Unpaired bases in superhelical DNA: kinetic evidence

R.J.Jacob*, J.Lebowitz*, M.P.Printz/≠

*Dep. of Biology, Syracuse University, Syracuse, New York, USA
/The Rockefeller University, New York, New York, USA

Received 14 January 1974

ABSTRACT

Kinetic analysis of the early, fast reaction of superhelical DNA with formaldehyde reveals that this region or regions is 56% "single strand like" in character. Hydrogen-tritium exchange studies coupled with other considerations show that this reaction is not due to a difference in conformational motility between form I and form II molecules, but is due to unpaired or weakly hydrogen bonded, localized region(s) of the form I allomorph of circular DNA.

INTRODUCTION

It has been reported that the covalently closed superhelical form of circular DNA (form I) has alterations or interruptions in its hydrogen bonded secondary structure[1]. This was partly demonstrated by an early, fast reaction with formaldehyde (HCHO) at 30° which was not observed for the open circular duplex (form II). Recent evidence from an analysis of the binding to DNA of CH_3HgOH, which acts as a chemical probe for unpaired bases[2], as well as results from the action of single strand specific endonucleases[3,4] (see below) strongly support the concept that "single strand like" regions exist in the superhelical but not in the nicked form of circular DNA.

Another interpretation of the above results could be that the enhanced chemical and enzymatic reactivity seen for superhelical DNA (compared to nicked DNA) is only a reflection of their difference in structural motility or breathing rate; namely an increased breathing rate of the entire superhelical DNA. Increased conformational motility might occur from the bending or torsional stresses imposed upon the secondary structure by the superhelical turns[5,6]. One possible way to distinguish the difference between chemical reactivity dependent on increased conformation motility of the whole molecule versus a reaction with localized "single strand-like region(s)" would be to measure the breathing rates of the two forms of circular DNA and see if these are consistent with the data obtained using a

chemical probe (HCHO) of DNA structure. It is the purpose of this paper to discriminate between these alternative proposals by examining both hydroxymethylation rate data and tritium exchange in terms of an appropriate kinetic model.

MATERIALS AND METHODS

The circular DNA used for this study was isolated from the Pseudomonas phage PM2 as described previously[1]. Both form I and form II DNA were used for this study. Tritium exchange was performed by the gel filtration technique, utilizing both one and two column methods[7].

The kinetic reaction[1] of HCHO with form I was monitored spectrophotometrically by a Cary 16 UV-Vis spectrophotometer coupled to a Lauda K-2/R constant temperature unit. The spectrophotometer was equipped with dual wavelength drive, automatic sample changer, recorder (multiple range, 0.1-2.0) and multi-zero suppression interfaces. All temperatures were monitored by a Digitec digital thermometer. The spectral changes at a long λ_m (270 nm) and short, λ_o (249 nm) wavelengths were recorded and subjected to dual wavelength analysis[8,9]. All data plotted as optical charges and fraction of helix measurements[9] will be reported in a later publication[15].

RESULTS

Recently von Hippel and Wong[8] and Utiyama and Doty[9] have applied the following model for the reaction of HCHO with duplex DNA and polynucleotides.

$$\underset{\text{paired DNA}}{\text{Closed base}} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{\text{DNA}}{\text{Unstacked Open Unpaired}} + \underset{\text{Probe}}{\text{Chemical}} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \underset{\text{Product}}{\text{Reacted}} \qquad (1)$$

CONFORMATION REACTION CHEMICAL REACTION

In the reaction scheme (1) k_1 and k_2 represent, respectively, the rate constants for the opening and closing reactions of the cooperative "breathing" unit (one or more base pairs), k_3 represents the second order rate constant of the reaction of the probe with the transiently open form, and k_4 represents the first order rate constant of the back reaction if it occurs. If $k_2 >> k_3$ and k_4 then it can be shown[10,11] that the observed rate constant equals the following expression.

$$k_{obs.} = \frac{k_1}{k_2} \cdot k_3 = K_{conf} \cdot k_3 \qquad (2)$$

Equation 2 simply states that the observed rate of reaction of a chemical probe is equal to the probability or fraction of open conformations

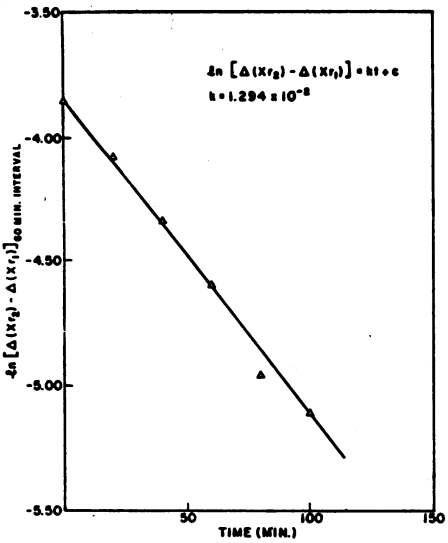

Figure 1. Pseudo first order rate (by Guggenheim analysis) of hydroxymethylation of the altered secondary structure in PM2 form I DNA.

