The Effects of Deoxyribonucleic Acid Secondary Structure on Tertiary Structure

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The secondary structure of supercoiled DNA was varied by changes in ionic strength. For $I = 0.075$ – 0.4 the structure remained in the previously established branched form with only minor alterations in molecular dimensions. In $4M$ -NaCl, which induces linear DNA to change its secondary structure to the C structure and brings about an increase in the superhelix density of the molecule, no extra branches were observed on the molecules. The limiting factors that dictate supercoil structure seem to be the number and position of potential branch points and the proximity with which the two intertwining DNA strands can approach each other on the arms of the branches. This value is close to 10 nm under the conditions described, and is 14 – 15 nm at $I = 0.2$. It is suggested that such values should be borne in mind when models of chromosome structure are being constructed.

Although many secondary structures have been proposed for DNA (Bram, 1971), there remain only three well-established helical forms believed or assumed to exist in solution. The evidence for the induction of the A structure in solution (Brahms & Mommaerts, 1964) remains to be verified, but the fact that DNA–RNA hybrids adopt this helical form provides circumstantial evidence for its existence. The B structure is well established as the solution structure at low ionic strength. The C form of DNA has been shown to predominate under conditions of low water activity which occur in solutions of high ionic strength (Tunis-Schneider & Maestre, 1970), in organic solvents (Green & Mahler, 1971) and in complexes of DNA with various basic proteins such as occur in nucleohistones *in vivo* (Hanlon *et al.*, 1974; Gottesfeld *et al.*, 1974). Consequently it seems likely that at any moment a significant proportion of cellular DNA exists in a helical structure which is not the conventional B structure. A simplified summary of some possible structures is shown in Fig. 1.

In addition to variations in orderly helical structure it has become apparent that DNA can form open structures by localized transient unwinding, sometimes referred to as ‘breathing’. This type of variation in secondary structure may be monitored by ^3H exchange (McConnell & Von Hippel, 1970) or by reaction with formaldehyde, and is most apparent in solutions of low ionic strength. When some restraint on the DNA structure favours this type of unwinding, specific regions of the DNA molecule may undergo extensive unwinding and so become susceptible to attack by nucleases specific for single strands or by unwinding proteins (Monjardino & James, 1975; Beard *et al.*, 1973).

Fig. 1. Secondary-structure transitions for DNA in solution. Reactions 1, 2 and 3 are assumed to occur for all DNA molecules, whereas reaction 4 only occurs in molecules where there is some degree of reverse repetition so that a palindrome may form.

In small supercoiled DNA molecules, such strong restraints are imposed on their conformations by virtue of their lack of free ends and their consequent inability to unwind normally. Such DNA molecules have been shown to contain single-stranded areas not present in the corresponding open-circular DNA, and since such molecules are clearly more flexible at these points it has been suggested that in superhelical DNA molecules with branched structures these early-unwinding regions are located at the ends of the arms.

We have been studying the conformation of superhelical DNA molecules in solution and have established that a branched structure is formed at normal superhelix densities (Jolly & Campbell, 1972a; Campbell & Jolly, 1973). The number and length of these branches has been shown to depend on the primary structure (Campbell & Eason, 1975) and on the size of the molecule (Campbell, 1976), but the effect of changes in secondary structure on molecular conformation remained to be established. Using circular dichroism, we showed that whereas open-circular bacteriophage ϕ X174 replicative-form DNA will move smoothly into the C structure, taking on 37 more superhelical turns, the transition of the corresponding superhelical DNA into this conformation only involves about 15 additional superhelical turns (Campbell & Lochhead, 1971). This seemed to indicate some sort of 'limiting' tertiary conformation of the molecule which prevented it from taking up more superhelical turns. The experiments described here were undertaken to determine the nature of this limitation.

