and III (up). The height of the bar on the right is *c*.

such as potassium and sodium.[20,21] This difference is attributed to the binding nature of the cations to the polysaccharide and formation of junction zones. Although still debated, experimental findings support the involvement of both single and double helices in developing the junction zones. While computer modeling studies[22] are biased to single strands, small angle X-ray[23] and neutron[24] scattering, scanning electron microscopy[25] and order–disorder conformation and molecular-weight distribution results[26] lean to double helices. Interestingly, conformational property studies,[27] multiangle laser light-scattering experiments,[28] and non-contact atomic force microscopy[29] favor both types. Optical rotation changes of these polysaccharides are well explained from the double helical arrangement.[30] The osmotic coefficients for sodium and potassium ι-carrageenans are nearly equal and almost double that of the calcium form and specific optical rotation suggests that an ordered conformation for ι-carrageenan is thermally more stable with calcium than monovalent ions.[31] Our X-ray structural results confirm the double helical form in the solid state. However, in very dilute solution, the breakdown of interchain hydrogen bonds in the double helix might nucleate the dissociation into single strands.

Some effects of sodium and calcium ions on the aggregation of ι-carrageenan helices are evident from their unit cell dimensions. The trigonal net in the sodium form has a side of 24.0 Å, but it is 23.6 Å in the calcium form, and the lateral contraction is compensated by an increase in the fiber repeat (12.9 vs. 13.2 Å). As a result, the calcium ι-carrageenan helices come 0.3 Å closer than in the sodium form thereby promoting stronger inter-helical interactions. Although both forms have similar packing schemes, changes are inevitable. Specifically, relative to the sodium setting, helices II and III of the calcium form are rotated by $-20°$ each and displaced from the basal plane by -1.8 and -2.9 Å, respectively. We believe that these movements are responsible for producing noticeable differences in the

intensity distribution in their diffraction patterns. In this constellation, the peripheral sulfate groups from adjacent helices are face to face so that each pair is snugly connected by a calcium ion. In contrast to this –Ca– bridge, the inter-helix communication at the sulfate groups in the sodium form is via a –Na–, –Na–*W*–Na– or –Na–*W*–*W*–Na– bridge. Thus the calcium ion not only connects and balances the charge on two sulfate groups from different helices, it also brings the helices closer while the sodium ion is bound to one sulfate moiety merely for charge balancing. The cumulative effect of all these subtleties is that divalent ions promote stronger interactions between ι-carrageenan helices than are possible with monovalent ions. This inference is in full accord with other experimental observations.[21]

On the other hand, surprisingly, potassium κ-carrageenan gels are stronger than those of its divalent form, suggesting a reversal in the above trend. And λ-carrageenan does not form gels with any salts. It is easy to argue that the absence of 2-sulfate group in the κ-carrageenan primary structure and the presence of three sulfate groups per disaccharide repeat in λ-carrageenan, should have a major impact on their secondary, as well as tertiary structures. However, at this juncture nothing could be predicted due to the non-availability of accurate molecular details for these polymers.

Our structural results have given the details of how cations and water molecules promote the aggregation of ι-carrageenan double helices and support the 'domain' model of gels proposed over two decades ago.[32] As inferred from our crystal structure, in the presence of calcium ions, the smallest junction zone could encompass three helices in which each pair, at its sulfate groups, is glued directly by a calcium ion and reinforced with some water molecules. An important observation is that the average value (2.4 Å) of the shortest Ca···O bonds (one per sulfate group) is less than the corresponding Na···O distance (2.6 Å). This difference and the above direct ionic inter helix bridges at the sulfate groups may be responsible for the stronger junction zones with calcium than with sodium ions. In reality, many helices might associate by cation–water links that mimic the trigonal packing arrangement to develop a cohesive network. If kinks preclude structural regularity, junction zones of smaller size only will be formed. Rees et al.[33] estimated the optical activity coefficients for the potassium ι-carrageenan from stereochemical information and noticed agreement with the experimentally observed values. Modeling calculation[34] on optical rotation suggested a reduced chain extension for the gel-state than in the fiber samples. As the precise molecular details of the mono and divalent salt forms are available now, advanced theoretical models have to be developed for a better estimation and understanding of the physical properties of ι-carrageenan.

