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Dehydrating agents sharply reduce curvature in DNAs containing A tracts

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ABSTRACT

The structural basis of DNA curvature remains elusive, because models for curvature based on crystallographic structures of molecules containing A tracts do not agree with any of the models for sequence-directed curvature based on solution studies. Here we demonstrate that the difference is probably due to MPD (2-methyl-2,4-pentanediol), the dehydrating agent commonly used in crystallography. One characteristic signature of curved DNA molecules is that they run anomalously slowly on polyacrylamide gels, appearing to be larger than they actually are. The gel anomalies of three curved DNAs from trypanosome kinetoplast minicircles drop monotonically with increasing MPD concentration, indicating that MPD straightens molecules that are curved in aqueous solution. This is not due to some non-specific effect of MPD on poly(dA) or polypurine tracts, because control molecules containing dA_{70} and dG_{43} run normally over the full range of MPD concentrations. Circular dichroism spectra are not affected by MPD, ruling out a conformational change to a structure outside the B-DNA family. The effect is not due to MPD-induced changes in phasing of the curved sequences, because MPD has virtually no effect on the linking numbers of relaxed plasmids containing either curved sequences or dA_{70}. At the concentrations of MPD used in X-ray crystallography, the curvature of DNAs containing A tracts is substantially lower than in solution, which probably explains the ongoing discrepancies between the crystallographic results and models based on solution studies.

INTRODUCTION

Sequence-directed DNA curvature was discovered in kinetoplast minicircle DNA from trypanosome mitochondria (1). The characteristic sequence of curved DNAs is of the form $(A_jX_k)_n$, where $j \approx 3$–7 and the sum of j and k must be ~ 10 or 11. A wide variety of studies have supported the original model of Marini *et al.* (1), that observable DNA curvature arises when small curves, kinks or junctions associated with the A tract are repeated in phase with the helix repeat (2–9). Hagerman (10) has provided an excellent review of this work.

Curved DNA molecules are characterized by anomalous migration rates on polyacrylamide gels. In fact, the original investigation (1) had been motivated by the earlier unexplained observation that some DNA molecules ran substantially more slowly on gels than marker DNAs of the same size (11,12). The degree of curvature is related to the R factor, $R = L_{app}/L_{true}$, where L_{app} and L_{true} are, respectively, the apparent and the true length of the molecule, measured in base pairs. Whereas random sequence DNA has an R factor of one, curved DNAs may have R factors as large as two or greater, depending on the extent of curvature, the location of curves within the molecule, the size of the molecule and the gel concentration.

The structural basis for curvature in DNAs containing A tracts remains an issue of contention. Solution studies produced two principal models for sequence-directed curvature. The original 'wedge model' (13,14) held that A tracts are curved and general sequence DNA is straight. The original 'junction model' (3,15) contended that both A tracts and general sequence DNA are essentially straight, but they differ in structure so that there are junctions between them and the deflection of the helix axis at these junctions leads to curvature. Differences between these models have narrowed in recent years, with the recognition that all base pair steps probably have non-zero roll angles; the primary distinction between these models today is a matter of definition of the helix axis and "far too much has been made of the essentially trivial differences between these two complementary ways of describing DNA curvature" (16). A third model came from theoretical studies (17) and the crystal structures of DNAs containing A tracts (18–21), which found that A tracts are not curved. These findings led to the 'curved general sequence model' (17,22–25), in which A tracts are straight, while curvature resides in non-A sequences.

