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Dynamics of the B-to-Z transition in supercoiled DNA

(Z-DNA/monoclonal antibody/plasmid DNA/filter binding assay)

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ABSTRACT **The sequence $(dC\text{-}dG)_{16}$, inserted into the polylinker of plasmid pUC8, adopts a lefthanded Z-DNA conformation at "natural" supercoil density. The radioactively labeled monoclonal antibody Z-D11, which has a very high affinity for this DNA conformation, provides a convenient sensitive tool to measure selectively the amount of Z-DNA. Chloroquine reversibly changes the supercoil density of plasmid DNA and thereby the equilibrium between right- and left-handed double-helical DNA. The time-dependent formation or disappearance of Z-DNA was measured by using the antibody either as a fast indicator of Z-DNA or as an additional effector of the B-to-Z equilibrium. In the middle of the transition, a relaxation time of about 1 hr is observed in 0.1 M NaCl at 22°C. The kinetic data are compatible with an all-or-none transition between the two conformations. The overall rate constant for Z-DNA formation, k_{BZ}, decreases with the square of the chloroquine concentration, while the reverse one, k_{ZB}, increases with about the fourth power.**

The change between the right-handed B form and the left-handed Z form of DNA has become one of the best-studied examples for the conformational flexibility of these molecules (for a recent review see ref. 1). Earlier experiments used high sodium chloride concentrations to shift the equilibrium between the two forms (2, 3). The kinetics of the transition in linear double-helical molecules of different length with the sequence $(dC\text{-}dG)_n$ were followed by spectroscopic methods (3). Although such an approach should also be feasible for supercoiled DNA (4, 5), the small amount of Z-DNA in plasmids creates considerable experimental problems.

Covalently closed circular DNA, as isolated, e.g., from *Escherichia coli*, has a deficiency of helical turns, which is compensated by a negative supercoiling of the molecule (e.g., see ref. 6). Such a DNA is in a state of high energy and a process that relaxes the supercoil density will be favored. One such process is the preferred binding of intercalating molecules, such as ethidium, to covalently closed circular (ccc) DNA, as compared to relaxed or linear DNA (7, 8). Another process is a change from a right- to a left-handed double-helical structure (9). The equilibrium of the B-to-Z transition of cloned $(dC\text{-}dG)_n$ in plasmid DNA has been measured mainly by gel electrophoresis (e.g., refs. 10, 11, and 16).

The elegant use and analysis by two-dimensional gel electrophoresis has provided a wealth of quantitative information on the energetics of this transition in ccc DNA (11).

In contrast, there are practically no quantitative data available on the dynamics of this process in supercoiled DNA. In view of a possible biological role of Z-DNA and with respect to the molecular mechanisms involved, the time dependence of the transition in torsionally stressed DNA is of interest. With monoclonal antibodies (mAbs) specific for Z-DNA, a convenient tool is available for measuring the amount of left-handed DNA in the presence of large amounts of B-DNA (12–14).

The use of the mAb Z-D11 for isolating potential Z-DNA from genomic DNA has been described previously (15). Here, this radioactively labeled antibody is used to monitor the kinetics of the conformational change of $(dC\text{-}dG)_{16}$ in ccc DNA in solution, after changing the supercoil density by varying the chloroquine concentration. The data were analyzed with respect to a simple reaction scheme.

MATERIALS AND METHODS

Chemicals. Chloroquine [4-amino-(*N*-1-methyl-4-diethylaminobutane)-7-chloroquinoline] was obtained from Sigma as the diphosphate and used without further purification. The concentration was measured by absorbance at 343 nm in potassium phosphate, pH 6, using $\varepsilon = 19.5\ mM^{-1} \cdot cm^{-1}$ (17, 18). Sepharose 4B was from Pharmacia. Calf thymus DNA and restriction enzymes were from Boehringer Mannheim. All chemicals were analytical grade commercial products.