TABLE 1

Comparison of the Conformational Constants of Linear Duplex DNAs and Superhelical DNAs Using HCHO as a Chemical Probe.

	DNA	Temp. C°	$K_{conf.}$	Ref.
1.	Calf Thymus	60	.01	(7)
2.	T7	58	.093	(8)
3.	Nicked PM2 and ØX-RF	30	not measurable	
4.	ØX-RF Superhelical	30	.61	Data of (1)
5.	Calf Thymus	30	$\sim 1.0 \times 10^{-7}$	Extrapolated data (7)
6.	+PM2 - Superhelical	30	.56	This study
7.	‡ØX-RF Single Stranded	30	1.0	(1)

Legend to Table 1

\+ pseudo first (4% HCOH) order $K_{obs} = 1.29 \times 10^{-2}$

‡ pseudo first (4% HCOH) order $K_3 = 2.30 \times 10^{-2}$

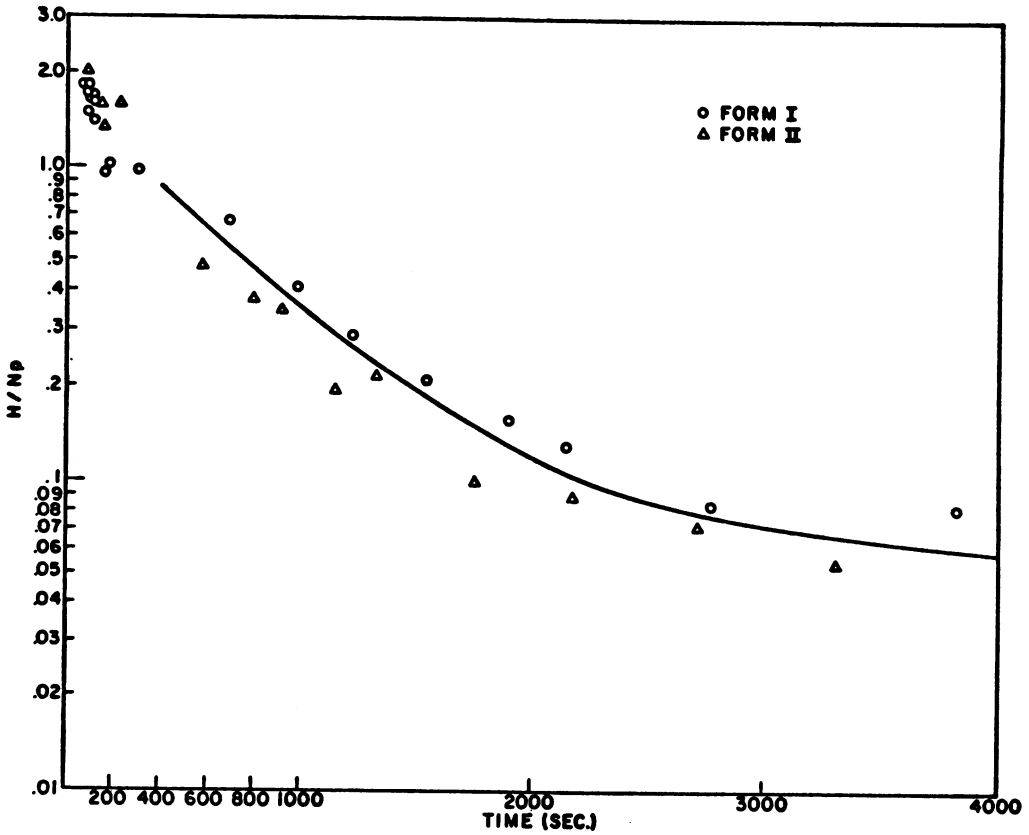

Figure 2 Hydrogen exchange curves for forms I and II. The ordinate is hydrogens remaining unexchanged per nucleotide pair. Tritiated solutions were prepared with preparations of PM2 form I or form II DNA at 1-2 mg/ml in 0·1M NaCl, 0·01M sodium cacodylate buffer, pH 7.6. Sufficient tritiated water was added to obtain a final tritium level of 5-10 mCi/ml. The samples were incubated for 2-3 hours at 0° and run using the two column technique[4,14]. All form I preparations contained from 5-15% form II and did not demonstrate significant conversions upon titration. Concentration of nucleic acid in the column effluents were determined spectrophotometrically using absorption at 260 nm and an $\epsilon_{1\,cm}$ = 6600. No significant difference in the molar extinction coefficient between forms I and II were noted. Levels of tritium were determined by liquid scintillation counting in Bray's solution.

