Supercoiling in Circular Triple-Helical Polysaccharides

Superhelicity, or supercoiling, in covalently closed circular DNA duplexes is experimentally well documented.[1] We report here on the first experimental finding of supercoiling in a polysaccharide. Electron micrographs of the polysaccharide schizophyllan, obtained from samples for which the native linear triple helix had been denatured in dimethyl sulfoxide, by high temperature, or at high pH and subsequently renatured, all revealed circular and linear morphologies as well as larger, more complex clusters. In preparations of high molecular weight polymer (>750 × 10^3) renatured schizophyllan displayed circular species in both their relaxed and supercoiled forms. Circular species showing partial separation of the three single strands of the helix were also detected. The interwound twisting of circular triple helices and the local partial unwinding of the triple helix are modes of compensation for torsional stress arising from a linking number different from that of the relaxed circular topology.

Schizophyllan is a linear (1→3)-β-D-glucan with periodic (1→6)-glucosyl side groups.[2] It adopts a rigid triple-helical structure in aqueous solution.[3,4] Electron micrographs of sample M-2 reveal almost rodlike structures (Figure 1A). Chain-length heterogeneity differs from area to area for each replica. The chosen electron micrograph (Figure 1A) represents a typical example. Control replicas with no polymer sample show the same appearance as the background in Figure 1. The isolation procedure is reported[2] to yield schizophyllan samples with residual nitrogen less than 0.01%. This practically excludes the possibility that the visualized structures are contaminating DNA rather than schizophyllan. The linear mass density (M_L) was found to be 2080 ± 200 nm^{-1} from the ratio between the weight-average molecular weight (M_w) in aqueous solution (437 × 10^3) and the weight-average contour length (210 nm, 177 molecules) measured in electron micrographs similar to Figure 1A. This value of M_L is consistent with the proposed triple-helical structure of schizophyllan,[5] although no direct observation of helicity can be made from the micrographs.

The schizophyllan triple helix can be denatured, i.e., dissociated, by dissolving in dimethyl sulfoxide (DMSO),[3] by heating in aqueous solution above 135 °C,[6,7] or by dissolving in alkaline aqueous solution at NaOH concentrations greater than 0.24 M.[2] Renaturation of sample M-2 at polymer concentrations $c \leq 1.0$ mg/mL yields mixtures of linear, circular, and branched structures (Figure 1B–D); higher concentrations lead to increased proportions of multichain clusters (microgels). The electron micrographs selected for the present figures show representative variation in polymer topology, but note that