This research was motivated by the suggestion of DiGabriele and Steitz (21) that differences between crystallographic and solution results might be due to the presence of dehydrating agents in the crystals. We hypothesized that A tracts might be curved in solution and that the dehydrating agent that is commonly used to grow crystals, 2-methyl-2,4-pentanediol (MPD), might cause the A tracts to straighten. If this is the case, increasing concentrations of MPD should reduce the observed gel anomaly for DNAs containing A tracts. We tested this hypothesis

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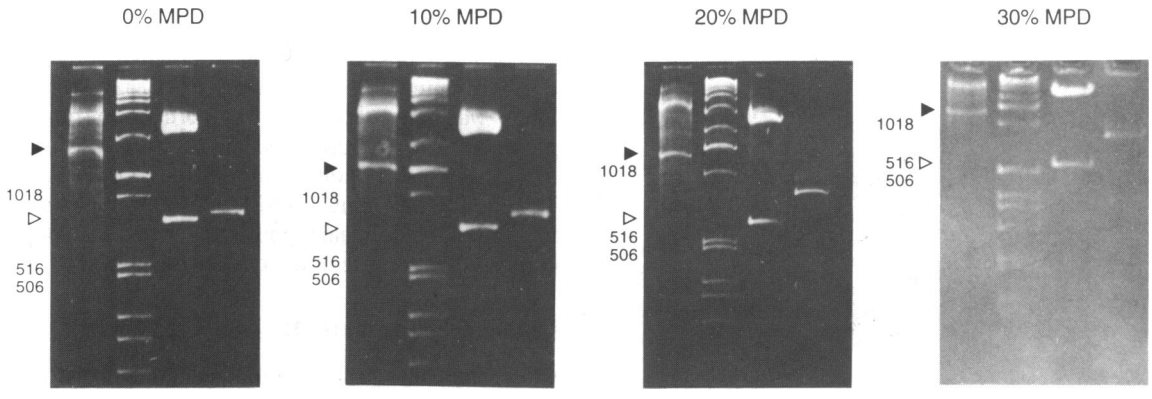

Figure 1. Gel electrophoretic mobilities of curved DNA fragments at various MPD concentrations. In each panel, the four lanes are, from left to right: the *Leishmania* fragment from pPE103 (L_{true} = 689 bp); the 1 kb size markers; the *Crithidia* fragment from pPK201/Cat (L_{true} = 219 bp); an 880 bp marker. The open and closed triangles at the left side of each panel mark the positions of the *Crithidia* and *Leishmania* fragments, respectively. For the *Leishmania* fragment, the reduction in anomalous mobility can be monitored most easily by comparison with the 1018 bp marker and the bright band just above it (1635 bp) in lane 2. Changes in mobility of the *Crithidia* fragment can be monitored by comparison with the 880 bp marker (lane 4) and the 506/516 doublet (lane 2). Note that the 506/516 doublet is not resolvable in the gel with 30% MPD.

in two well-known curved fragments from trypanosome kinetoplast minicircles, one from *Crithidia fasciculata* and the other from *Leishmania tarentolae*. In addition, we examined a curved 976 bp fragment from the kinetoplast minicircle of *Bodo caudatus*, a free-living, non-parasitic trypanosome.

MATERIALS AND METHODS

Chemicals and enzymes

Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and were used according to the manufacturer's instructions. Wheat germ topoisomerase I was obtained from Epicentre Technologies (Madison, WI). MPD was obtained from Eastman Kodak (New Haven, CT). Acrylamide and Bis (both ultrapure grade) were from Boehringer Mannheim (Indianapolis, IN).

DNA fragments

The construction and sequences of plasmids pPK201/Cat (containing a curved region from *C.fasciculata*) and pPE103 (containing a curved region from *L.tarentolae*) have been described elsewhere (1,2,7). These plasmids were generously provided by Paul Englund (Johns Hopkins University). Digestion of pPK201/Cat with *Bam*HI produced a 219 bp fragment with the original *Crithidia* 211 bp curved insert (7). Digestion of pPE103 with *Bam*HI and *Sal*I yielded a 689 bp fragment containing the original 414 bp curved sequence from *Leishmania* (1,2).

