Stabilization of (dG-dC)n.(dG-dC)n in the Z conformation by a crosslinking reaction

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ABSTRACT

(dG-dC)n.(dG-dC)n was converted to the Z conformer by heating in the presence of Mn++. Reaction of this preparation with the crosslinking reagent, DL-diepoxybutane (DEB), stabilized this conformer so that it retained its structure even when returned to conditions that favored reversion to the B conformation. Treatment of the crosslinked Z conformer with periodate caused scission of the crosslink, allowing reversion to the B conformer. Reaction of (dG-dC)n.(dG-dC)n in the B conformation with DEB did not prevent conversion to the Z conformer in 4M NaCl; dialysis of the high salt solution against low ionic strength buffer allowed return to the B conformer. The Z—B transitions were followed by circular dichroism studies and immunochemical procedures. The results suggest the feasibility of stabilizing Z sequences of DNA in chromatin by crosslinking, so that they could then be identified after DNA isolation.

INTRODUCTION

The ability of polynucleotides to assume a left-handed, double-stranded helical conformation was first suggested by Pohl and Jovin1 upon observing an inversion of the circular dichroism (CD) spectrum of (dG-dC)n.(dG-dC)n under conditions of high ionic strength. Subsequent proof of the existence of a left-handed or Z helix came from the crystallographic studies on (dG-dC)n.(dG-dC)n of Wang et al.2,3 and Drew,et al4,5 and from fiber diffraction data6. NMR7,8 and laser Raman studies9 provided supporting evidence. It was subsequently shown that (dG-dm5C)n.(dG-dm5C)n and (dC-dA)n.(dG-dT)n could also assume the Z conformation under appropriate conditions10,11,12,13.

All of the nucleotide sequences cited above are present in genomic DNA14,15. An important question, therefore, is whether they assume the Z conformation in the cell and, if so, how they contribute to gene expression. Various experiments with antibodies
specific for Z DNA have given results consistent with the presence of Z DNA in cells. Positive results were reported for polytene chromosomes\textsuperscript{16,17,18}, for the macronuclei of Stylonychia mytilus\textsuperscript{19}, and the nuclei of several types of rat cells\textsuperscript{20}.

Although immunochemical experiments generally yield reliable data, there is always the possibility that anti-Z antibodies are recognizing macromolecules that share epitopes with Z DNA, rather than Z DNA, itself. The most direct evidence for its presence in cells would be by isolation of DNA and demonstration of component sequences in the Z conformation. Since conditions for the isolation of DNA can disrupt the Z conformation, procedures must be developed to stabilize it prior to isolation.

We report here the successful stabilization of (dG-dC)\textsubscript{n}.(dG-dC)\textsubscript{n} in the Z conformation through the use of a crosslinking reagent, which, subsequently, can be cleaved to allow return of the polynucleotide to the B conformation.

**EXPERIMENTAL**

Circular dichroism (CD) measurements were made on (dG-dC)\textsubscript{n}.(dG-dC)\textsubscript{n} and its derivatives at a concentration of 42.5 ug/ml using a Jasco J-40 spectropolarimeter.

**Reaction of B Conformer with Diepoxybutane (DEB)** - (dG-dC)\textsubscript{n}.(dG-dC)\textsubscript{n} at a concentration of 42.5 ug/ml was allowed to react with 10\% (v/v) DEB (Sigma) in 10mM triethanolamine, 1mM EDTA, pH 8.0 for 2 hr at 37\°C. The reaction mixture was then dialyzed at 4\°C against 1000 volumes of 1mM triethanolamine, 2mM EDTA, pH 8 for 16 hr followed by dialysis for 3 hr against 1000 volumes of 1mM KH\textsubscript{2}PO\textsubscript{4}-K\textsubscript{2}HPO\textsubscript{4} buffer, pH 7.4 containing 0.5mM EDTA (designated below as "buffer").

**Reaction of Z Conformer with DEB** - (dG-dC)\textsubscript{n}.(dG-dC)\textsubscript{n} at a concentration of 47.2 ug/ml in 1.1mM MnCl\textsubscript{2}, 11.1mM triethanolamine buffer, pH 8 was heated at 60\°C ± 0.2\°C for 15 min. The solution was then cooled to room temperature and DEB was added such that the final concentrations of reactants were 10\% (v/v) DEB, 42.5 ug/ml of (dG-dC)\textsubscript{n}.(dG-dC)\textsubscript{n}, 1mM MnCl\textsubscript{2}, and 10mM triethanolamine. The reaction was allowed to proceed for 2 hr at 37\°C, after which the reactive mixture was cooled to room temperature and dialyzed in the same manner as the B conformer after its reaction with DEB.
(above). In this case, the first dialysis against the TEA buffer removes Mn++ which, if present, would precipitate in the phosphate buffer.

