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Native ordered structure of welan polysaccharide: conformational transitions and gel formation in aqueous dimethyl sulphoxide

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ABSTRACT

Welan, in aqueous solution, has "weak gel" properties analogous to those of ordered xanthan but, unlike xanthan, shows no evidence of conformational change between 0 and 100°C. When the polymer is dissolved in dimethyl sulphoxide (Me_2SO) rather than in water, however, there is a massive decrease in viscosity and total loss of gel-like character. In mixtures of the two solvents, the change in rheology occurs over a narrow range of composition (~ 85–90% v/v Me_2SO for 0.5% welan). On heating and cooling in a solvent close to the lower end of the critical range (86% Me_2SO), the polymer shows typical order–disorder and disorder–order transitions [as monitored by optical rotation, differential scanning calorimetry, and temperature-course of rheological change]. When solutions of disordered welan in Me_2SO are poured into excess water they form cohesive strings of gel. We interpret these results as showing that: (1) the stable conformation of welan in water is the double helix structure identified by X-ray fibre diffraction in the solid state; (2) in native welan, as biosynthesised, the strands are perfectly paired, and ordered along their full length; (3) on exposure to high concentrations of Me_2SO, the native structure is dissociated into disordered coils; (4) rapid renaturation from the disordered state gives shorter helices, with exchange of partners to form a stable cross-linked network.

INTRODUCTION

Welan (formerly known as S-130) is one of a family of structurally related bacterial polysaccharides developed by the Kelco Division of Merck & Co. Inc[1]. The parent member of the series is the linear anionic polysaccharide gellan, which has[2,3] a tetrasaccharide repeating sequence of: $\rightarrow$ 3)-β-D-Glc*p*-(1 $\rightarrow$ 4)-β-D-Glc*p*A-(1 $\rightarrow$ 4)-β-D-Glc*p*-(1 $\rightarrow$ 4)-α-L-Rha*p*-(1 $\rightarrow$. Gellan is biosynthesised with an L-glyceryl substituent on O-2 of the 3-linked glucose and, in at least a proportion of the repeat units, an acetyl group on O-6 of the same sugar[4], but both substituents are removed by alkaline hydrolysis in normal commercial extraction[5].

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Deacylated gellan forms thermally reversible gels with monovalent or divalent counterions[5,6], and the disorder–order and order–disorder transitions accompanying[7], respectively, gelation and melting, persist under non gelling conditions of polymer concentration or ionic environment[7–9]. The ordered structure has been identified by X-ray fibre diffraction analysis[10] as a co-axial double helix with the two strands staggered by 180°.

Other polysaccharides in the gellan series have the same polymer backbone, but with monosaccharide or disaccharide side-chains at different points of attachment to the repeating unit. In welan (which is produced by *Alcaligenes* ATCC 31555) the side-chain is a single sugar at O-3 of the 4-linked glucose[11]. The sugar may be either α-L-rhamnose or α-L-mannose, in the approximate ratio 2:1. At least 85% of the repeat units also have an acetyl substituent at O-2 of the 3-linked glucose[12] (the same position as the glyceryl group in native gellan).

In contrast to gellan, aqueous solutions of welan show no indication of gel formation or of conformational change[7,8] on heating and cooling between 0 and 100°C. One obvious interpretation of such behaviour would be that the polymer exists in solution as a disordered coil, as has indeed been suggested[8]. In the present work, however, we present direct evidence that the normal conformation is a stable ordered structure, by monitoring its dissociation and regeneration in response to changes in solvent environment and temperature.

MATERIALS AND METHODS

Welan (batch number 88074A) was kindly supplied by Kelco. Dimethyl sulphoxide (Me_2SO) was purchased from Sigma (minimum purity 99.5%). Solutions were prepared by mechanical stirring at ambient temperature. Samples for optical rotation were clarified by centrifugation (30 min at 15000*g*) and filtration (Whatman GF/F glass fibre filters; nominal pore size 0.7 μm). For determination of intrinsic viscosity, a stock solution of welan at ~ 0.7% w/v in Me_2SO was dialysed to equilibrium against twice its own volume of Me_2SO, and the dialysate was used for all subsequent dilutions.