Materials and Methods

PM2 bacteriophage DNA was isolated by the method of Espejo & Canelo (1968) and bacteriophage ϕ X174 double-stranded intracellular DNA was isolated as described before (Jolly & Campbell, 1972a). The transition to C structure was induced by dialysis of the DNA into a buffer containing 6mM- Na_2HPO_4 /2mM- NaH_2PO_4 /1mM-disodium EDTA/4M-NaCl, pH 6.8 (4M-NaCl/BPES). The ϵ_p of the DNA in this buffer was found to be 7067 for bacteriophage ϕ X174 DNA and 7260 for bacteriophage PM2 DNA. All other buffers described contained the phosphate and EDTA and varied only in NaCl concentration.

Light-scattering experiments were performed with a helium or neon laser as light-source as described before (Campbell, 1976) and the appropriate corrections were made for the change in refractive index and refractive increment induced by the high-salt buffer. The refractive index was determined as 1.367 and the refractive increment, which was determined over a concentration range of 20–200 $\mu\text{g/ml}$ on a Bryce Phoenix differential refractometer by using the laser

as light-source, was $0.142 \pm 0.03 \text{ ml/g}$. For low-salt experiments the refractive index was 0.165 ml/g .

Some uncertainty remains as to the exact value of the calibration constant. The routine calibration of the instrument with Ludox colloidal silica from Du Pont Industrial Chemical Division, Wilmington, DE, U.S.A., cannot be performed at high ionic strength because the Ludox precipitates, and the calibration was therefore performed at a refractive index of 1.337. This difference is very small compared with the refractive-index difference in organic solvents and had no detectable effect on the measured molecular weights.

Computer-generated interference curves were obtained as described before (Campbell & Jolly, 1973; Campbell, 1976). In more recent work (Campbell & Eason, 1975; Campbell, 1976) it has been necessary to take account of some uncertainties about the degree of unwinding caused by ethidium bromide, and in the present paper we have used the value of 26° at physiological ionic strengths (Wang, 1974), to permit the exact calculation of the diameter of the arms of the branched supercoil. However, in high-salt solution the value for ethidium binding is almost certainly different. Wang (1969) pointed out that by using ethidium titration to measure the effect of salt on the winding of the double helix he obtained a much smaller value than did Tunis-Schneider & Maestre (1970) using circular dichroism. The reason for this may well be the co-operative effects of ethidium binding in high-salt solutions (Pohl *et al.*, 1972), which could have localized effects on the B-to-C structure transition. The value therefore used here for the number of superhelical turns in bacteriophage ϕ X174 DNA is 24 superhelical turns, which is the previous value (Jolly & Campbell, 1972a) corrected for the new angle. In addition 15 superhelical turns have been added to allow the 30% transition into the C structure. A variation of ± 3 superhelical turns on this value has virtually no effect on the conclusion of the present paper.

Results

Increase in branching

Upholt *et al.* (1971) have shown by electron microscopy that at high superhelix densities there is a considerable increase in the number of branches seen in the superhelical DNA. However, electron microscopy introduces many changes in molecular flexibility, as we have discussed before and may not give a true picture of solution structure (Campbell & Jolly, 1973). Nevertheless there is evidence to indicate that SV40 DNA has a site that becomes accessible to nucleases specific for single strands when I is lowered from 0.2 to 0.075 (Beard *et al.*, 1973), suggesting that a tertiary conformational change may take place. We have therefore studied the effect of changes in ionic

strength on the tertiary structure of SV40 DNA and PM2 bacteriophage DNA in the range from 0.075 to 0.4M. The Zimm plots were almost identical with those already published (Campbell & Eason, 1975; Campbell, 1976) over this range. Below I 0.4 there is very little C-structure transition, and the only expected variable is in the 'breathing' of the DNA, which is susceptible to salt-concentration changes whether or not the salt raises or lowers the T_m ('melting' temperature) (McConnell & Von Hippel, 1970). One possible reason for the lack of structural change is that reaction 4 in Fig. 1 occurs and that the change of ionic strength makes such regions more susceptible to single-strand nucleases (Beard *et al.*, 1973), even though it does not alter the basic three-dimensional structure. In general there is reason to believe that the ends of the supercoil arms contain palindromic regions rather than non-specific AT regions (see the Discussion section).