Plasmid DNA. Plasmid pUC8 (19) was cut with *Bam*HI, treated with phosphatase, and purified by gel electrophoresis in low-melting agarose. Plasmid pLP32 with a $(dC\text{-}dG)_{16}$ insert (20) was digested with *Bam*HI and the two fragments were separated on a small Sepharose 4B column. After ligation of the small fragment with the vector and transformation into *E. coli* JM 83 cells, individual recombinant clones were characterized. The resulting plasmid with 2751 base pairs, pFP 332, with $(dC\text{-}dG)_{16}$ in the polylinker, was grown and the ccc DNA was prepared as described (21). More than 95% was ccc DNA. The DNA concentration was measured after linearization with *Eco*RI by the fluorescence increase of an ethidium bromide solution (21, 22), using calf thymus DNA as standard. The supercoil density was estimated from the topoisomer distribution, by agarose gel electrophoresis in the presence of chloroquine.

Iodinated Antibody. The mAb Z-D11 from ascites fluid was purified to homogeneity and labeled with $Na^{125}I$ to about 5000 cpm/ng as described (14, 15). Up to 90% of the radioactivity bound specifically to Z-DNA after labeling. The half-life of the ability of the labeled antibody (^{125}I-mAb) to form a complex was 30 days.

Filter Binding Assay. To measure formation of the complex between ^{125}I-mAb and ccc DNA an ion-exchange membrane (NA45, Schleicher & Schüll) was used; this membrane quantitatively binds DNA but does not bind the antibody to any appreciable extent. The membrane was fixed in a homemade filtration device and washed with TAES buffer (10 mM Tris acetate/1 mM EDTA/100 mM NaCl, pH 8.0), supplemented with bovine serum albumin at 0.1 mg/ml. DNA and antibody were mixed in a volume of 0.05 ml of TAES buffer for a given time and filtered. The filter was washed immediately with 0.5–1.0 ml of TAES buffer and the radio-

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Abbreviations: ccc DNA, covalently closed circular DNA; mAb, monoclonal antibody.

activity bound to it was measured with a γ counter. (The background of the assay is indicated in Figs. 2, 3, and 5 by the distance between abscissa and zero values.)

Kinetic Measurements. In one set of experiments the labeled antibody was used as a "fast" indicator of the amount of Z-DNA present at different times after changing the amount of chloroquine bound to the ccc DNA.

(i) *Dilution experiments.* pFP332 DNA was equilibrated for 30 min at room temperature in TAES buffer containing, e.g., 250 μM chloroquine. At time zero 9 vol of TAES buffer was added, the solution was mixed, and at different times 45 μl was withdrawn and mixed with 5 μl of antibody solution (11 nM). After further 10-min incubation, the solution was filtered and the amount of complex formed—in this case at 25 μM chloroquine—was determined.

(ii) *Salt jump.* Plasmid DNA (0.35 nM) was incubated at room temperature with TAE buffer (TAES buffer lacking sodium chloride) containing, e.g., 25 μM chloroquine. At time zero the salt concentration was increased to 100 mM by adding 5 M NaCl to dissociate chloroquine from the DNA. At different times, aliquots were mixed with antibody for 10 min and the Z-DNA formed was measured by the filter assay.

(iii) *Chloroquine jump.* The DNA in TAES buffer was mixed at time zero with a 10 times concentrated chloroquine solution and the disappearance of Z-DNA was measured again after 10-min incubations with antibody.

In another set of experiments advantage was taken from the shift of the B-to-Z equilibrium upon binding of the high-affinity antibody. The ccc DNA was equilibrated at various chloroquine concentrations in TAES buffer at room temperature for 30–60 min. At time zero, a molecular excess of labeled antibody was added. At various times the amount of complex formation was determined by the filter assay.

RESULTS

Binding of mAb Z-D11 to Plasmid pFP332. The titration of the labeled mAb with plasmid DNA is shown in Fig. 1. Due to the high affinity of this antibody to $(dC\text{-}dG)_{16}$ in the Z form, practically stoichiometric binding of the DNA is observed at nanomolar concentrations of reactants. One plasmid molecule binds 2 ± 0.2 antibody molecules with high affinity. At an excess of antibody, the linearity of the assay allows quantitative determination of the amount of Z-DNA present in the solution. The low background and the linearity of the filter binding assay provide a sensitive alternative to the usual nitrocellulose filter assays used in quantitating protein–DNA interactions. The association rate constant of the antibody to Z-DNA was determined as $k_{12} = 2 \cdot 10^7\ M^{-1} \cdot s^{-1}$ at room temperature (data not shown). Therefore, at nanomolar antibody concentration the complex formation is completed within 5–10 min.