Optical rotation was measured at 436 nm on a Perkin–Elmer 241 polarimeter, using a jacketed cell of path-length 10 cm. Temperature was controlled by a circulating water bath and measured using a thermocouple in the neck of the cell. Readings were taken after thermal equilibration at each temperature (typically 5 min). Differential scanning calorimetry (DSC) measurements were made using a Setaram microcalorimeter at a scan rate of 0.5 deg/min. Sample and reference cells were balanced to within 0.5 mg, using the same solvent (Me_2SO–water mixture) in both.

Dilute solutions were characterised on a Contraves Low-Shear 30 viscometer, using cup-and-bob geometry (inner and outer radii 5.5 and 6.5 mm). Measurements at higher viscosity were on a Sangamo Viscoelastic Analyser, using cone-and-plate geometry of diameter 50 mm and cone angle 2°. Rheological response to low-amplitude oscillatory shear was measured on a sensitive prototype rheometer designed and constructed by one of us (RKR), using 50 mm cone-and-plate geometry with cone angle 0.02 rad. Temperature dependence was monitored at a fixed heating or cooling rate of 1°/min, controlled by a circulating water bath and recorded by a thermocouple attached to the stationary element. The exposed periphery of the sample was coated with light silicone oil to minimise loss of solvent at high temperature and absorption of atmospheric moisture by Me_2SO (which is extremely hygroscopic).

RESULTS

Solvent-dependence of solution rheology.—Fig. 1 shows the frequency-dependence of rheological response for a 1% w/v solution of welan in distilled water at ambient temperature (300 K). The mechanical spectrum is similar to that of a gel[13,14]. Elastic response (storage modulus, G') exceeds viscous flow (loss modulus, G'') and both moduli show only slight dependence on frequency (ω). Complex dynamic viscosity $[\eta^* = G^*/\omega = (G'^2 + G''^2)^{1/2}/\omega]$ decreases steeply with increasing frequency, giving an essentially linear plot of log η^* vs. log ω, again similar to the behaviour of a gel network.

The steady-shear viscosity (η) of the same solution is lower (by a factor of ~ 1.5) than η^* at equivalent numerical values of shear-rate ($\dot{\gamma}/s^{-1}$) and frequency (ω/rad s^{-1}). This contrasts with the behaviour[13,14] of normal polymer solutions and other simple fluids, where $\eta(\dot{\gamma}) = \eta^*(\omega)$, a generality known as the Cox–Merz rule[15]. The form of shear-thinning (decrease in η with increasing $\dot{\gamma}$) is also different from that of normal solutions of conformationally disordered polysaccha-

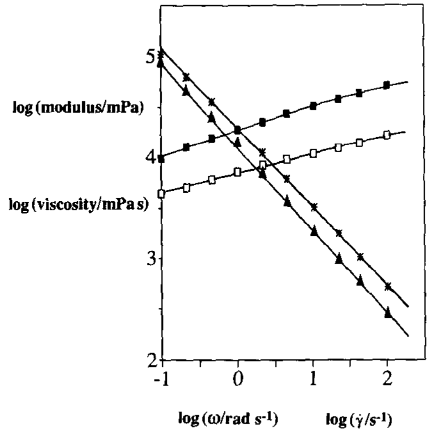

Fig. 1. Solution rheology of welan (1% w/v; 300 K) in water, showing the frequency (ω) dependence of G' (■), G'' (□), and η^* (*), measured at 2% strain, and the shear-rate ($\dot{\gamma}$) dependence of η (▲).

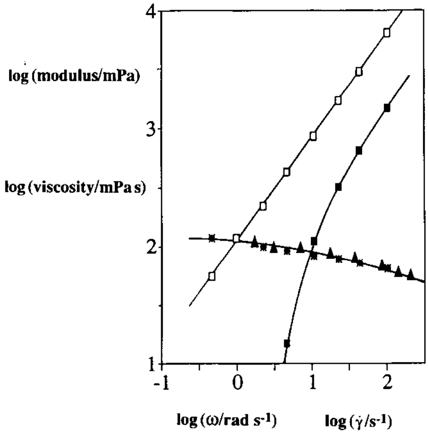

Fig. 2. Solution rheology of welan in Me_2SO; conditions and symbols as in Fig. 1.

ride coils[16]; log η varies linearly with log $\dot{\gamma}$ and shows no indication of any levelling-off towards a "Newtonian plateau" at low shear rate.