When the DNA was induced to move to its limiting structure by the use of 4M-NaCl, the molecule showed no change in the number of arms. The Zimm

Fig. 2. Zimm plot for bacteriophage ϕ X174 DNA in 4M-NaCl/BPES buffer

All extrapolations and calculations were as described in Campbell (1976). K represents the optical constant, c the concentration, and R_9 the intensity of scattered light corrected for viewing angle.

Fig. 3. Potential structures for bacteriophage ϕ X174 DNA in high salt and low salt

The value for low salt has changed from previous publications from 12 to 24 superhelical turns as discussed in the Materials and Methods section, to account for the new ethidium bromide unwinding angle. (a) 0.2M-NaCl; (b) 4M-NaCl; (c) 0.2M-NaCl, toroidal structure. A toroidal structure with 39 superhelical turns and a radius of gyration of 79 nm corresponding to the 4M-NaCl structure has a very much steeper curve, being off the scale at an abscissal value of 0.25. $P(\theta)^{-1}$ represents the reciprocal particle-scattering factor.

plot is shown in Fig. 2 and the curve fit in Fig. 3. The structure at $I=0.2$ is shown for comparison [corrected for the new ethidium bromide-unwinding value from the structure of Campbell & Eason (1975)]. The same conclusion was reached for bacteriophage PM2 DNA, which retained its four arms and gave a Zimm plot almost identical with that previously published (Campbell, 1976).

Bacteriophage PM2 DNA, being larger than bacteriophage ϕ X174 DNA and SV40 DNA, might well have been expected to have had more potential unwinding sites. We therefore conclude that an increase in superhelix density does not change the number of arms on these molecules and that they remain positionally specific over a wide range of conditions. We have previously shown that a decrease in the number of superhelical turns has no striking effect on the three-armed structure of bacteriophage

ϕ X174 DNA until the number of superhelical turns has fallen to a very low value (Campbell & Jolly, 1973). The lack of a change in branching in bacteriophage in PM2 DNA is not in fact very surprising, since simple topological consideration shows that if a new branch is to be formed, an old one must disappear, as the old site cannot remain at the end of a branch. The dimensions of the two molecules are shown in Table 1.

Unacceptable molecular condensation

It could be argued that the increase in the number of superhelical turns could in general make a molecule so compact that it was not structurally stable. However, the measured radius of gyration of bacteriophage ϕ X174 DNA was 79 nm and we have been able to induce looped toroidal structures with a radius of gyration of 60 nm, and it is therefore evident that such molecules can exist in highly condensed states. Any form of looped toroid with such a number of superhelical turns would, however, have a scattering curve totally different from that measured (Fig. 3). Clearly therefore, the molecule must remain in the

branched structure, in which further molecular condensation is unacceptable.

Topological requirements of the branched structure

The 'segment length', which is the length of one superhelical loop in the branched structure is calculated by eqn. (1):

$$\text{Segment length} = \sqrt{\left(\frac{L}{2N}\right)^2 - \left(\frac{\pi D}{2}\right)^2} \quad (1)$$

where L is the contour length, N the number of superhelical turns, and D the molecular diameter. It is clear that if L is fixed, an increase in N must result in a decrease in D . Bacteriophage ϕ X174 DNA in the C conformation should contain a full 60 superhelical turns, and if these were all incorporated into the structure the resultant molecule would look like Fig. 4(a), with a diameter of 8 nm or less and a segment length of 2 nm or less. For the 39 superhelical turns which the molecule actually contains, the minimum diameter is 13 nm, corresponding to a segment length also of 2 nm. This is shown in Fig. 4(b). However, the molecule does not take up this possible minimum diameter but assumes a diameter of 10 nm, and this gives rise to a very much less compact molecule with a measured radius of gyration of 79 nm. Two topological reasons could be advanced for the chosen

