The helical repeat of DNA at high temperature

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ABSTRACT

The increasing number of studies on thermophilic organisms addressed the question of DNA double helix parameters at high temperature. The present study shows that the helix rotation angle per base pair \( \theta \) of an unconstrained DNA decreases linearly upon temperature increase, up to the pre-melting range. In the ionic conditions tested, this rule extends to temperatures up to 85°C, which is a common growth temperature for many hyperthermophilic organisms. In addition, the torsional constant \( K \) of DNA decreases with temperature, indicating that the energy required to modify the DNA twist is lower at high temperature. These findings have several implications for people working on the structure and enzymology of DNA at high temperature.

INTRODUCTION

Until recently, experimental studies of the DNA double helix at high temperatures essentially focused on its melting and reassociation properties, since temperatures above 45°C were considered as non-physiologic. During the past ten years, a number of biochemical experiments have been undertaken on a variety of thermophilic organisms, mainly Archaebacteria (1), whose temperature growth is in the range 80–105°C. Despite these extreme conditions, DNA is necessarily transcribed, replicated, repaired and recombined in these living cells (2). It then appeared of importance to get insight into the DNA structure and topology at high temperature. Successive discovery of reverse gyrase (3, 4), an enzyme that promotes positive supercoiling of DNA at 80°C, and of naturally occurring positively supercoiled DNA in an archaebacterial virus (5) suggested that this type of DNA structure was a possible response to exceedingly high growth temperatures. Recently, several DNA metabolizing enzymes, including RNA (6) and DNA polymerases (7, 8), topoisomerases (9), and histone-like proteins (10) were isolated from thermophilic Archaebacteria and Eubacteria. Theirs in vitro interactions with DNA and enzymatic activities were tested up to 90°C. One of the basic questions was the structural state of the DNA substrate in these conditions. Although it was known that in reaction mixtures used for polymerization or topoisomerization the DNA was not melted at these temperatures, nothing was known about its helical repeat. At low temperature, Depew and Wang (11) found that the double helix was unwound linearly with increasing temperature. In the present paper, we show that this finding extents to high temperatures: in unconstrained (form II) DNA, the average helix rotation angle per base pair linearly decreases with temperature. In addition, the torsional constant of DNA, which measures the energy necessary to change the twist of the double helix by a unit angle, also decreases with temperature.

MATERIALS AND METHODS

Preparation of DNA substrates

Supercoiled plasmids pBR 322 and pXG 540 were prepared as previously described (12). pXG 540 is a generous gift of Dr. D.M.J. Lilley (Dundee, UK). It was obtained by insertion of a 540 bp fragment of Xenopus laevis, containing a (AT)_{54} sequence, between the EcoRI and Bam HI sites of plasmid pAT 153 (13). For both plasmids, the corresponding form II DNAs containing a single nick per circle were prepared by incubation of form I with DNAse I in the presence of a saturating concentration of ethidium bromide (14). The quality of the DNA products (form II) was checked by alkaline gel electrophoresis that separates full length linear from circular single-stranded DNAs.

Enzymes

DNAse I was purchased from Boehringer (Mannheim) and used at a final concentration of 2 µg/ml. Proteinase K was obtained from Merck (Darmstadt). Thermophilic ligase HB 8 from Thermus thermophilus purified to near homogeneity (15) was kindly provided by M. Takahashi (Mitsubishi Kasei Institute of Life Sciences, Tokyo). The enzyme was active from about 15 to 85°C, with an optimum about 65°C (16). It was diluted in 10 mM Tris–HCl pH 7.4, 100 mM KCl and 2% glycerol. A preliminary kinetic experiment was performed to determine the conditions of ligation: 10^{-3} U/µg DNA is sufficient to ensure a quick closure of form II at 70°C (less than 1 min). In the case of lower or higher temperatures, up to 10^{-2} U/µg DNA is used to obtain a similar efficiency. In all cases, the low amount of ligase used prevented a possible stoichiometric effect of this enzyme on the DNA helical repeat.
Covalent closure by ligase

Reaction mixtures (24 µl each in siliconized tubes) contained a final concentration of the following components: 30 mM Tris–HCl, pH 7.8; 5.5 mM MgCl₂; 30 mM KCl; 11 mM DTT; 20 µg/ml DNA form II; 30 µg/ml bovine serum albumin; 0.7 mM NAD⁺; 2% glycerol; and 5. 10⁻⁴ to 5. 10⁻³ units of thermophilic ligase (HB8) from *T. thermophilus*. Before incubation, ligase was introduced as a 2 µl drop on the inner wall of the tube placed horizontally in order to avoid mixing with the 22 µl mixture placed at the bottom. The tube was incubated horizontally in an ethylene glycol bath at the desired temperature for 15 min. Ligation was initiated by quick mixing of the drop with the rest of the mixture. Incubation was prolonged for 5 min and the reaction stopped by quick cooling on ice, followed by a 20 sec centrifugation and an addition of 2 µl EDTA 0.5 M. After addition of SDS up to 1% and proteinase K up to 0.5 mg/ml, the mixture was incubated for 30 min at 55°C, phenol extracted, and the DNA was precipitated with ethanol.