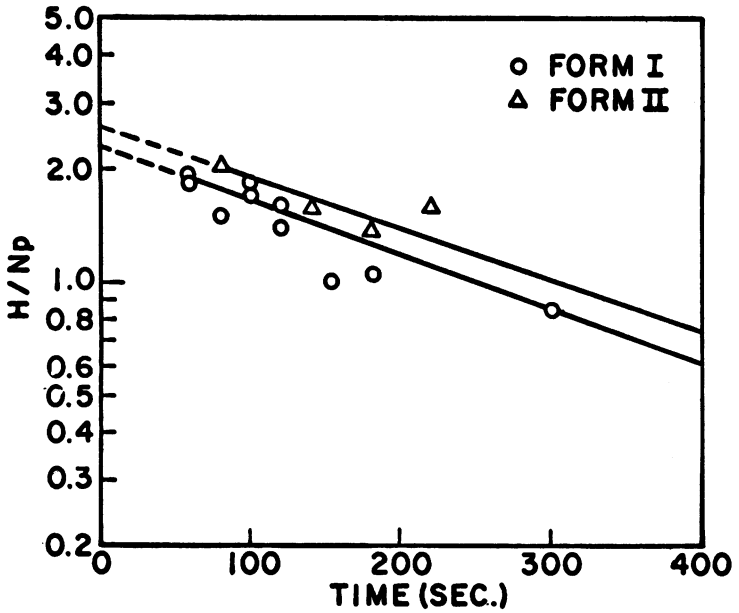

Figure 3. Expanded plot of the early time data from figure 1.

(open/closed) times the rate of reaction of the unpaired state with the probe, i.e. the rate of reaction with single stranded DNA. Therefore a separate measurement of k_{obs} and k_3 for duplex and denatured DNA respectively allows one to measure K_{conf}, the extent of transiently open conformations.

Table 1 presents some of the data from the studies in references 8 and 9 with the application of eq. 2 in calculating K_{conf} for superhelical ØX-RF and PM2 DNAs. The latter PM2 calculation employs the more refined dual wavelength analysis used in the studies of duplex DNA and polynucleotides[8,9]. This approach allows us to separate out the relative spectral contributions due to unstacking and hydroxymethylation. The rate of pure hydroxymethylation (k_{xr}) is $1.29 \times 10^{-2} min^{-1}$ by Guggenheim analysis as shown in figure 1. Therefore the K_{conf} for PM2 is considered to be the more reliable estimate of the fraction of "open regions" in this superhelical DNA. Correction of ØX-RF data would undoubtedly give a comparable result from the previous kinetic data[1].

When the K_{conf} of the HCHO reaction for superhelical ØX-RF and PM2 are compared with other DNAs (Table 1) it is readily seen that the former molecules are highly unusual. They react with HCHO as if 55-60% of the molecules were in a transiently open conformation at 30°. The K_{conf} value obtained

from dual wavelength analysis on PM2 DNA, estimates that this region or regions exist on the average as 56% open "single strand like" structure. Obviously if this is the case we would expect an enormous motility or breathing in H^3 exchange studies.

Comparison of Exchange Curves: The results are plotted in Fig. 2. It can be seen that the kinetics of the hydrogen exchange-out process for both forms are similar if not identical. The points at times less than 300 seconds can be represented by a straight line and considered as a single kinetic class (Fig. 3). The initial linear region extending from 50 seconds to 300 seconds can therefore be represented as a single first order kinetic class with an apparent, uncorrected half time of exchange of 125 seconds. As can be seen this region is the same for both forms I and II. The extrapolation of this early time data to zero time ordinate indicates that the form I equilibrium value is lower than that of form II. The equilibrium value for form II is in good agreement with that calculated from the 42% G + C nucleotide composition of the molecule (2.42 H/np for data with exchange times in excess of 100 seconds[16]). At times greater than 300 seconds the curve for form I is paralleled by that for form II. Because of this parallel decay on a semi-logarithmic plot, both curves reflect a similar distribution of kinetic classes. The lower value of the form I curve indicates slightly fewer hydrogens in these slower classes of hydrogens. The experimental scatter that is present at long times of exchange is accentuated by the semi-logarithmic plot. The upward curvature of the curves at long times suggests a very small (0.01 H/np) contribution from a class of hydrogens with a very long half time of exchange. Such data have been observed previously and probably reflect an artifact of the gel filtration procedure[12].