Kinetoplast DNA from *B.caudatus* was electrophoresed on 0.75% agarose and the 10 kb minicircle DNA was excised and electroeluted into a solution containing 40 mM Tris, 20 mM sodium acetate, 1 mM EDTA (pH 7.7). Gel-purified 10 kb circles were then digested with *Hae*III and the fragments separated on 1.5% agarose gels. Restriction fragments of 2.4, 0.8, 0.65 and 0.4 kb were purified and cloned into the plasmid Blue Scribe (Promega, Madison, WI). Migration of the fragments was compared on agarose and 6% polyacrylamide gels. The 2.4 kb fragment, pBC1, showed the highest degree of anomaly on polyacrylamide gels. Sequencing identified a 976 bp *Xho*I–*Dde*I restriction fragment consisting of a series of five nearly perfect successive 140 bp repeat sequences, separated by ~80 bp of divergent sequence. (The full 976 bp sequence has been submitted to the EMBL/GenBank Data Library under accession number L24561.) Curvature within this region was examined by gel electrophoresis, electron microscopy and molecular modeling (Milner *et al.*, to be published), all of which localize the curvature to the 47 bp consensus sequence AAAATTTRTGATTTT-GRCTATTTTTGGGGTAAAATRCRCTTATTTT (R = purine), which occurs in each of the 140 bp repeats. (The underlined regions identify the curved tracts that are closely phased with the helix repeat.) The 976 bp fragment was cloned into the polylinker of Blue Scribe to form pBC16, which was digested with *Eco*RI and *Hin*dIII, yielding a 1027 bp fragment that was used in the experiments described here.

To verify that the effects of MPD are specific for curved A tract DNAs and are not a non-specific effect associated with poly(dA) or polypurine tracts, two controls were tested. One of these contains the sequence dA_{70} in a 168 bp fragment from the plasmid pRW47 and the other contains the sequence dG_{43} in a 54 bp fragment from pRW39; these plasmids were kindly provided by Robert D. Wells (Institute of Biosciences, Texas A&M University). The preparation of these plasmids and the fragments of interest is described elsewhere (26).

Gel electrophoresis

Polyacrylamide gels consisted of 8% monomeric acrylamide and 0.27% Bis. MPD was included in both the gel matrix and running buffer at concentrations of 0, 10, 20 or 30% (v/v). Electrophoresis was performed at 100 V in 1× TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) using a Mini-Protean cell (BioRad, Melville, NY). The time required to resolve a particular fragment depended on both the size of the fragment and the concentration of MPD. It ranged from 2.5 h in 0% MPD for the pPK201 insert to 17 h in 30% MPD for the pBC16 insert. Gels were stained with ethidium bromide, destained for several hours and photographed under UV illumination with Polaroid type 667 film.

Markers for molecular size (1 kb ladder) were purchased from Gibco-BRL (Gaithersburg, MD). In addition, a mixture of several