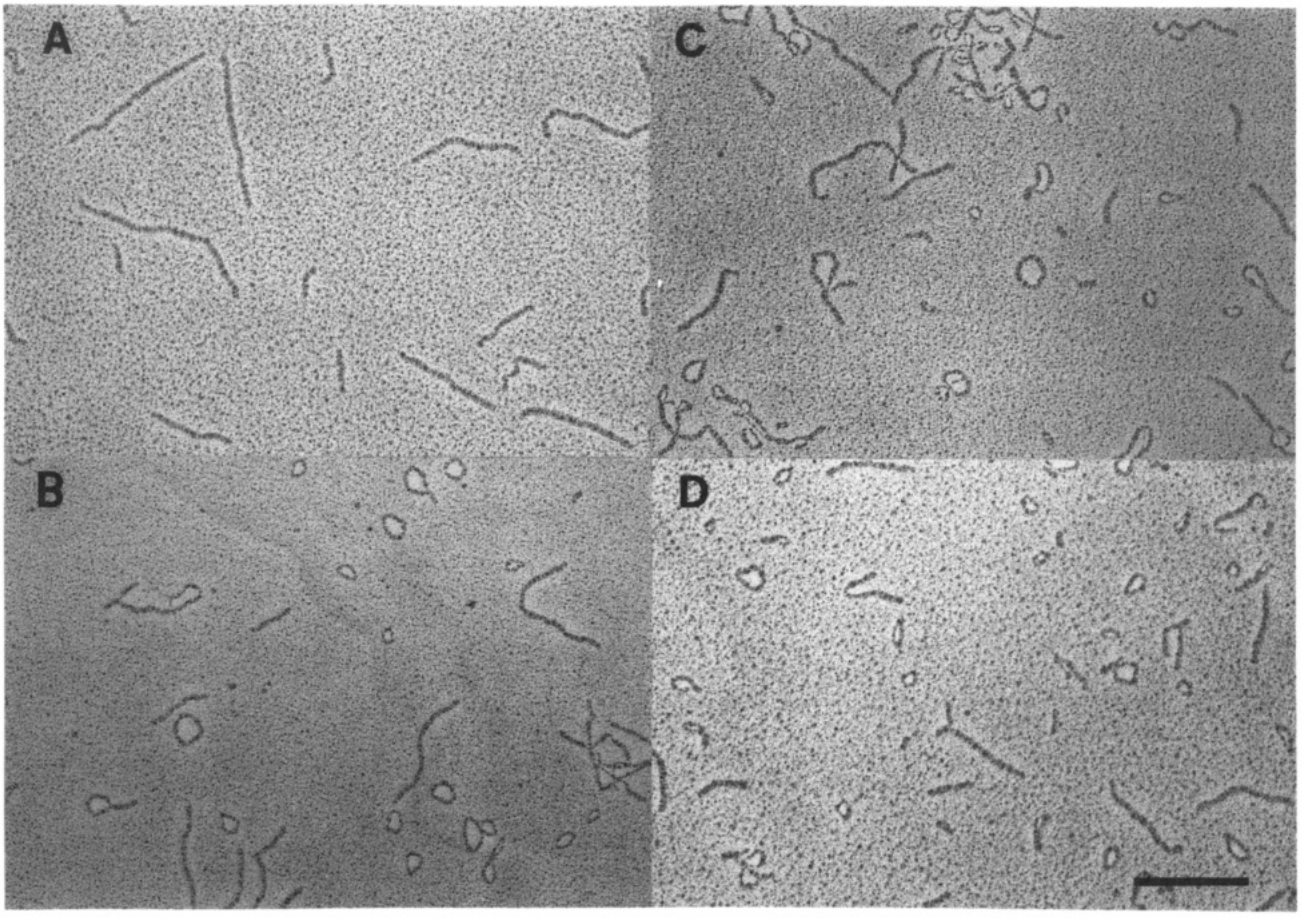

Figure 1. Electron micrographs of vacuum-dried aqueous solutions of schizophyllan M-2 prepared by (A) dissolution in pure water, (B) dissolution at c = 1.0 mg/mL in DMSO and subsequent renaturation by exhaustive dialysis against distilled water followed by annealing at 115–120 °C for 2 h and slow cooling to room temperature, (C) dissolution in 0.3 M NaOH with renaturation by neutralization with aqueous HCl, exhaustive dialysis against distilled water, and subsequent annealing at 115–120 °C for 2 h, and (D) heating of an aqueous solution to 150 °C with renaturation by slow cooling to room temperature. Schizopyllan sample M-2 was prepared by ultrasonic depolymerization and subsequent fractional precipitation of the native polymer material.[21] Replicas for electron microscopy were prepared by vacuum drying of small droplets of a 50% glycerol/water solution containing 5–15 μg/mL of schizophyllan on freshly cleaved mica. Dried specimens were first rotary shadowed with a 0.7-nm-thick film of 95%/5% platinum/carbon deposited at an angle of 6° and finally with a 7-nm-thick carbon support film deposited at an angle of 90°.[22] Scale bar = 200 nm.