Influence of Chloroquine on the B-to-Z Transition. The interaction of dyes, such as ethidium or chloroquine, with ccc DNA provides a convenient way to change the supercoil density of the molecule in a fast and reversible way. Chloroquine was used here instead of ethidium, since it has a lower affinity for DNA and therefore the free and total concentration of these effector molecules will be practically the same under the conditions of the experiments. This simplifies the analysis considerably. Decreasing the supercoil density of this plasmid leads to the conversion of the Z-DNA to right-handed B-DNA. Fig. 2 shows the relative amount of Z-DNA present in pFP332 DNA as a function of the total chloroquine concentration. The plasmid DNA was equilibrated at various chloroquine concentrations and incubated with an excess of labeled antibody for 10 min and 74 hr, respectively, and filtered through an ion-exchange membrane to determine the amount of complex formed. At short incubation time, the results reflect the B-to-Z equilibrium. For the plasmid used here, a roughly sixth-power dependence on the chloroquine concentration is observed. The midpoint of the transition (CQ_m) depends strongly on the supercoil density of the DNA and the salt concentration but only weakly on the temperature. If the DNA is incubated with the antibody for a long time, a change of the B-to-Z equilibrium due to the preferred binding is observed (Fig. 2).

Relaxation Times for the B-to-Z Transition. By changing, e.g., the chloroquine concentration suddenly and adding the antibody for a *short* time period, the kinetics of the conformational change can be followed directly. Fig. 3*A* shows the time dependence of the disappearance of Z-DNA upon increasing the chloroquine concentration to various levels. Within the accuracy of the present measurements the formation of B-DNA can be described by a simple exponential relaxation curve.

The reverse reaction, namely the formation of Z-DNA from B-DNA, was measured by equilibrating the DNA at high chloroquine concentration and then diluting the solution with buffer. At different times, a brief incubation with antibody reveals the amount of Z-DNA formed (Fig. 3*B*). Another way to measure the rate of Z-DNA formation is to incubate the DNA at low salt concentration with chloroquine and then add salt to dissociate the dye. Again, a reasonable description of the kinetics, after a short incubation with antibody, is by simple exponential relaxation curves. The corresponding relaxation times at 0.1 M NaCl and room

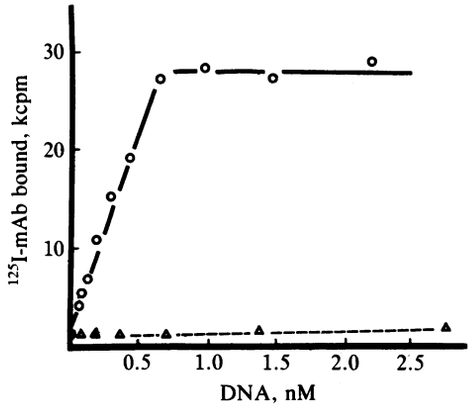

FIG. 1. Titration of ^{125}I-labeled monoclonal antibody Z-D11 (1.1 nM) with supercoiled plasmid molecules pFP332 (○) and pUC8 (△). Both were incubated at room temperature in TAES buffer and the amount of complex formed was determined by filtration through an ion-exchange membrane.

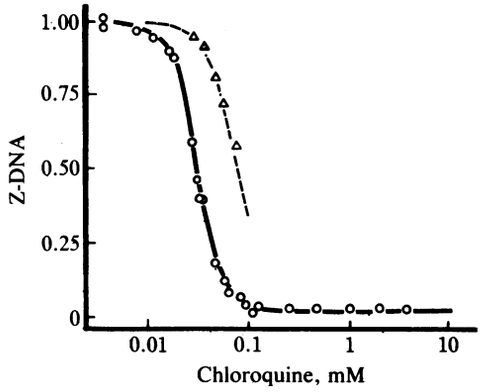

FIG. 2. Relative amount of Z-DNA of plasmid pFP332 as a function of the chloroquine concentration in TAES solution, as determined by the filter assay. ccc DNA (0.35 nM) was equilibrated with chloroquine at room temperature for 30 min and then incubated with 1.1 nM antibody for 10 min (○) or 74 hr (△) before filtration.