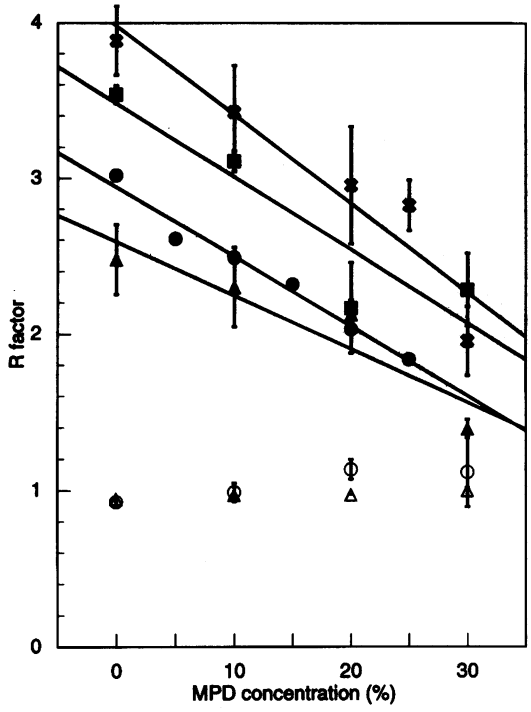

Figure 2. *R* factors for various curved and control DNA fragments as a function of MPD concentration. *R* factors were measured in a series of polyacrylamide gels similar to those shown in Figure 1. Each data point is the mean of two to four measurements made under identical conditions. Error bars correspond to ± 1 standard error. The curved fragments analyzed are: crosses, 219 bp from *Crithidia* pPK201/Cat; closed squares, 1027 bp from *Bodo* pBC16; closed triangles, 689 bp from *Leishmania* pPE103. Controls are: open triangles, dA_{70} in a 168 bp fragment from pRW47; open circles, dG_{43} in a 54 bp fragment from pRW39. Also shown (closed circles) are the *Leishmania* data in ethanol (2) for the 414 bp subfragment from pPE103. Note that this subfragment is smaller than our pPE103 fragment and that no error bars were provided in the original paper. Linear regression of *R* factor versus MPD concentration gives the four lines shown. These are, from top to bottom, *Crithidia*, *Bodo*, *Leishmania* in ethanol (2) and *Leishmania* in MPD (our data).

purified pBR322/*Hae*III restriction fragments was used as markers in the very low molecular weight range.

Apparent size determinations

To determine the apparent length (L_{app}) of each DNA fragment under the various gel conditions, subsets of the 1 kb marker bands, run on the same gel, were used as standards. The migration of four to five marker bands closest to the curved fragment was used to establish the parameters of a best fit exponential calibration curve. L_{app} was then calculated by inserting the observed mobility of the fragment under investigation into the equation. This procedure was necessary because the relative migrations of the marker bands are not constant, but vary slightly with MPD concentration (Fig. 1). We examined alternative equations for deriving calibration curves from the positions of the marker bands and we examined the effects of using different numbers of marker bands in the calibration procedure, but neither of these affected the fundamental conclusions drawn from the experiments. *R* factors were calculated according to $R = L_{app}/L_{true}$, where L_{true} is the true length of the molecule. Both L_{app} and L_{true} are measured in base pairs.

Linking number assays

To quantitate possible DNA helix unwinding by MPD, we used a standard linking number assay (27,28). Supercoiled plasmid DNA was relaxed with wheat germ topoisomerase I in the presence of increasing concentrations of MPD. The resulting topoisomer distributions were resolved in 1.2% agarose gels (1 × TBE buffer, including 0.5 μM chloroquine, 58 V, 41 h) and the migration of individual topoisomers within each population were compared with those of the other populations.

Circular dichroism (CD)

CD spectra in 1 × TBE buffer containing 0–40% MPD were measured with a Jasco J500A spectropolarimeter. Molar ellipticities Δε were calculated using published extinction coefficients (29).

RESULTS

As far as we are aware, there has been only one previously published measurement of the effect of dehydrating agents on the gel mobility of a DNA containing curved A tracts. Marini *et al.* (2) showed that ethanol reduces the anomalous mobility of a 414 bp fragment from *Leishmania*. They also found that the CD spectrum of this same fragment is unaffected by ethanol concentrations up to 44%, indicating that the molecule remains in the general conformation of B-DNA, so the reduced anomaly cannot be attributed to conversion to a non-B conformation.

Since MPD is the dehydrating agent commonly used in DNA crystallography, we carried out a series of experiments on different curved sequences at different concentrations of MPD. As shown in Figures 1 and 2, MPD causes a notable reduction in the gel anomalies of curved fragments from *Crithidia*, *Leishmania* and *Bodo* and the effect is comparable with that of the Marini ethanol experiment. The *Bodo* fragment was examined because its curved sequence is unusual in two ways. First, it contains five separate curved 47 bp repeats that are separated by uncurved sequences, each ~170 bp long. Secondly, some of the successive A tracts in the 47 bp consensus sequence have reversed polarity; where *Leishmania* and *Crithidia* have repeat sequences resembling $(A_5N_5A_5N_5)_n$, the *Bodo* sequence is of the form $(A_5N_5T_5N_5)_n$. Evidently, sequences with repeated A tracts and fragments with successive A tracts of reversed polarity both respond to MPD in the same way. Further, the effects of MPD on the gel anomalies is not particularly sensitive to the size of the total fragment or to the arrangement of curved tracts within the fragment.