Table 1. Structure of bacteriophage ϕ X174 DNA and bacteriophage PM2 DNA in high and low salt

Note that bacteriophage PM2 DNA has a much smaller diameter, as it has a much higher natural superhelical density even in low-salt solutions. Thus although its size is only twice that of bacteriophage ϕ X174 DNA, it has 2.5 times the number of superhelical turns, and the number of superhelical turns for bacteriophage PM2 DNA in high salt has been obtained by doubling that of bacteriophage ϕ X174 DNA, as discussed in the Materials and Methods section and adjusting upwards so that an integral number of superhelical turns can remain in each arm without alteration of arm-length ratios. This means that it may have a high error. However, between the possible values of 73 and 83 superhelical turns the diameter and segment length compatible with the measured radius of gyration are in the range 9.6–10.5 nm. It is noteworthy that in low salt the diameters and segment sizes are not equal for bacteriophage ϕ X174 DNA.

	ϕ X174 DNA		PM2 DNA	
	Low salt	High salt	Low salt	High salt
Radius of gyration (nm)	95	79	125	123
Number of superhelical turns	24	39	60	80
Number of arms	3	3	4	4
Arm lengths (nm)	185	134	168	160
	185	134	168	160
	185	134	252	240
Segment length (nm)	23.1	10.3	14.0	10.0
Diameter (nm)	15.0	10.1	14.0	10.2

Fig. 4. Potential structures of bacteriophage ϕ X174 DNA undergoing the C-structure transition

(a) The most compact possible structure for a molecule which took on the full 60 superhelical turns. The diameter can be decreased. The molecule would have an R_g (radius of gyration) of 22 nm. (b) The most compact possible model for the structure which took on the correct value of 39 superhelical turns. Again the R_g is 22 nm, but the diameter is increased. (c) A possible structure for the molecule to assume if DNA curvature was the limiting factor. The diameter is 8 nm and the segment length 16 nm. (d) The correct structure for bacteriophage ϕ X174 DNA at its limiting position in 4M-NaCl. The diameter and segment length are equal.

diameter being 10nm. The first is that the bending energy required to make the more compact superhelix in Fig. 4 is too great and the second is that the proximity of the two DNA strands leads to too great an interaction-energy barrier. By extending the arms the DNA molecule is able to lessen bending. We have previously discussed the importance of bending energy in superhelix formation (Campbell & Jolly, 1973). The persistence length of DNA at this ionic strength has not been measured, but it will obviously be below the value of 41nm measured at $I = 0.2$ (Jolly & Campbell, 1972b). Even so a stretch of DNA about 36nm long will require to make a sharp curvature on each side of each segment.

If the bending energy were the determining force, it should be possible for the molecule to be extended as shown in Fig. 4(c) with a much smaller radius. Since this does not happen, we conclude that the proximity of the two DNA strands is the final determining factor and this view is supported by the fact that the diameter of the supercoil is exactly the same as the segment length, and the molecule therefore appears like that shown in Fig. 4(d) with a large number of almost completely symmetrical circles set in a line when viewed in only two dimensions. At no point are the two DNA strands any closer than 10nm from one another. This 'interaction energy' has always been assumed to be small (Davidson, 1972) under normal conditions, but is clearly one of the main determining factors in the present system.