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References

1. Therkelsen, G. H. In *Carrageenan*; Whistler, R. L.; BeMiller, J. N., Eds. Industrial Gums. Polysaccharides and their Derivatives, 3rd ed.; Academic Press: New York, 1993; pp. 145–180.
2. Piculell, L. In *Gelling Carrageenans*; Stephen, A. M., Ed. Food Polysaccharides and their Applications; Marcel Dekker: New York, 1995; pp. 205–244.
3. Franz, G.; Pauper, D.; Alban, S. In *Pharmacological Activities of Sulfated Carbohydrate Polymers*; Paulsen, B. S., Ed. Bioactive Carbohydrate Polymers; Kluwer Academic: Boston, 2000; pp. 47–58.
4. Yamada, T.; Ogamo, A.; Saito, T.; Uchiyama, H.; Nakagawa, Y. *Carbohydr. Polym.* **2000**, *41*, 115–120.
5. Janaswamy, S.; Chandrasekaran, R. *Carbohydr. Res.* **2001**, *335*, 181–194.
6. Chandrasekaran, R.; Radha, A.; Lee, E. J.; Zhang, M. *Carbohydr. Polym.* **1994**, *25*, 235–243.
7. Arnott, S.; Scott, W. E.; Rees, D. A.; McNab, C. G. A. *J. Mol. Biol.* **1974**, *90*, 253–267.
8. Smith, P. J. C.; Arnott, S. *Acta Crystallogr., Sect. A* **1978**, *34*, 3–11.
9. Chandrasekaran, R.; Radha, A.; Okuyama, K. *Carbohydr. Res.* **1998**, *306*, 243–255.
10. Chandrasekaran, R.; Radha, A.; Lee, E. J. *Carbohydr. Res.* **1994**, *252*, 183–207.
11. Chandrasekaran, R.; Radha, A. *J. Biomol. Str. Dynam.* **1992**, *10*, 153–168.
12. Cael, J. J.; Winter, W. T.; Arnott, S. *J. Mol. Biol.* **1978**, *125*, 21–42.
13. Bezou, C.; Nonat, A.; Mutin, J.-C.; Christensen, A. N.; Lehmann, M. S. *J. Solid State Chem.* **1995**, *117*, 165–176.
14. Hagiya, K.; Kusuka, K.; Ohmasa, M.; Lishi, K. *Acta. Crystallogr., Sect. B* **2001**, *57*, 271–277.
15. Bayley, S. T. *Biochim. Biophys. Acta* **1955**, *17*, 194–205.
16. Anderson, N. S.; Campbell, J. W.; Harding, M. M.; Rees, D. A.; Samuel, J. W. B. *J. Mol. Biol.* **1969**, *45*, 85–99.
17. Millane, R. P.; Mitra, A. K.; Arnott, S. *J. Mol. Biol.* **1983**, *169*, 903–920.
18. Mitra, A. K.; Arnott, S.; Atkins, E. D. T.; Isaac, D. H. *J. Mol. Biol.* **1983**, *169*, 873–901.
19. Chandrasekaran, R. *Adv. Carbohydr. Chem. Biochem.* **1997**, *52*, 311–439.
20. Morris, V. J.; Belton, P. S. *J. Chem. Soc., Chem. Commun.* **1980**, 983–984.
21. Morris, V. J.; Belton, P. S. *Proc. Food. Nutr. Sci.* **1982**, *6*, 55–66.
22. Questel, J. L.; Cros, S.; Mackie, W.; Pérez, S. *Int. J. Biol. Macromol.* **1995**, *17*, 161–175.
23. Denef, B.; Mischenko, N.; Koch, M. H. J.; Reynaers, H. *Int. J. Biol. Macromol.* **1996**, *18*, 151–159.
24. Mischenko, N.; Denef, B.; Mortensen, K.; Reynaers, H. *Physica* **1997**, *B234–236*, 283–285.

25. Lee, I.; Atkins, E. D. T.; Miles, M. J. *Ultramicroscopy* **1992**, *42–44*, 1107–1112.
26. Hjerde, T.; Smidsrød, O.; Stokke, B. T.; Christensen, B. E. *Macromolecules* **1998**, *31*, 1842–1851.
27. Hjerde, T.; Smidsrød, O.; Christensen, B. E. *Biopolymers* **1999**, *49*, 71–80.
28. Viebke, C.; Borgström, J.; Piculell, L. *Carbohydr. Polym.* **1995**, *27*, 145–154.
29. McIntire, T. M.; Brant, D. A. *Int. J. Biol. Macromol.* **1999**, *26*, 303–310.
30. McKinnon, A. A.; Rees, D. A.; Williamson, F. B. *Chem. Commun.* **1969**, 701–702.
31. Rinaudo, M.; Karimian, A.; Milas, M. *Biopolymers* **1979**, *18*, 1673–1683.
32. Morris, E. R.; Rees, D. A.; Robinson, G. *J. Mol. Biol.* **1980**, *138*, 349–362.
33. Rees, D. A.; Scott, W. E.; Williamson, F. B. *Nature* **1970**, *227*, 390–392.
34. Schafer, S. E.; Stevens, E. S. *Carbohydr. Polym.* **1996**, *31*, 19–22.