Could the effect of MPD on gel anomalies be the consequence of some non-specific effect? To investigate this, we determined the CD spectra of the *Bodo* fragment and of $dA_n \cdot dT_n$. No major spectral changes occur in MPD up to concentrations of 40% (Fig. 3), so MPD does not cause a transition to the A-DNA conformation. This is not unexpected, since the same thing was observed for the *Leishmania* fragment in ethanol (2) and molecules containing A tracts crystallize in the B-form at 30–45% MPD (18–21). We can also rule out a non-specific effect on A tracts or polypurine tracts, because the fragments containing dA_{70} and dG_{43} both run normally at MPD concentrations up to 30% (Fig. 2).

The reduced gel anomalies could, in principle, be due to MPD-induced changes in the helical repeat of the DNA. This possibility required a close examination, because of experiments

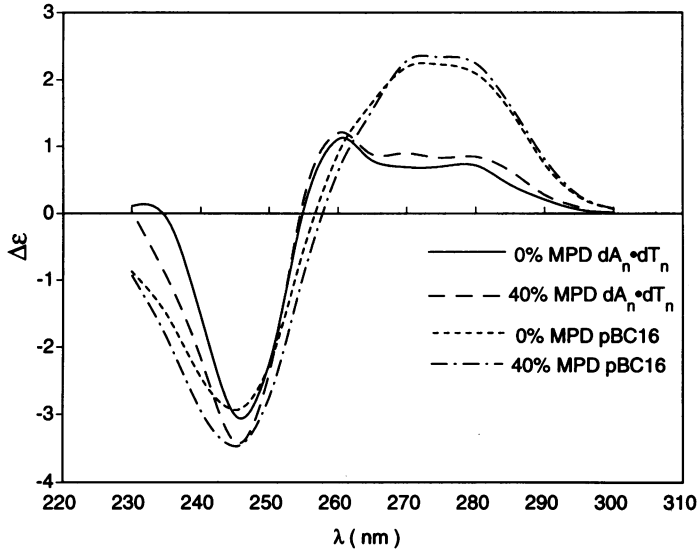

Figure 3. Circular dichroism spectra of $dA_n \cdot dT_n$ polymer at 0% MPD (———) and 40% MPD (— —) and plasmid pBC16 at 0% MPD (– – – –) and 40% MPD (– — –).

carried out by Dan Zimmer and Don Crothers (personal communication). They investigated the effects of MPD on gel mobilities using the ligated multimer assay, with a monomer repeat of 21 bp. In 20–30% MPD, multimers up to 84 bp do show reduced gel anomalies, in agreement with our result. For multimers longer than 132 bp, however, there is actually some increase in the *R* factor. They suggested that their result might have been due to a reduction in curvature, coupled with improved phasing due to changes in helical repeat. We tested this idea by examining the effects of MPD on the linking number of relaxed plasmids. Plasmids were treated with topoisomerase I in the presence of increasing amounts of MPD. Figure 4 shows the data for pPK201/Cat containing a highly curved sequence; identical results were obtained for pBR322 and for a plasmid containing the dA_{70} tract. For all DNAs tested, there were no measureable changes in either the positions of the topoisomer populations or the relative intensities of individual topoisomer bands within each population. This indicates that the centers of the distributions, and thus the helix repeat, are unaffected by MPD up to 10%. At higher MPD concentrations the enzyme loses its activity. Note that this is a very sensitive assay, since a difference as small as 0.3 supercoils will produce a clearly measureable shift in band intensities. In a plasmid as large as pBR322 (4363 bp), this means that 10% MPD causes a change in twist angle of <0.3°/helical turn at 10% MPD. Thus changes in phasing are not responsible for the reduction of gel anomalies by MPD, at least at low concentrations of MPD.

We conclude that the MPD-induced reduction of gel anomalies for DNAs containing A tracts is specifically due to reduced curvature of these molecules.

DISCUSSION

Solution studies and crystallography have produced quite different viewpoints about the origins of DNA curvature in molecules containing A tracts.