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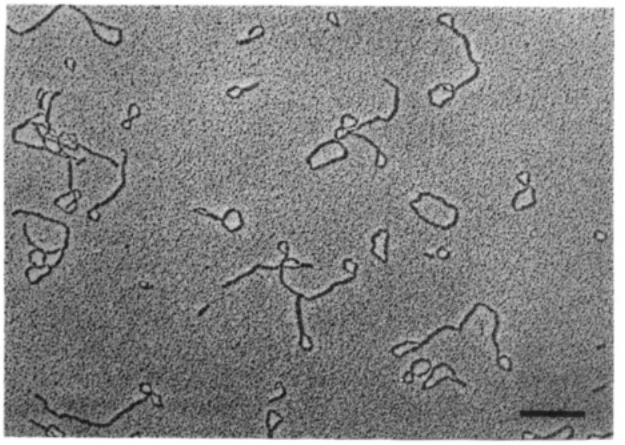

Figure 2. Electron micrograph of high molecular weight schizophyllan ($M_w = 1100 \times 10^3$) after renaturation/annealing at c = 0.4 mg/mL as in Figure 1B. The structural diversity ranges from purely linear and circular to twisted circular and more complex morphologies in larger clusters. Scale bar = 200 nm.

other areas of the replicas generally show different size and conformation distributions for the same types of species. Renaturation and annealing of sample U-1 ($M_w = 134 \times 10^3$) as in the preparation of Figure 1B yield only the reconstituted linear triple-helical species, as evidenced by electron microscopy and chromatography. Thus, circular renatured species appear to occur only for molecular weights above some critical value.

Thermodynamic equilibrium arguments[8] suggest that the energetic penalty, if any, associated with bending the triple helix into a circle is compensated by configurational degeneracy of the circles: Equienergetic triple-stranded circular structures reconstituted from homogeneous single strands exist for many possible relative locations of the chain ends along the circumference. Only one linear triple helix, completely in register, realizes the energetic benefit of full interchain valence saturation. The degeneracy of the circles, which modulates the probability of circle formation,[9] increases for the triple-stranded circles as the second power of the chain length. Further studies with longer schizophyllan chains were therefore undertaken.

High molecular weight schizophyllan ($M_w = 1100 \times 10^3$), renatured as for Figure 1B, displays macromolecular morphologies in addition to the homogeneous linear and circular triplex trimers expected at thermodynamic equilibrium.[8] In particular, structures with hairpin loops at both ends are observed (Figure 2). Similar structures were observed for 750×10^3, 1350×10^3, and 2350×10^3 schizophyllan samples. Careful examination of the latter species, utilizing a different shadowing angle that enable us to resolve more detailed features, revealed numerous examples of interwound (i.e., twisted) circular species. Figure 3 illustrates interwound circles with various twist numbers. Application of techniques previously described for analysis of polysaccharide stiffness from electron micrographs[10] suggests that the twisted structures are not easily rationalized in terms of deformation artifacts. We suggest, therefore, that they are superhelical or supercoiled circular structures arising from differences between the actual (α) and fully relaxed (α_0) linking numbers of the circular triplex. Here we generalize the concept of linking number commonly used in descriptions of duplex circules in DNA.[1]

The proposed occurrence of supercoiling in schizophyllan circular triple helices, which are not closed by the covalent linkage of chain ends, implies that constraints to relaxation of torsional stress in these species are kinetic rather than topological.[1] If a circular triplex is formed at