**Buffers:**

- TEP is 36 mM Tris, 30 mM NaH₂PO₄, 1 mM EDTA, pH 7.8.
- TAE is 40 mM Tris-acetate, pH 7.8, 1 mM EDTA.

**Monodimensional electrophoreses:** four types of electrophoreses have been used in this study:

(i) standard 1.3% agarose gels in TEP buffer.

(ii) gels containing magnesium acetate (5 mM) in TAE buffer. Magnesium increases the twist of DNA, which results in a more negatively supercoiled DNA and higher mobility during electrophoresis.

(iii) Netropsin containing gels in TEP buffer. Netropsin is a minor groove binder that also increases the DNA twist.

(iv) Chloroquine containing gels in TEP buffer. Chloroquine intercalates between adjacent base pairs and decreases the DNA twist, resulting in a loss of negative supercoils and eventual formation of positive supercoils.

Concentration of the various agents is indicated in figure legends. In all cases, electrophoresis was performed for 20h at 1.5 V/cm at 5°C.

**Bidimensional electrophoresis:** the gel was 1.3% agarose in TEP. In the first dimension, DNA was run for 17 h at 1.2 V/cm and at 4°C in the presence of 1.5 µg/ml chloroquine. The second dimension was carried out for 9h at 1.2 V/cm in the presence of 6 µg/ml chloroquine.

Expression of the data

Closure by ligase generates a Gaussian distribution of topoisomers separated by gel electrophoresis (Fig. 1A). Densitometric profiles of each lane of the different electrophoreses were generated from a video image by using 'Scan Analysis' software program developed by Orkis, Marseille, France. An example of such a profile is shown in Fig. 1B. The surface of the different peaks was automatically calculated by the program, and is proportional to the concentrations Cₘ, Cₘ+i, ..., Cₘ+i of the various topoisomers, where Cₘ is the concentration of the major topoisomer. The ratio Cₘ+i/Cₘ is related to the fraction of turn ωₜ between the center of each Gaussian distribution and the major topoisomer as follows (11): the difference of free energy ∆G° between the major topoisomer Lₖₘ and any other topoisomer Lₖₘ+i is given by:

\[ \Delta G° = G°(Lₖₘ + i) - G°(Lₖₘ) = K \cdot (\omegaₜ + i)^2 - K \cdot (\omegaₜ)^2 \]  (1)

where K is the torsional constant of the double helix. This equation indicates that the torsional energy of a given topoisomer is proportional to the square of the distortion imposed to the structure. The free energy ∆G° is also related to the concentrations Cₘ+i and Cₘ by the expression:

\[ \Delta G° = -RT \ln \left( \frac{Cₘ+i}{Cₘ} \right) \]  (2)

It follows from equations (1) and (2) that:

\[ \Delta G° = K \cdot i \cdot (2 \omegaₜ + i) = -RT \ln \left( \frac{Cₘ+i}{Cₘ} \right) \]  (3)

or:

\[ 1/i \cdot \ln \left( \frac{Cₘ+i}{Cₘ} \right) = -K/RT \cdot (2 \omegaₜ + i) \]  (4)

Representation of 1/i . Ln [Cₘ+i]/[Cₘ] as a function of i is expected to be a straight line with a slope -K/RT. For i = -2 ωT, the ordinate is zero and [Cₘ+i] = [Cₘ], center of the Gaussian.
Figure 2. Electrophoretic analysis of the DNA after closure by ligase, as a function of temperature. A. Temperatures range of 21 to 57°C. Each of the 6 samples was run on three different gels, all containing 1.3% agarose. Lanes 1-6, in the presence of 5mM magnesium acetate in TAE buffer; lanes 7-12, in 3.4 µg/ml netropsin in TEP buffer; lanes 13-18, in 3 µg/ml chloroquine in TEP buffer. Topoisomers in lanes 1 to 12 are negatively supercoiled, while topoisomers in lanes 13-18 are positively supercoiled. Temperature of closure is 21°C (lanes 1, 7, 13); 29.1°C (lanes 2, 8, 14); 35.4°C (lanes 3, 9, 15); 42.8°C (lanes 4, 10, 16); 50.5°C (lanes 5, 11, 17) and 57.0°C (lanes 6, 12, 18). B. Temperatures range of 44 to 79°C. Each sample was run on two different gels. Lanes 1-7, in the presence of 5mM magnesium acetate in TAE buffer; lanes 8-13, standard gel in TEP buffer. Temperatures of closure are: 44.5°C (lanes 1 and 8); 52.0°C (lanes 2 and 9); 58.5°C (lanes 3 and 10); 65.3°C (lanes 4 and 11); 72.5°C (lanes 5 and 12) and 79.3°C (lanes 6 and 13). Lane 7 is form II DNA substrate in the absence of ligase.