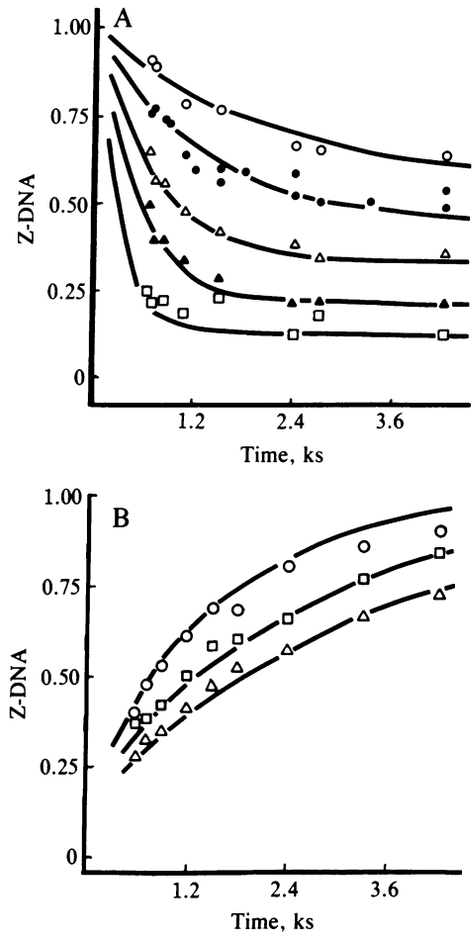

FIG. 3. Time dependence of the relative amount of Z-DNA in plasmid pFP332 (0.35 nM) after a sudden change of the chloroquine concentration and incubating aliquots of the solution for 10 min with antibody (1.1 nM). (*A*) Disappearance of Z-DNA upon increasing the chloroquine concentration to 30 (○), 35 (●), 40 (△), 45 (▲), or 50 (□) μM. (*B*) Formation of Z-DNA upon dilution with 9 vol of TAES buffer, starting with 200 (○), 240 (□), or 280 (△) μM chloroquine.

temperature are summarized in Fig. 4. A strong dependence of the relaxation time on the final chloroquine concentration in the solution is observed. A sharp maximum at the middle of the transition with a relaxation time of roughly 1 hr was obtained, compatible with previous qualitative results (10, 23).

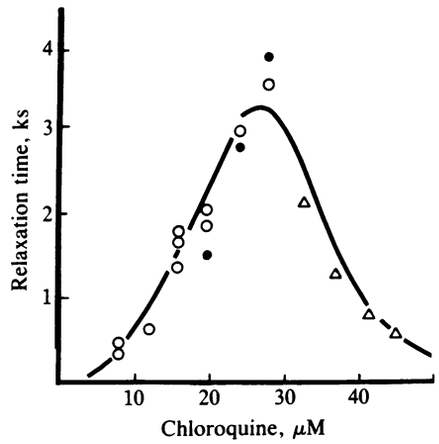

FIG. 4. Average relaxation time of the B-to-Z transition as a function of the final chloroquine concentration in TAES buffer at about 22°C. △, Chloroquine jump (Fig. 3*A*); ●, dilution experiment (Fig. 3*B*); and ○, salt jump, by increasing the sodium chloride concentration from 0 to 100 mM.

Shifting the B–Z Equilibrium upon Antibody Binding. The mAb Z-D11 has a high affinity for the Z form of $(dC\text{-}dG)_{16}$ but no measurable affinity for the B form. Therefore, upon binding it will shift the conformational equilibrium to the Z form. Such a coupled equilibrium also provides kinetic information, especially if the isomerization is slow compared to the binding process. Fig. 5 shows results of such an approach. The DNA was equilibrated at different chloroquine concentrations and at time zero excess antibody was added. Samples were withdrawn at different times and the amount of complex formed was measured with the filter assay. The fast binding process is not resolved in these experiments and the slow relaxation curve was again fit with a single exponential.