The rheology of welan in Me_2SO at the same concentration and temperature (Fig. 2) is entirely different. Normal liquid-like response now dominates, with $G'' \gg G'$ throughout the accessible frequency range, close Cox–Merz superposition of η and η^*, and a Newtonian plateau at low frequency and shear-rate. The greatest difference, however, is that the viscosity in Me_2SO is far lower than in water (by more than two orders of magnitude at 1 s^{-1}).

Fig. 3 shows the variation in viscosity with solvent composition for welan (0.5% w/v; 300 K) in mixtures of Me_2SO and water. The characteristic gel-like properties (Fig. 1) and associated high viscosity persist to a mixing ratio of about 5:1 Me_2SO–water, but on further reduction of water content the viscosity drops steeply towards the much lower value observed in pure Me_2SO. Most of the

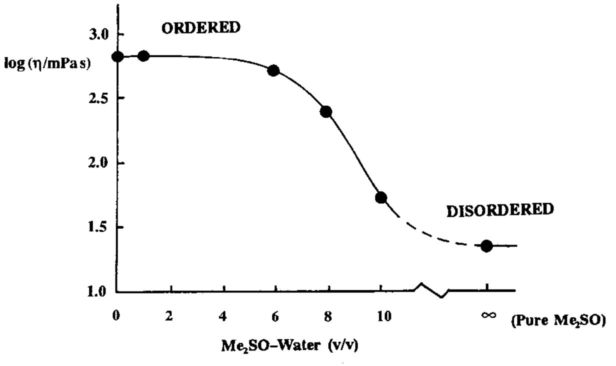

Fig. 3. Steady-shear viscosity (10 s^{-1}; 300 K) of welan (0.5% w/v) in water, Me_2SO, and mixtures of the two solvents.

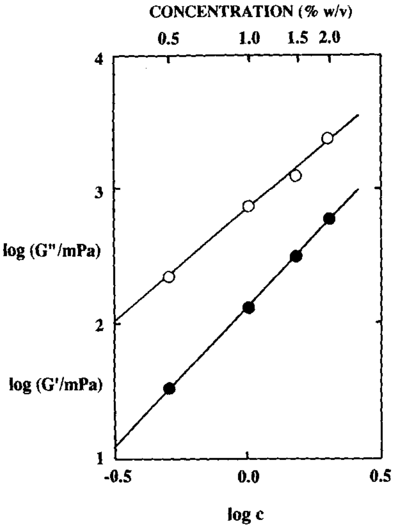

Fig. 4. Concentration-dependence of G' (●) and G'' (○) for welan in Me_2SO; measurements were made at 10 rad s^{-1}, 2% strain and 300 K.

change occurs between about 85 and 90% Me_2SO, so that the viscosity within this narrow range is critically sensitive to slight variations in solvent composition.

Controlled "renaturation".—Regeneration of gel-like character was achieved by controlled manipulation of solvent composition within the critical range. As an illustrative example of the procedure used, a solution of welan was prepared at 0.6% w/v in 10:1 mixture of Me_2SO–water. As shown in Fig. 3, this solvent gives behaviour close to that in pure Me_2SO. The solution was then mixed in equal proportions with an appropriate Me_2SO–water mixture, to give 0.3% w/v welan in 6:1 Me_2SO–water, which is close to the "pure water" end of the critical range of solvent composition (Fig. 3).

The time-course of recovery was monitored using small-deformation measurements of G' and G'' (at 10 rad s^{-1} and 2% strain). For comparison, moduli were also recorded for 0.3% welan in pure water. The corresponding values in pure Me_2SO were too low to be measured directly. However, G' and G'' at higher concentrations of welan (2.0, 1.5, 1.0, and 0.5% w/v) yielded linear plots of log modulus vs. log concentration (Fig. 4), allowing the values at 0.3% to be estimated by extrapolation.