DISCUSSION

It is clear from this exchange data that (1) the secondary structure of both forms of circular DNA may be characterized by the same breathing rate for tritium exchange (at 0°), and that (2) the superhelical molecule appears to extrapolate to a slightly lower equilibrium number of H/np at zero time. On the other hand, the reaction with HCHO at 30° gives a K_{conf} of .56 which is effectively a DNA molecule that appears to be half denatured by initial hydroxymethylation kinetics.

In the introduction we proposed two models for the reaction of chemical probes with superhelical DNA. 1. The DNA contains a localized region or regions of interrupted or altered secondary structure. These regions are reactive because they contain unpaired bases and behave with substantial

"single strand" character. In this model K_{conf} is a reflection of these regions and not of the whole molecule. 2. The alternative model is that the higher free energy of the superhelical molecule promotes an increase in the breathing rate of the secondary structure due to the bending or torsional stresses produced by supercoiling. In this case K_{conf} reflects the whole molecule and we would observe increased reactivity of all the bases in the molecule. Any chemical reaction must produce a coupled loss of duplex and superhelical turns with a corresponding decrease in stress and K_{conf}.

It should be possible to discriminate between the alternative proposals by examining the predictions one would make for each case. The first model predicts the absence of rapid exchange for the normal secondary structure. The only region(s) undergoing rapid exchange would be the altered secondary structure. This leads to the prediction that some of the hydrogens have been shifted into a faster exchange (instantaneous with regard to the time scale of these experiments) class, and the remaining hydrogens exchange at the rate that is normal for nicked PM2 DNA.

An examination of the experimental data in this report as well as previously published data clearly supports this model. There is indeed an absence of rapid exchange for the superhelical molecule relative to the nicked molecule for all the detectable exchanging hydrogens. Our previous hyperchromicity data suggest that only approximately 4% of the bases can react readily with HCHO at a rate one half of that of single strand DNA (see also Table 1). It is important to note that the extrapolated H/nP is well within the range expected for a small fraction of rapidly exchanging hydrogens that are not detectable, i.e. instantaneous exchange class. The error of the exchange procedure precludes using this approach as an accurate estimate of the unpaired bases. However the value for the region(s) total size of 3.5-4.0%, is supported by recent studies of PM2 DNA[2].

The measurement of K_{conf} in this study was performed at 30°C whereas the H^3 exchange was performed at 0°C. Naturally, it would have been advantageous to have compared respective studies at identical temperatures either 0° or 30°C. Unfortunately the nature of the different chemical events involved in H^3 exchange relative to hydroxymethylation engender experimental limitations preventing such a comparison. Hydrogen exchange proceeds quite rapidly on the time scale of a gel filtration method. At 30°C, the H^3 exchange would increase approximately 10 to 30 fold[7] and this rate would be far too rapid for the columns employed in this study. On the other hand, the reaction of formaldehyde with DNA is virtually quenched at 0°C and we could not obtain any meaningful rate data

at this temperature. This is not unexpected since we would estimate a decrease of k_3 by a factor of 20 and a decrease in K_{conf}[8].

However, the lack of measureable HCHO reactivity at 0°C does not eliminate the possibility of a small fraction of hydrogens exchanging instanteously based upon the following considerations. McConnell and von Hippel[17] have demonstrated that at least two types of fluctuations occur in native DNA that result in different open conformations. One type is formed non co-operatively with low energy of activation and involves little or no unstacking and is effectively sensed by the H^3 exchange probe. The second type involves the cooperative unstacking of bases and is a local and transient manifestation of the usual melting process which occurs in the helix to coil transition zone of DNA. The latter is sensed by HCHO[8]. A partial but permanent opening of unstacked bases would naturally show considerably initial HCHO reactivity at temperature well below T_m and would be counted as a type II fluctuation. However we would anticipate that these regions would fold and stack more readily as the temperature decreased to 0°C with the possibility of forming additional intra-strand base-pairs. This would reduce the rate of reaction with HCHO as the transient open unstacked conformations diminished in frequency. However a small number of hydrogens would still be capable of exchanging rapidly via permanent stacked but unpaired bases in these altered regions. Hence a model in which K_{conf} reflects only the altered secondary structure is compatible with rapid hydroxymethylation at 30° and very diminished reactivity at 0° coupled with a small fraction of rapidly exchanging hydrogens.