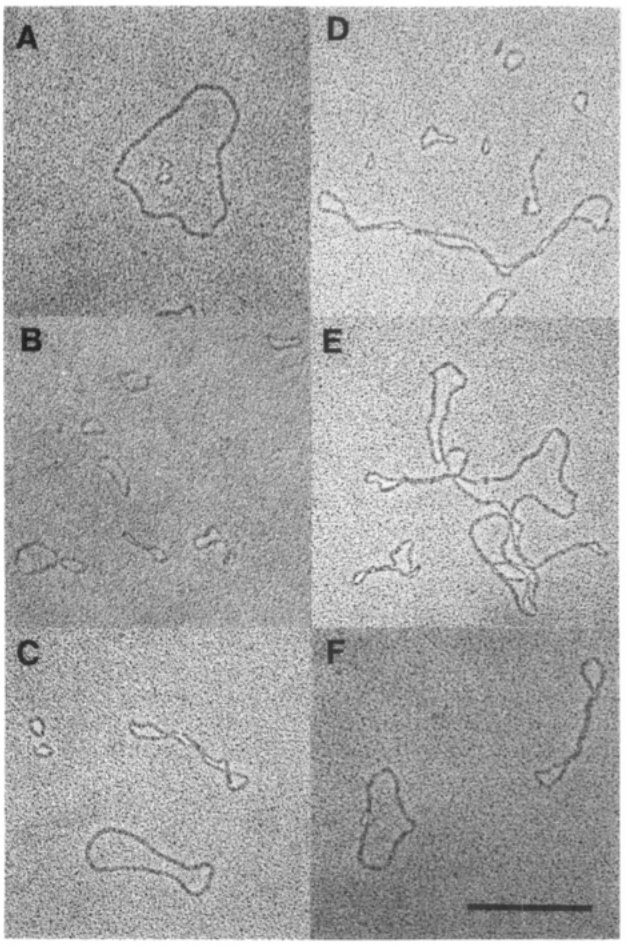

Figure 3. Electron micrographs of circular schizophyllan species differing in apparent supercoiling density. Sample preparation as in Figure 1B; preparation for electron microscopy as in Figure 1, except for shadowing to film thickness 0.7 nm with 95%/5% platinum/carbon from an angle of 9°. The micrographs show (A) an apparently fully relaxed circle, (B–E) interwound circles with various twist numbers, and (F) a circle with partial strand separation revealing three schizophyllan single strands. The electron micrographs were obtained on schizophyllan samples with $M_w = 750 \times 10^3$ (B), 1350×10^3 (E), and 2400×10^3 (A, C, D, and F). Scale bar = 200 nm.

or near the annealing temperature, we may assume the linking number α is that characteristic of the fully relaxed structure at that temperature. Subsequent cooling to room temperature may quench further relaxation to the corresponding linking number α_0, so that a finite linking number difference $\alpha - \alpha_0$ persists at room temperature despite the open character of the circular triplex. In DNA α_0 is known to be temperature dependent,[11] and a similar temperature dependence of α_0 for schizophyllan must be anticipated. The high temperature (>135 °C) required to disrupt the schizophyllan triple helix in aqueous solution betokens strong interchain interactions in the triplex. The activation energies associated with relaxation of triple-strand intertwining are expected to be correspondingly large, so that quenching of the relaxation process by cooling is entirely plausible. We cannot rule out the possibility that quenching of the relaxation process occurs, not upon cooling from the annealing temperature to room temperature but upon further evaporative cooling or exposure to increasing glycerol content during electron microscopic sample preparation.[10] X-ray fiber diffraction experiments on curdlan fibers suggest that curdlan changes from a 6-fold to a 7-fold triple-helical structure when subjected to an annealing procedure similar to the one used herein.[12] This evidence obtained on the unsubstituted (1→3)-β-D-glu-

can suggests that the helical pitch may depend on temperature for this class of polysaccharides.

The response of closed circular duplex DNA to a nonzero linking number difference is not restricted to supercoiling. Among other possibilities, disruption of short helical segments can occur.[1] Figure 3F shows a schizophyllan circle with a disrupted helical segment in which three single strands are well resolved. This micrograph provides direct evidence, previously lacking, that circular schizophyllan is indeed triple-stranded.

We are not currently aware of any biological significance for supercoiling in schizophyllan that might be analogous to its role in the packing of duplex DNA into nucleosomes. We have previously reported the circular morphology in lentinan,[8] a (1→3)-β-D-glucan of similar structure.[13] The latter polymer is of interest as a biological response modifier.[14-17] Both lentinan and schizophyllan currently have clinical applications in Japan for treatment of certain cancers.[18,19] The possible implications for lentinan and schizophyllan activity of higher order structure, including macrocyclization and superhelicity, remain at present an unresolved question. Direct evidence showing that supercoiling of other polysaccharides exists and is important for their function in vivo is lacking at present, but the finding that supercoils also exists among the polysaccharides opens up such a possibility. Interaction between low molecular weight substances and polysaccharides forming supercoils provides a potential mechanism for control of hydrodynamic volume that could be of importance, e.g., for bacterial expulsion of polysaccharides or for packaging of the polysaccharides of the extracellular matrix.[20]

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