Isomerization Rate Constants k_{BZ} and k_{ZB}. By using the antibody at high concentration [A] with respect to the binding sites [Z], that is under pseudo-first-order conditions, and assuming an all-or-none transition of the $(dC\text{-}dG)_{16}$ insert between [B] and [Z], a good first approximation is the following reaction scheme:

$$B \underset{k_{ZB}}{\overset{k_{BZ}}{\rightleftharpoons}} Z \underset{k_{21}}{\overset{k'_{12}[A]}{\rightleftharpoons}} ZA, \qquad [1]$$

which in general will give rise to two relaxation times (24, 25).

In the first set of experiments (Figs. 3 and 4), the antibody is used as a "fast" indicator of the amount of Z-DNA, and the relaxation time of the intramolecular conformational change is given by

$$1/\tau_0 = k_{BZ} + k_{ZB}. \qquad [2]$$

Together with the "equilibrium" data from Fig. 2, where $K_{BZ} = [B]/[Z] = k_{ZB}/k_{BZ}$, the two rate constants can be calculated.

In the second case (Fig. 5) the conformational change is a slow isomerization coupled to a fast binding step, resulting in two relaxation times (25):

$$1/\tau_1 = k_{21} + k'_{12}([A] + [Z]) \qquad [3a]$$

and

$$1/\tau_2 = k_{BZ} + k_{ZB}\,(k_{21}/k'_{12} + [Z])/ (k_{21}/k'_{12} + [Z] + [A]). \qquad [3b]$$

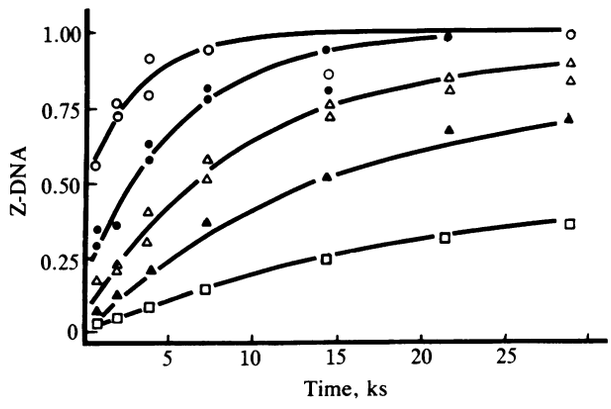

FIG. 5. Change of the relative amount of complex formed between labeled antibody and pFP332 DNA with time. ccc DNA (0.35 nM) was equilibrated with 30 (○), 40 (●), 50 (△), 60 (▲), and 80 (□) μM chloroquine for 30 min and antibody (1.1 nM) was added at time zero. At different times, aliquots were removed and filtered, and the bound radioactivity was measured.

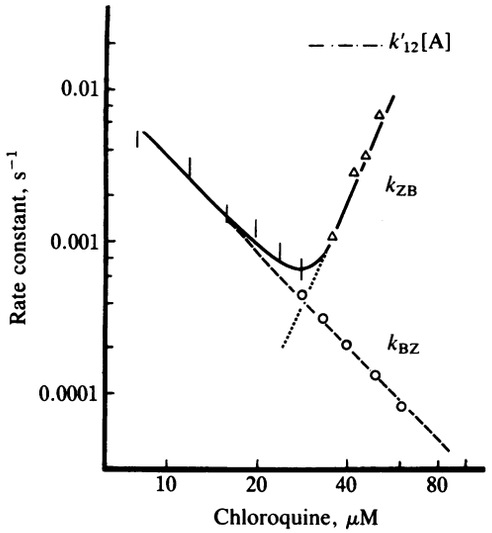

FIG. 6. Overall rate constants for the formation of Z-DNA (k_{BZ}, ---) and B-DNA (k_{ZB}, △·····△) in supercoiled plasmid DNA as a function of the chloroquine concentration in a double-logarithmic plot. The solid line is the reciprocal of the relaxation time τ_0 from Fig. 4 and the open symbols are the relaxation times τ_2 from Fig. 5 (○). The approximate value of the pseudo-first-order rate constant for the antibody binding is also shown (·—·—·). [The removal of 3 (6) negative superhelical turns corresponds to about 10 (40) μM chloroquine.]