If the second model were operative K_{conf} would be a reflection of the breathing of the whole molecule due to superhelical tension. If this unwinding stress or tension makes all base-pairs equally susceptible to hydroxymethylation it should make them all equally susceptible to rapid exchange. As we have indicated above these probes are sensitive to different fluctuations and are utilized at different temperatures due to experimental limitations of the kinetic measurements. In light of this consideration we can not say that our inability to detect rapid exchange rules out this model unequivocally. The temperature decrease should diminish the breathing for all base pairs and it is difficult to envision a mechanism that shifts the bulk of the hydrogens to a normal exchange rate while leaving a small fraction that appears to exchange instanteously.

Also, in this latter model we must have a mandatory coupled loss of superhelical and duplex turns. An analysis of the variation of the super-

helical content vs. hydroxymethylation does not support this prediction, i.e. there is no coupled loss of duplex and superhelical turns[1]. Instead initial formylation, under identical conditions used in this study, produces an increase in superhelical turns[1]. This has been confirmed by another approach with HCHO (Chaudhuri and Lebowitz, unpublished data) and also occurs for the case of CH_3HgOH[2].

Lastly, we turn our attention to the study of the action of the S1 single strand specific endonuclease on superhelical SV40 DNA. Beard, Morrow and Berg have shown that this enzyme cleaves SV40 specifically in only 2 regions of the DNA[3]. These same locations plus one additional site have been confirmed by using a water soluble carbodiimide as a chemical probe of SV40 DNA (Lebowitz, Chen, Garon & Salzman, unpublished data).

The results of this report coupled with our previously superhelical data[1] and the very recent nuclease studies cited above offer conclusive evidence in our minds for localized region(s) of interrupted or altered secondary structure in superhelical DNA.

The structural details and mechanism for the creation of these regions is currently under investigation. A preliminary discussion of structural models that could explain the observed superhelical changes has been offered[2] and this will be expanded upon in the future. It is tempting to speculate on the possible role this structure could play in replication and transcription as well as other cellular processes but this is beyond the scope of this report.

ACKNOWLEDGEMENTS

The authors would like to thank Mrs. F. Nehmadi and Miss H. Williams for their superior technical assistance during this work. We would also like to acknowledge Dr. M. Rush for assistance in centrifugation and to Dr. M. Gutai and Mrs. G. Ventura for help in manuscript preparation. This work was supported by a grant from the National Cancer Institute CA12784, and one of us, J. L., is a recipient of a Career Development Award CA7051402 from the National Cancer Institute, U. S. Public Health Service.

REFERENCES

1 Dean, W. W. and Lebowitz, J., (1971) Nature New Biology. 5, 231-238.
2 Beerman, T. A., and Lebowitz, J. (1973) J. Mol. Biol. 79, 451-470.
3 Beard, P., Morrow, J. F., Berg, P. J. of Virology, 12, 1303-1313.
4 Kato, A. C., Bartok, K., Fraser, M. J. and Denhardt, D. T. (1973) Biochem. Biophys. Acta, 308, 68-78.
5 Wang, J. C. (1969) J. Mol. Biol. 43, 25-39.
6 Bauer, W. & Vinograd, J. (1970) J. Mol. Biol. 47, 419-435.
7 Printz, M. P., and Von Hippel, P. H. (1968) Biochemistry, 7, 3194-3206.
8 Von Hippel, P. H., and Wong, K. (1971) J. Mol. Biol. 61, 587-613.

9 Utiyama, H., and Doty, P. (1971) Biochemistry, 10, 1254-1264.
10 McConnell, B. and von Hippel, P. H. (1970) J. Mol. Biol. 50, 297-316.
11 Hvidt, A. and Nielsen, S.O. (1966) Advan. Protein Chem. 21, 287-386.
12 Rosenfeld, A. R., Stevens, C. L. and Printz, M. P. (1971) Biochemistry 9, 4971-4981.
13 Hanson, C. V. (1971) J. Mol. Biol. 58, 847-863.
14 Printz, M. P. (1970) Biochemistry 9, 3077-3086.
15 Jacob, R. J., Lebowitz, J. (1973) Manuscript in preparation.
16 Englander, J. J., von Hippel, P. H. (1972) J. Mol. Biol. 63, 171-177.
17 McConnel, B. and von Hippel, P. H. (1970) J. Mol. Biol. 50, 317-332.

≠ Division of Pharmacology, Department of Medicine, Room 2042, Basic Science Bldg., UCSD, La Jolla, California 93027, USA