The point of view from solution studies has been elaborated primarily by Ed Trifonov and Don Crothers. In its current form, Trifonov's wedge model is based on solution measurements of the helical twist angles (30) and the direction and magnitude of wedge angles (31) for all possible base pair steps. In this model A tracts curve toward the minor groove. Crothers' junction model (3,15) was originally based on the many observations that $dA_n \cdot dT_n$ has a unique structure that is different from that of random sequence B-DNA (32). It held that curvature arises from deviations of the direction of the helix axis at junctions between the A tracts and the non-A tracts when these are repeated in phase with the twist of the double helix. The wedge and junction models have both evolved over time and the only difference between them now is how one chooses to define the helix axis (16). Most important for our discussion, both models are based on all the available solution data and they agree on the direction of

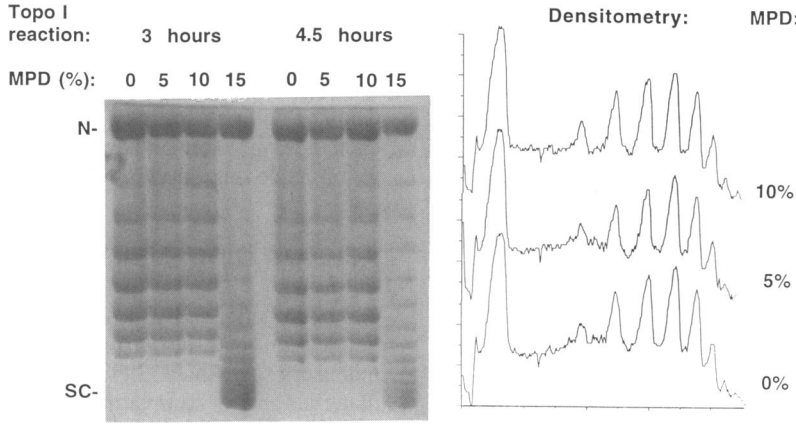

Figure 4. Topological assay to determine the effects of MPD on the DNA helix repeat. Plasmid pPK201 was relaxed with topoisomerase I in the presence of increasing amounts of MPD. Reactions were carried out for either 3 or 4.5 h, to ascertain that equilibrium had been reached. (Left panel) Separation of topoisomer products in a 1.2% agarose gel in the presence of 0.5 μM chloroquine. N, nicked circular DNA; SC, native supercoiled DNA. At 15% MPD, the topoisomerase enzyme has lost much of its activity, resulting in incomplete relaxation of the DNA; note that the topoisomers in the 15% lane are thus still negatively supercoiled, while in the other lanes they are positively supercoiled, due to the intercalation of chloroquine in the buffer and in the gel. (Right panel) Densitometric tracings of the 4.5 h set of lanes for 0, 5 and 10% MPD.

curvature, which is toward the minor groove at the center of the A tract. Independent analysis of the experimental data by De Santis *et al.* (33) supports this result.

The second viewpoint comes from X-ray crystallography, which ideally should be able to explain the origin of curvature in molecules containing A tracts. Several such molecules have been crystallized and analyzed (18,20,21). In all of these the A tracts are straight and uncurved. Similar results occur in crystals containing AATT (34,35) and AAATTT sequences (19); these sequences are also known to generate curves in solution when repeated in phase with the helix repeat (36). Curves are found outside the A tracts, but the patterns are so irregular that crystallographers have not been able to agree on a set of sequence–structure rules for non-A tracts, nor about the origin of curvature. There are ongoing debates about the extent to which crystal packing effects are responsible for the observed patterns. Three recent papers define and discuss the issues related to crystal packing in B-DNAs (21,37,38).

The lack of curvature in crystal structures of A tracts has led Dickerson and co-workers to argue for the curved general sequence model (23–25,38). The essence of this model is that A tracts are straight and non-A tracts are curved, primarily by positive roll angles that compress the major groove. This model predicts curvature in the same direction as the wedge and junction models and it has been pointed out by both Crothers (15,16) and by Dickerson (25) that the solution data are not sufficiently sensitive to determine the detailed basis of curvature at the base pair level and that the critical issue is the overall direction of curvature. On this issue, the junction, wedge and curved random sequence models all agree.