Cleavage of the Crosslink with Periodate - Periodate oxidation was carried out with 0.015N NaIO₄ in 1mM KH₂PO₄-K₂HPO₄ buffer, 0.5mM EDTA, pH 7.4 for 16 hr at 20°C in the dark. (The periodate buffer solution was freshly prepared for each oxidation reaction.) The reaction solution was then dialyzed against two changes of 1000 volumes of 1mM KH₂PO₄-K₂HPO₄, 0.5mM EDTA, pH 7.4 for a period of 4 hr.

RESULTS

Shown in Fig. 1 are the CD spectra of (dG-dC)ₙ, (dG-dC)ₙ in "buffer," and in 1mM MnCl₂ after heating to 60°C for 15 minutes and cooling to room temperature. With respect to the latter, the CD spectrum is typical of the Z conformer with a maximum at about 270nm and a minimum at 295nm. The polymer in "buffer" shows the characteristic CD of the B conformer.

Crosslinking with Diepoxbutane (DEB) - The Z conformer produced by heating for 15 minutes at 60°C in 1mM MnCl₂ (above) was allowed to react with 10% DEB at 37°C for two hours. It was then

![CD spectra](image)

**Fig. 1.** CD spectra of (dG-dC)ₙ, (dG-dC)ₙ in "buffer" (---) and after heating at 60°C for 15 min in the presence of 1mM MnCl₂ (----).
Fig. 2. CD spectra of (dG-dC)\textsubscript{n}.(dG-dC)\textsubscript{n} after heating at 60° for 15 min in the presence of 1mM MnCl\textsubscript{2} and subsequent reaction with DEB (--); the same preparation after reaction with periodate for 16 hr (---).

exhaustively dialyzed. Its CD spectrum, in "buffer," shown in Fig. 2, indicates that the crosslinked polynucleotide did not revert to the B conformation despite the fact that the solution conditions favored the conversion. The CD spectrum has the typical peaks seen for the Z conformer in Fig. 1, although the peak at 270nm is somewhat diminished in intensity.

Conversion of the Crosslinked Z Conformer to the B Conformer by Scission of the Crosslinks - Shown in Fig. 2 is the CD spectrum of the crosslinked Z conformer after cleavage of the crosslink by 16 hours exposure to 0.015N NaIO\textsubscript{4} at 20° C (see discussion). The CD spectrum is characteristic of the B conformer, closely resembling uncrosslinked poly(dG-dC)\textsubscript{n}.(dG-dC)\textsubscript{n} in "buffer" (Fig. 1). Reaction of DEB with the B-conformer - Reaction with 10% DEB was also carried out on (dG-dC)\textsubscript{n}.(dG-dC)\textsubscript{n} in 10mM triethanolamine, 1mM EDTA, pH 8, i.e. on the B-conformer. The CD spectrum of the product closely resembled that of the uncrosslinked preparation (Fig 3). The spectrum remained unchanged by 16 hr. treatment with periodate (not shown). Upon bringing the solution of the crosslinked polymer to 4M NaCl (by dialysis), its CD spectrum became typical of that of a Z-conformer and only slightly different from that of (dG-dC)\textsubscript{n}.(dG-dC)\textsubscript{n} in 4M NaCl (Fig 4). Thus, reaction of the B conformer with DEB did not prevent its conversion to the Z conformer. Dialysis of the Z conformer in 4M
Fig. 3. CD spectra of (dG-dC)ₙ.(dG-dC)ₙ in 10mM triethanolamine 1mM EDTA before (---) and after (——) reaction with DEB.

NaCl against "buffer" allowed return to the B conformer as determined by CD (not shown).

Immunological Studies - The CD studies were confirmed by immunological methods using antibodies specific for Z-DNA in a double antibody competition assay with [³H]AAF-(dG-dC)ₙ.(dG-dC)ₙ as the tracer. Inhibition of binding was seen with the preparation crosslinked after conversion to the Z conformation by heating to 60°C for 15 min. in 1mM MnCl₂. No inhibition was seen after the 16 hr. periodate treatment, nor was inhibition seen with

Fig. 4. CD spectrum of (dG-dC)ₙ.(dG-dC)ₙ after reaction with DEB and subsequently brought to 4M NaCl (——). CD spectrum of unreacted polynucleotide in 4M NaCl (---).
the DEB crosslinked B conformer, either before or after periodate treatment.