Such a representation is shown in Fig. 1C. In this case, \( i = 0.726 \) and \( \omega_T = -0.363 \). The center of the topoisomer distribution is at 0.36 turn on the right of the major topoisomer.

Finally, the linking difference (\( \Delta L_k \)) in fraction of turns between the distribution centers obtained for various temperatures was plotted as a function of temperature. The slope \( s \) of the corresponding diagram was expressed as follows:

\[
s = -\frac{\Delta L_k}{\Delta \theta} = -\frac{n \Delta \Omega}{360 \Delta \theta}
\]

where \( n \) is the number of DNA base pairs, and \( \Delta \Omega \) the variation of the average helix rotation angle per base pair for a variation \( \Delta \theta \) of temperature. For instance, in the case of pBR 322:

\[
\Delta \Omega/\Delta \theta = -360 \text{ s/4363 angular degrees per } ^\circ C
\]

RESULTS
DNA unwinding as a function of temperature
This measure is based on the quick closure of a form II DNA by ligase (11). Plasmid pBR 322 form II DNA containing a single nick per circle was incubated at various temperatures with thermophilic ligase from *Thermus thermophilus* in the conditions described in Materials and Methods. Each reaction mixture was divided in 2 to 3 aliquots and the DNA analyzed in agarose gels containing various agents which modify the helical repeat of DNA. Fig. 2A shows the effect of increasing temperatures of closure on the position of the Gaussian distribution of topoisomers from 21°C to 57°C. Electrophoreses were performed in the presence of magnesium (lanes 1 to 6), netropsin (lanes 7 to 12), and chloroquine (lanes 13 to 18). The same type of experiment was performed from 44°C to 79°C and the DNA analyzed in a standard gel, or in a magnesium-containing gel (Fig. 2B).

Electrophoreses of aliquots of the same sample in various conditions allow to obtain topoisomers distributions exploitable for calculation of the distributions centers. For instance, a magnesium-containing gel does not resolve the topoisomers produced at 21°C (Fig. 2A, lane 1), while netropsin (lane 7) and chloroquine (lane 13) gels do. In the case of the experiment described in fig.2A, standardization between the different gels is obtained by comparing the distributions in lanes 4, 10, and 16.
A number of experiments similar to those described in figure 2 were performed within various temperature ranges: the data were exploited as described in Materials and Methods (see fig. 1): the center of each gaussian was determined according to Depew and Wang's method (11) and the corresponding linking number difference ΔLk was plotted against the temperature of closure (θ). As shown in fig. 3, DNA unwinding appears to be a linear function of temperature: this finding is applicable to a wide range of temperatures, from 21 to 83°C. The slope s of this straight line allows to determine the temperature dependence ΔΩ/Δθ of the helix rotation angle Ω per base pair:

$$\Delta\Omega/\Delta\theta = -0.0105 \text{ degrees/}^\circ\text{C . bp}$$

This value is comparable to the previous electrophoretic data of Depew and Wang (11), using PM2 DNA, who found a ΔΩ/Δθ of −0.011 to −0.012 in a temperature range between 0 and 29°C. Thus, the law of thermal DNA unwinding determined by these authors extends to high temperatures and remains valid up to 80°C. In terms of DNA helical repeat (h = 360/θ), if h is taken as 10.4 bp/turn at 25°C in the presence of low magnesium, one can estimate a value of about 10.6 at 83°C in the same ionic conditions.

Variations of the torsional constant K as a function of temperature

The torsional constant of the DNA molecule K represents the average energy necessary to increase or decrease the DNA helix rotation angle by a unit value. As pointed out by Depew and Wang (11), this constant may be deduced from the slope of the straight line such as the one described in the plot of fig. 1C. This slope represents $-K/RT$ (see Materials and Methods) where T is the temperature in the Kelvin scale. From topoisomers distributions obtained by closure at various temperatures, we have determined the slopes of the Depew and Wang's diagrams (see Materials and Methods). The corresponding torsional constant K was plotted as a function of temperature (fig. 4). K appears to decrease linearly with temperature, from 2.2 at 36°C to 1.1 Joule. mole-1. degree-2 at 83°C, indicating that the torsional rigidity of the molecule is lower at high temperature.
Finally, we have also determined for pBR 322 the product $nK$ (where $n$ is the number of base pairs) in RT units:

$$nK = 1320 \text{ RT at } 35^\circ \text{C},$$

a value in good agreement with the data published for other circular DNAs of different sizes (1000 to 1500 RT) (11).