The sample was loaded onto the rheometer immediately after mixing, and the first readings were obtained within 2 min. By this stage the moduli were already substantially higher (Fig. 5) than the values estimated for 0.3% welan in pure Me_2SO. Formation of an elastic network (by the criterion of $G' > G''$) occurred within 30 min. Measurements were continued for 3 h after mixing, by which time the moduli were virtually constant, and slightly higher than those observed for welan dissolved directly in water.

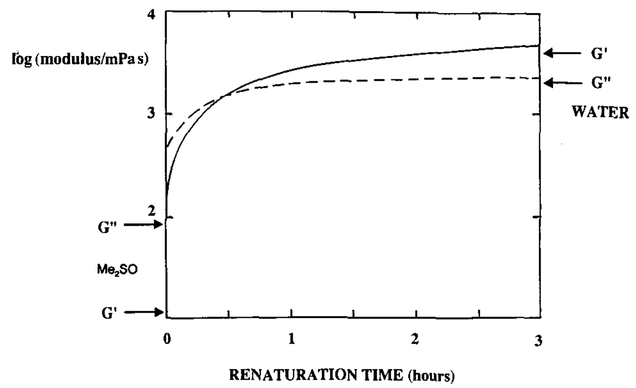

Fig. 5. Development of G' (———) and G'' (— — —) after a rapid change of solvent conditions from 10:1 to 6:1 Me_2SO–water. Measurements were made at 10 rad s^{-1}, 2% strain, and 300 K, at a final welan concentration of 0.3% w/v. The corresponding moduli for 0.3% welan in water and in Me_2SO are shown by horizontal arrows on, respectively, the right-hand and left-hand axes.

Analogous experiments at higher concentrations of welan (0.5 and 0.8% w/v) showed a progressive increase in the rate of network formation, and in the amount by which the final moduli exceeded those of the corresponding aqueous solutions (Fig. 6). The increase in rate with increasing concentration is demonstrated by the progressive convergence of moduli recorded 20 min and 3 h after changing the solvent environment. The ratio of the "3 h" value to the modulus obtained for the same concentration of welan in water is ~3.5 for the 0.8% solution, ~2.5 at 0.5%, and ~1.5 at 0.3%.

Rapid "renaturation".—On direct addition of water to solutions of welan in Me_2SO the polymer immediately formed a gelatinous precipitate. The converse experiment of pouring a solution in Me_2SO into a large excess of water gave

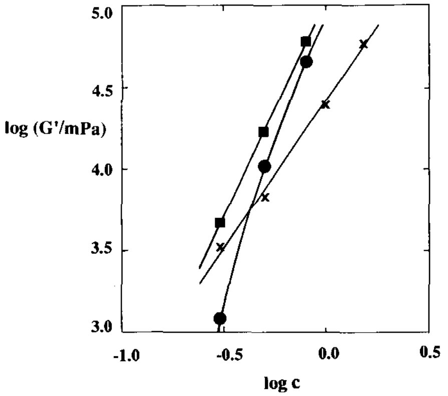

Fig. 6. Concentration-dependence of G' (10 rad s^{-1}; 2% strain; 300 K) for welan in water (+) and in 6:1 Me_2SO–water 20 min (●) and 3 h (■) after a rapid change in solvent environment from 10:1 Me_2SO–water. Polymer concentration (c) is expressed in % w/v.

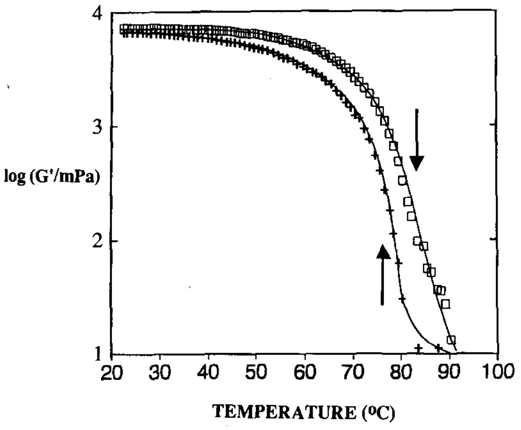

Fig. 7. Temperature-dependence of G' (1 rad s^{-1}; 2% strain) for welan (0.5% w/v in 86% Me_2SO) on heating (□) and cooling (+). Vertical arrows show the midpoint temperatures of the associated thermal transitions from DSC (Fig. 8).

cohesive strings of gel, which remained intact on prolonged soaking or washing with a further large excess of water. Since welan, although showing some gel-like character in aqueous solution, does not normally give "true" gels, this was a surprising observation, and its likely molecular origin is discussed later.