However, substantial differences do exist between the solution and crystallographic results. The Dickerson group argues that the curved random sequence model "must be regarded as the only consistent model for A tract bending" (25). Crothers and co-workers (16) point out that "this interpretation requires a significant revision in the structure of generic or canonical B-DNA", because it would necessitate a roll angle of ~6° for every base pair. They regard this model as "a formal possibility", but argue that "the available structural data for a variety of sequences do not support such a hypothesis."

Following the suggestion of DiGabriele and Steitz (20) that these differing viewpoints might reflect the effects of dehydrating agents, we have investigated the extent to which MPD affects the structure of curved DNAs in solution. Our experiments clearly show that gel anomalies are markedly reduced by MPD concentrations of 30% and that this effect is specific for curved molecules. No major conformational change (such as a B to A transition) is seen by CD and linking number assays indicate that MPD causes no detectable changes in helix winding angle, so MPD does not affect phasing of the curved tracts, at least at low concentrations. We thus conclude that MPD reduces DNA curvature.

Finally, what have we learned about the structural basis of DNA curvature? By themselves, our data cannot rule out any of the competing models, since MPD could exert its effects through changes to the A tracts, to the non-A tracts, to the junctions or to some combination of these. But support for the curved general sequence model is severely compromised by the experiments reported here, because the model rests largely on the straight A tracts found in the crystal structures. This conformation may well be the result of dehydrating conditions in the crystals. Nor does the theoretical study of Calladine *et al.* (22) provide independent support for the curved general sequence model. These authors are candid that they can only determine the *difference* in roll angles between A tracts and non-A tracts, not the actual values. They based their zero roll angle for AA steps on the crystal structures, so their model does not require that A tracts are straight. They could have rationalized the gel mobility data just as well if they had chosen an AA roll angle of –6.6°, a TA roll angle of –3.3° and zero roll angles for all other dinucleotide steps. Their model would then agree with the wedge model.

The foregoing considerations strongly question the validity of the curved general sequence model and they raise the possibility that crystallography may not be able to explain the origin of sequence-directed DNA curvature, because curvature is much reduced under the dehydrating conditions necessary for crystal formation. Crystal packing effects are another source of concern.

Conformational energy calculations (17,39–44) have been used to examine the sequence dependence of DNA helicoidal parameters and flexibility. The results have been varied, depending on the parameters of the energy function, the details of the model (e.g. whether or not backbone atoms were included) and on the protocols used (energy minimization, grid searches, Monte Carlo methods, etc.). But two common features have emerged. Virtually all studies agree that it is easier to deform the double helix by roll angle variations than by variations in tilt angle, and several studies, particularly the more recent, have found asymmetries in the resistance to roll angle deformations. The latter observation led Wilma Olson and her collaborators to suggest a simple explanation for the discrepancy between solution and crystallographic studies on DNA curvature (45): if the equilibrium roll angle of AA steps is near zero, then this is the value that would be observed in crystallography; and if the double helix is anisotropic with respect to roll angle deformations, then the mean roll angle will be non-zero in solution studies. This point of view could be rationalized with our results if MPD increases the stiffness of DNA, because the mean roll angle in solution would then move closer to the equilibrium value of zero. This is a logical possibility, since MPD would be expected to reduce solvent screening of electrostatic repulsions between backbone phosphate groups.

If we are correct that dehydrating agents reduce DNA curvature, they should affect a range of experimental properties of DNA containing A tracts, including behavior in oligomerization and cyclization experiments, NMR, electron microscopy, hydrodynamic measurements and patterns of hydroxyl radical cleavage. To understand sequence-directed DNA curvature in detail, it will be necessary to examine the effects of dehydrating agents, temperature and divalent cations, all of which are known to influence migration of DNA in polyacrylamide gels.

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