**DNA unwinding in the premelting range**

Examination of DNA unwinding between 49 and 83°C is shown in fig. 5A. Up to 83°C (lane 6), topoisomers distribution was regularly shifted towards negative supercoils with increasing temperature, indicating a regular decrease in the helix rotation angle. However, as shown in the densitometry of lane 6 (fig. 5C), a new population of fast migrating DNA appears after closure at 83°C, in addition to the normal distribution of topoisomers. This population, which was not present at 76°C (fig. 5B) is interpreted as due to the melting of some AT rich regions in pBR 322. Such a melting would dramatically decrease the DNA linking number after closure by ligase: for instance, melting of only 10 bp would directly produce a $\Delta Lk$ of about $-1$ turn. Indeed, a few regions of pBR 322 are AT rich and possibly melted at 83°C in the ionic conditions used: 11 sequences in pBR 322 genome, totaling 97 bp are susceptible of melting in these conditions. The consequence in an experiment of ligation would be a linking number deficit of 97/10.4, about 9 turns, which might be consistent with the DNA population observed in fig. 5 C.

To determine whether an AT rich region would melt in the temperature range around 80°C in our experimental conditions, we have used a plasmid containing a (AT)$_m$ sequence: the topoisomers distribution obtained by closure of this plasmid (named pXG 540) respectively at 68.6 and 78.8°C was analyzed in a 2D gel (fig. 6). A shift of about 7 turns was observed between the centers of the distributions shown in figures 6A and 6B (as determined by band counting) instead of 1.3 turn predicted by standard unwinding between 68.6 and 78.8°C (see fig. 3). The difference (about 6 turns) is consistent with the specific melting of the 68 bp of the insert (13). Indeed, a second distribution, centered at about the most relaxed topoisomer, possibly exists at 79°C in the electrophoresis of figure 6B (upper right of form II), which likely corresponds to unmelted population of DNA. This distribution may correspond to the expected shift of about 1.3 turn with respect to the population of figure 6A (69°C). In the case of 'wild type' pBR 322, no abnormal shift occurred between 65 and 79°C (see fig. 2B lanes 11 to 13 and fig. 5 B).

In this case, melting of the small A + T rich regions (maximum 15 bp long) likely occurred above 80°C.

**DISCUSSION**

The experiments described in the present paper indicate that the double helix is linearly unwound upon temperature increase. Indeed, this applies to unconstrained DNA (form II) where, before closure by ligase, the DNA ends of one strand are free to rotate around the other strand. The unwinding ratio, defined by $\Delta H/\Delta T$ appears constant in a wide range of temperatures, up to the premelting region. It is important to note that in this region (above 80°C in our ionic conditions), two different DNA populations seem to coexist—a Gaussian population of topoisomers which follows the unwinding rule normally—a population of DNA where the less stable (AT rich) sequences are likely melted. This reflects the fact that at the time of closure, some DNA molecules have their AT sequences melted and others have not. Interestingly, when a long AT region is artificially inserted, the major population of DNA at 79°C, was composed of molecules with the inserted sequence melted (fig. 6B), while no melting occurred in the short AT regions of the natural plasmid. In addition, there is no intermediate distribution visible in figure 6B, indicating that molecules with a partly melted AT insert are not present, and consistent with a highly cooperative melting.

In addition to the helix rotation angle $\Theta$, the torsional constant $K$ of DNA also decreases with temperature. This result indicates that the energy required to modify the DNA twist is lower at high temperature. A possible implication of this finding is that DNA supercoiling would also require less energy in these conditions. Perhaps processes such as replication, transcription, or recombination, which need the unwinding of the DNA would be facilitated at high temperature.

Finally, the present data have several applications for people working on the stability of DNA duplex (17–19) and on the enzymology of DNA at high temperature. For instance: (i) it is possible to determine the twist of a DNA at any temperature below the melting point from its value at a given temperature, (ii) in the case of a covalently closed circular DNA, one can deduce the actual degree of supercoiling in the test tube at high temperature from electrophoretic data obtained in agarose gel at laboratory temperatures. (iii) It is possible to estimate the $\text{in vivo}$ actual superhelical density (linking number difference) of viral or plasmid DNA extracted from thermophilic organisms (5, 20, 21). (iv) It is possible to define the relaxed state for covalently closed circular DNA at high temperature.

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*Figure 6. Bidimensional gel electrophoresis of plasmid pXG 540 in the premelting temperatures range. Conditions of electrophoresis are described in Materials and Methods. A. Plasmid pXG 540 closed by ligase at 68.6°C B. pXG 540 closed at 78.8°C. The intense band in A and B is form II DNA. Immediately on the upper right of this band is the most relaxed topoisomer.*
REFERENCES