Thermal transitions in mixed solvent.—Having explored the effect of changes in solvent at fixed temperature (300 K), we next examined the effect of changing temperature under fixed solvent conditions. The solvent chosen was 86% v/v Me_2SO (~ 6:1 Me_2SO–water) which, at ambient temperature (Fig. 3), gives behaviour close to that in water.

As shown in Fig. 7, the gel-like response (rigidity modulus, G') of welan (0.5% w/v) in this solvent shows little change on heating to ~ 60°C, but at higher temperatures it drops sharply to the very much lower values typical of welan in Me_2SO. The change is fully reversible on cooling, but displaced to lower temperature (by ~ 5°C).

Melting and regeneration of the "weak gel" network are accompanied by, respectively, endothermic and exothermic transitions in DSC (Fig. 8). The magnitude of heat-change is the same, to within experimental error, on heating and cooling ($\Delta H \approx 3.5$ J/g), and the transition-midpoint temperatures (T_m values) correspond closely to the temperatures at which rheological change is greatest (Fig. 7).

The rheological and thermal transitions (Figs. 7 and 8) are also accompanied by sigmoidal changes in optical rotation (Fig. 9), indicative[17] of a co-operative change in chain conformation between low and high temperature. Indeed, the form of the optical rotation change is closely similar to that observed[18] for kappa carrageenan (K^+ salt form), which converts from a disordered coil conformation to a co-axial double helix on cooling, and reverts to the coil form at somewhat higher tempera-

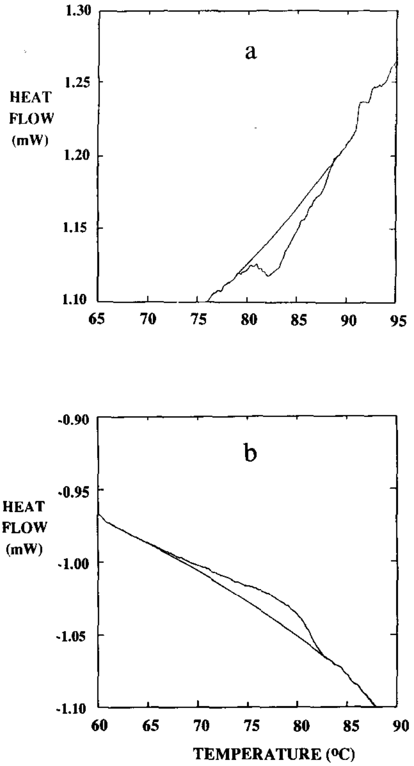

Fig. 8. DSC transitions for welan (0.5% w/v in 86% Me_2SO) on (a) heating and (b) cooling at a scan rate of 0.5°C/min. The interpolated baselines used in calculation of ΔH are shown.

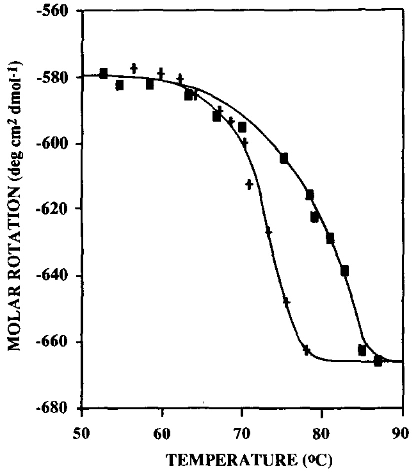

Fig. 9. Temperature-dependence of optical rotation (436 nm) for welan (0.5% w/v in 86% Me_2SO) on heating (■) and cooling (+).