Under the experimental conditions used in Fig. 5 the following simplifications are justified at the present level of accuracy: From the high affinity (Fig. 1) and the excess antibody used, it follows that [A] >> [Z] >> k_{21}/k'_{12}. Therefore

$$1/\tau_1 \approx k'_{12}[A] \qquad [4a]$$

and

$$1/\tau_2 \approx k_{BZ}(1 + K_{BZ}[Z]/[A]) \approx k_{BZ}. \qquad [4b]$$

The resulting dependence of the overall rate constants for the isomerization of the DNA in the plasmid as a function of the chloroquine concentration are shown in a double logarithmic plot in Fig. 6. The rate constant for forming Z-DNA in a supercoiled plasmid k_{BZ} decreases with the square of the chloroquine concentration; the one for the formation of B-DNA increases with about the fourth power of dye concentration.

DISCUSSION

The energetics of the B-to-Z transition of plasmid pF332, as derived from the analysis of two-dimensional gels, can be described by the same parameters as obtained previously for the larger plasmid pLP332 (ref. 11, unpublished observations). Upon changing from the B form to the Z form of the inserted $(dC\text{-}dG)_{16}$, about six superhelical turns are released. The specificity of the labeled mAb Z-D11 can be used with advantage for measuring quantitatively the amount of Z-DNA present in solution. Its use as a "fast" indicator or as a reagent for shifting the conformational equilibrium in ccc DNA, together with a simple filter assay, allows kinetic measurements in the presence of large amounts of B-DNA.

Although less is known about the details of binding of chloroquine to DNA (e.g., see refs. 17 and 26) as compared to ethidium, it is a convenient reagent to change the supercoil density of ccc DNA, which varies with the square root of the free chloroquine concentration. Since the concentration of binding sites in the DNA is much smaller than the chloroquine concentration, the free and total chloroquine concentration will be roughly the same at the chosen experimental conditions (17).

Such a reaction system, involving ccc DNA, where about 1.2% of the base pairs undergo a conformational transition, together with the binding of chloroquine, ions, and antibodies, will in general require a rather complicated analysis of the kinetics. By choosing the experimental conditions carefully, a considerable simplification is possible.

At present, the use of the simple reaction scheme 1 appears to be justified. Using the antibody in excess of the binding sites results in pseudo-first-order kinetics and in general two relaxation times are expected for such a scheme. But it should be kept in mind that the DNA represents a mixture of a small number of topoisomers. Therefore, one could expect a spectrum of relaxation times for the isomerization reaction and it is surprising that a single, average, relaxation time and the corresponding overall rate constants k_{BZ} and k_{ZB} are such a good description of the present experimental results.

The time domain in which the transition between right-handed B-DNA and left-handed Z-DNA occurs in ccc DNA is rather similar to results obtained previously with linear $(dC\text{-}dG)_n$ at high salt concentration (2, 3). But this can also be an accidental result, since the molecular mechanism of the transition in short linear and supercoiled DNA, respectively, might be somewhat different. The transition in pFP332 becomes faster at lower salt concentration (data not shown). This may explain the absence of smearing effects in electrophoresis experiments (11), since the transition will be considerably faster than the 20 hr typical for a separation of topoisomers. But at higher salt the two time scales may become comparable (20).

The nucleation of Z-DNA within the B-DNA of the plasmid requires at least the free energy for the formation of two junctions, that is about 10 kcal (42 kJ)/mol (11), while no such nucleation barrier is expected for the reverse reaction. k_{BZ} increases strongly with the increase of the number of negative superhelical turns, as deduced from the inverse square dependence on the chloroquine concentration. For a plasmid with a small segment being in either a right- or a left-handed conformation the total number of binding sites for chloroquine will be roughly the same. But since the number of superhelical turns will be lower in topoisomers with Z-DNA than in those without, the apparent affinity of chloroquine will also be lower. This change of the affinity may in part be responsible for the observed dependence of k_{BZ} on the chloroquine concentration. This effect should be less pronounced for larger plasmids containing $(dC\text{-}dG)_{16}$.

A more detailed molecular interpretation of the kinetics of this isomerization reaction is possible by making measurements with plasmids of various lengths and also with different $(dC\text{-}dG)_n$ inserts, using the approach outlined here.

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