DISCUSSION

The crosslinking reagent used in these studies is a racemic mixture of D- and L- DEB, with the following structure:

Lawley and Brooks\textsuperscript{23} have shown that native DNA can be crosslinked by DEB as a result of alkylation of the N7 positions of neighboring guanine residues on opposite strands. According to Otvos and Elekes\textsuperscript{25}, L- DEB is the more active isomer. Lawley and Brookes\textsuperscript{23} were able to isolate the following diguanyl derivative:

This derivative is a trans vicinal glycol which can be cleaved with periodate\textsuperscript{24}. The distances between the N7 positions of various pairs of G residues in B and Z DNA are shown in Table I.

It would appear that a 4 carbon crosslinking reagent could react with the G\textsuperscript{3} and G\textsuperscript{9} pair in both Z and in B DNA, although some distortion of the double helix would be necessary. Reaction with the G\textsuperscript{3} and G\textsuperscript{11} pair is unlikely. On the other hand, neither Lawley and Brookes\textsuperscript{23}, Otvos and Elekes\textsuperscript{25} nor we, have eliminated the possibility of the presence of dimer in the DEB reagent. It is also important to note that x-ray crystallographic data reflect only one of the conformations in which there is a local energy minimum\textsuperscript{26}. The molecule is in a dynamic equilibrium with many such forms, especially in solution. Molecular dynamics simulation studies\textsuperscript{27} on phenylalanine t-RNA (which mimic conditions in solution) show that the root mean square amplitude of atomic motion ranges from about 0.5 Å to about 3 Å. Thus it is not unreasonable that a four carbon crosslinking agent could bridge guanine residues on opposite strands of DNA.

The concentration of DEB used by us in the crosslinking
TABLE I. Distances between N7 atoms of G Residues in B and Z Conformers of (dG-dC)\(_6\).(dC-dG)\(_6\)

|                  | B (Å)\(^a\) | Z (Å)\(^a\) |
|------------------|-------------|-------------|
| \(G^3\) to \(G^5\) | 8.6 ± 0.4   | 10.4 ± 0.1  |
| \(G^3\) to \(G^9\) | 6.55 ± 0.8  | 8.1 ± 0.4   |
| \(G^3\) to \(G^{11}\) | 9.1 ± 0.3   | 13.65 ± 0.1 |

\(^a\)The data for the distances between N7 groups of guanines in the B conformation were calculated from published coordinates of a dodecamer\(^\text{30,31}\). The data for the Z conformation were calculated from coordinates generated from the expressions given in the paper by Wang et al.\(^\text{3}\). The calculations were done by S.C. Harvey of the U. of Alabama, Birmingham, Alabama.

A single crosslink between neighboring strands sufficed for their purposes\(^\text{26}\). We were interested in obtaining a degree of crosslinking high enough to constrain the DNA molecule in its Z conformation. Although we are still in the process of finding optimal conditions, we have succeeded in "fixing" (dG-dC)\(_n\).(dG-dC)\(_n\) in the Z conformation by the crosslinking reaction. This has been established by CD studies as well as by immunochemical procedures. Moreover, we have shown that periodate oxidation and cleavage of the crosslink allowed reversion to the B conformer.

When the B conformer was allowed to react with DEB, the resulting product could still be converted to the Z conformer by 4M NaCl as easily as was unreacted (dG-dC)\(_n\).(dG-dC)\(_n\), and subsequent dialysis against "buffer" allowed return to the B conformation.

The experimental results described in this paper suggest the possibility that (dG-dC)\(_n\).(dG-dC)\(_n\) sequences in cellular DNA or in isolated chromatin can be stabilized in the Z conformation by crosslinking procedures. If so, the DNA could then be isolated free of other components and examined for the presence of...
(dG-dC)ₙ.(dG-dC)ₙ sequences in the Z conformation. Presumably other sequences, such as (dG-dm₅C)ₙ.(dG-dm₅C)ₙ and (dC-dA)ₙ.(dG-dT)ₙ, could be treated similarly. Crosslinking of (dG-dC)ₙ.(dG-dC)ₙ sequences present or inserted into supercoiled plasmids should also be possible.

Another outgrowth of these experiments is the possibility of using physical methods and electron-microscopy to search for forms intermediate between the B and Z conformers that might be generated by controlled crosslinking and scission of the crosslinks using various reaction conditions. These studies are now in progress and will be the subject of another publication.

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