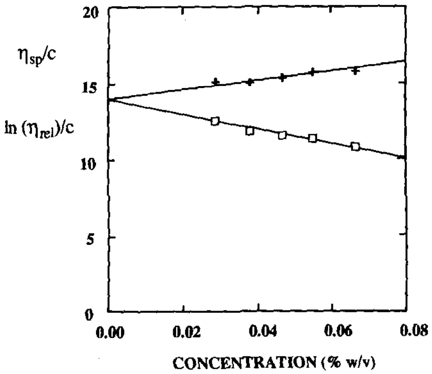

Fig. 10. Determination of the intrinsic viscosity of welan in Me_2SO (300 K) by combined Huggins (+) and Kraemer (□) extrapolation to zero concentration.

ture on heating, due to stabilisation of the ordered structure by cation-mediated helix–helix aggregation[19].

Intrinsic viscosity.—The intrinsic viscosity of welan in Me_2SO was obtained from measurements of relative viscosity in the approximate range $1.4 < \eta_{rel} < 2.0$ ($\eta_{rel} = \eta/\eta_s$ where η_s is the viscosity of the solvent). A stock solution was prepared at a relative viscosity just above the required range, and dialysed to equilibrium against pure Me_2SO. The dialysate was then used to dilute the stock solution to appropriate concentrations for viscosity measurements. Results were extrapolated to infinite dilution by combined use of Huggins and Kraemer plots (respectively η_{sp}/c vs. c and $\ln(\eta_{rel})/c$, where η_{sp} is the specific viscosity, $(\eta - \eta_s)/\eta_s = \eta_{rel} - 1$, and c is concentration)[14]. As shown in Fig. 10, the two plots gave a common intercept of $[\eta] \approx 14$ dl/g. By comparison, previous measurements of the same welan sample in aqueous solution yielded $[\eta] \approx 32$ dl/g, with virtually no change on varying ionic strength[7].

DISCUSSION

Rheological properties closely similar to those illustrated in Fig. 1 ($G' > G''$; $\eta^* > \eta$; linear variation of log η with log $\dot{\gamma}$) are well documented for solutions of another commercial microbial polysaccharide, xanthan[20]. Under most conditions of temperature and ionic environment, xanthan exists in solution in a rigid, ordered conformation[21], and its characteristic "weak gel" properties are attributed to side-by-side association of the ordered chains to give a tenuous intermolecular network[20].

The most direct evidence for the ordered structure of xanthan is that, at sufficiently low ionic strength, its dissociation and regeneration can be monitored as sharp co-operative processes when solutions of the polymer are heated and cooled[21]. Although no such transitions have previously been observed for welan, conformational order has been inferred from its "weak gel" rheology[7] and from the insensitivity of its intrinsic viscosity to salt[7,22] (in contrast to the behaviour of conformationally-disordered polyelectrolytes, whose coil dimensions collapse with increasing ionic strength, due to progressive shielding of intramolecular electrostatic repulsion[23]). The present investigation gives direct confirmation of these earlier conclusions by showing that, under appropriate solvent conditions, welan undergoes conformational transitions analogous to those of xanthan.

Recent studies by X-ray fibre diffraction analysis[24] have yielded a detailed and definitive description of the ordered structure of welan in the solid state. It is a co-axial double helix closely similar to that of gellan, but with the additional incorporation of sidechains within the ordered assembly. Our present results clearly indicate that this rigid structure persists under hydrated conditions and gives rise to the useful functional properties of welan in aqueous solution.

The extreme thermal stability of the welan helix in comparison to that of gellan can be attributed to the side-chain–main-chain interactions identified by X-ray. The ability of Me_2SO to disrupt the ordered structure is consistent with previous evidence that it acts as a better solvent than water for neutral polysaccharides such as amylose, pullulan, and dextran[25], and with its effectiveness in destabilising the uncharged triple helix of schizophyllan[26,27]. An additional factor that may be implicated in dissociation of the charged welan helix is the lower dielectric constant of Me_2SO ($\epsilon = 48$, in comparison with $\epsilon = 80.4$ for water), which will increase the electrostatic repulsion between ionised carboxyl groups on the two participating strands:

$$F = q_1 q_2 / \epsilon \epsilon_o r^2$$

where F is the force between charges q_1 and q_2 at separation r, and ϵ_o is absolute permittivity in vacuo (where $\epsilon = 1$).