Inspection of the data for bacteriophage PM2 DNA (Table 1) shows that its characteristics are similar to those for bacteriophage ϕ X174, with the interesting distinction that there is no contraction in the arms of bacteriophage PM2 DNA in high salt. At low salt concentrations, however, the segment length and diameter are equal at 14nm. This phenomenon only became apparent when the new ethidium bromide unwinding value was published (Wang, 1974) and suggests that for bacteriophage PM2 DNA, interaction energy is a major factor in the determination of supercoil structure, even at low ionic strength. There is a clear distinction between bacteriophage PM2 DNA and ϕ X174 DNA, which at low salt concentrations has a segment length much longer than its diameter. It should be noted that bacteriophage PM2 DNA is only twice the length of bacteriophage ϕ X174 DNA but that it has 2.5 times the number of superhelical turns and it has therefore a much higher natural superhelix density. This may well reflect the organism's unusual growth conditions (Espejo & Canello, 1968). The bacteriophage ϕ X174 DNA must have its structure determined by bending energy, as discussed before (Campbell & Jolly, 1973). The data suggest that whereas at high salt the two strands of DNA may be separated by as little as 10nm, at low salt the acceptable distance is 14 or 15nm.

From the above results it appears that when a small

superhelix is constrained to change its secondary structure to one with a tighter twist it may be prevented from doing so for the following reasons. (1) It contains a very small number of highly specialized sites which can be placed at the end of branches and that this number does not alter throughout a wide range of solution conditions. (2) The degree to which such sites unwind cannot be changed. (3) the extent to which a molecule can accommodate extra superhelical turns within its existing tertiary structure is limited by the proximity of the two DNA strands in the arms. The closest proximity acceptable to two DNA strands in high-salt buffer is 10nm and that in low-salt buffer is 14 or 15nm. Such values may be useful when models for chromosome packaging are constructed, since relatively exposed regions of double-stranded DNA are known to be present in the cell nucleus (Axel, 1975; Sollner-Webb & Felsenfeld, 1975).

Discussion

The experimental data reported in this paper have shown that the three-dimensional structure of the supercoil, which we have already shown to be unique for each molecule, is remarkably stable. This provides information that can be used to consider the regions at the ends of the arms. Until now we have not attempted to discriminate between the possibility of these regions being early unwinding regions because they were rich in AT base pairs or palindromes. However, the lack of variation in arm number over a wide range of ionic strength suggests that if they are simple early-denaturing regions they must have a very high relative density of AT base-pairs in comparison with the rest of the molecule. The alternative suggestion, that such areas have some degree of inverted repetition, has been reinforced by the discovery of three double-stranded regions in single-stranded bacteriophage ϕ X174 DNA (Bartok *et al.*, 1975). The fact that such sites are not perfect palindromes may naturally make them more susceptible to nucleases specific for single strands and also to reagents such as formaldehyde. However, there are two major differences between such experiments and the light-scattering technique. Chemical and enzymic reagents may act in a co-operative fashion, and thus once an unwinding protein has been bound to a site on the DNA it may hold the neighbouring base-pairs open for further binding. These techniques might therefore be expected to detect on average fewer sites than the light-scattering conformational analysis. The second difference is that for an almost perfect palindrome, its susceptibility to single-strand-specific chemical and enzymic agents may be very low and again fewer sites would be detected. In SV40 DNA, where we have found three sites and other methods have only detected two, the discrepancy is therefore easily

undersood. Bacteriophage Φ X174 DNA has undergone less extensive analysis and consequently comparisons are not possible. Controversy exists over the number and position of sites on bacteriophage PM2 DNA, both four (Brock *et al.*, 1975) and eight (Jacob *et al.*, 1974) have been suggested. Obviously a molecule with eight branches would be much more compact than bacteriophage PM2 superhelical DNA.

One of the underlying assumptions in the binding of chemicals or proteins to superhelical DNA has been that there will be some sort of special site only present on the superhelix which may be detected. However, it is clear that in the superhelical form of DNA processes such as opening out and forking are simply further potentiated. This has already been shown to be highly significant in terms of protein-DNA interactions, though its functional significance in the cell remains to be proven. The existence of many palindromic regions in eukaryotic chromatin suggests that such regions could be easily forked for the purposes of chromosome condensation, or indeed for more interesting functional reasons, if a slight superhelical torsion was induced by protein molecules. Suggestions that such structures may exist have been made (Ide *et al.*, 1975).

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