The difference in intrinsic viscosity between native, ordered welan ($[\eta] \approx 32$ dl/g) and the disordered polymer in Me_2SO ($[\eta] \approx 14$ dl/g) is far less than might have been anticipated from the massive change in solution rheology at higher concentration (Figs. 1 and 2). This can be partially explained by the two-fold increase in the number of individual species present when double helices dissociate into separate coils, but the difference in net hydrodynamic volume is still surprisingly small. A likely interpretation is that the charged coils are highly expanded by intramolecular electrostatic repulsion[23]. Although the lower dielectric constant of Me_2SO will promote association of counterions to the welan polyanion, and may therefore reduce the overall charge-density of the polymer, it will also increase the repulsive forces between the carboxyl groups that remain dissociated, with consequent expansion of coil dimensions.

Aqueous solutions of welan show little change in their "weak gel" properties over wide ranges of ionic environment[7,8] indicating that, with water as solvent, there is little tendency to cation-mediated association of the individual helices. There is, however, substantial thermal hysteresis between the disorder–order and order–disorder transitions observed (Figs. 7–9) in 6:1 Me_2SO–water. It is generally accepted from studies of other gelling polysaccharides that hysteresis arises from helix–helix aggregation[19], which stabilises the individual helices to temperatures above those at which they will form in isolation on cooling from the disordered state. This evidence of enhanced aggregation in a solvent consisting predominantly of Me_2SO can again be explained by the lower dielectric constant, with attractive forces between the negatively-charged helices and their positively charged counterions being substantially stronger than in water. The ability of welan helices to form ordered assemblies with site-bound counterions is demonstrated in the preceding paper[24], by detailed analysis of X-ray diffraction from oriented fibres.

Finally, the most striking result from this investigation was the observation that when solutions of disordered welan in Me_2SO were poured into water they formed strings of gel, which remained intact on prolonged soaking in a large excess of water. It is clear from the above discussion that stable cross-linking in an aqueous environment cannot be attributed to helix–helix aggregation. Instead, we suggest that welan, as biosynthesised, consists of helical dimers with the participating chains fully ordered along their entire length (i.e., exact pairing of the individual strands). Denaturation in Me_2SO separates each strand into two disordered coils. Rapid "quenching" to the ordered form, by exposure to water, promotes multiple nucleation of helix structure within individual chains, leading to formation of a cross-linked network by exchange of chain-partners (Fig. 11). Species approximating to "perfect" dimers can be prepared by extensive sonication of ordered xanthan; a marked enhancement in "weak gel" properties when such preparations are heated to the disordered state and re-cooled has recently been interpreted in a closely analogous way[28].

Since the welan double-helix is highly stable in water, the gels produced by sudden exposure of the disordered polymer to an aqueous environment show corresponding stability. The formation of much weaker networks when re-ordering is induced more slowly, by altering the ratio of Me_2SO–water in a mixed-solvent system (Fig. 5), indicates formation of fewer, longer, helices, thus driving the

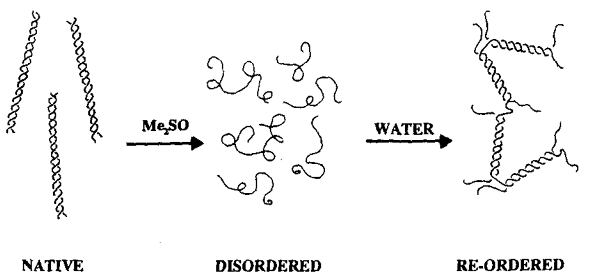

Fig. 11. Schematic representation of the proposed changes in conformation of welan on dissolving in Me_2SO and on mixing the resulting solution with excess water.

polymer back towards its initial, native form. It is possible, however, that the enhancement of G' observed (Figs. 5 and 6) after denaturation and re-ordering may reflect some limited crosslinking of chains by the mechanism outlined in Fig. 11, in addition to the cation-mediated aggregation discussed above.

If the proposal of exact chain-pairing in native welan is correct, it may well extend to other members of the gellan series and, indeed, to other biopolymers that are biosynthesised under conditions where their stable conformation is an intermolecular ordered structure.

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