The Influence of the Thymine C5 Methyl Group on Spontaneous Base Pair Breathing in DNA

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Sequences of four or more AT base pairs without a 5′-TA-3′ step, so-called A-tracts, influence the global properties of DNA by causing curvature of the helix axis if phased with the helical repeat and also influence nucleosome packaging. Hence it is interesting to understand this phenomenon on the molecular level, and numerous studies have been devoted to investigations of dynamical and structural features of A-tract DNA. It was early observed that anomalously slow base pair-opening kinetics were a striking physical property unique to DNA A-tracts (Leroy, J. L., Charretier, E., Kochoyan, M., and Gueron, M. (1988) Biochemistry 27, 8894–8896). Furthermore, a strong correlation between DNA curvature and anomalously slow base pair-opening dynamics was found. In the present work it is shown, using imino proton exchange measurements by NMR spectroscopy that the main contribution to the dampening of the base pair-opening fluctuations in A-tracts comes from the C5 methylation of the thymine base. Because the methyl group has been shown to have a very limited effect on the DNA curvature as well as the structure of the DNA helix, the thymine C5 methyl group stabilizes the helix directly. Empirical potential energy calculations show that methylation of the tract improves the stacking energy of a base pair with its neighbors in the tract by 3–4 kcal/mol.

To rationalize the sequence-specific binding of proteins and ligands to DNA it is important to gain a proper understanding of the relationship between the sequence of bases in DNA and the stability and structure of the DNA helix (1, 2). An example is the nucleosome packaging in chromatin, which is modulated by sequence-directed nucleosome positioning (3–6). Furthermore, it has been shown that properly positioned adenine tracts increase the accessibility of nearby promoter regions by their intrinsic tendency to oppose nucleosome binding (6, 7). Hence, alterations of the nucleosome packaging affect the accessibility of promoters and change expression. In this context, it is important to understand the structural and dynamical properties of A-tract DNA.

An example of A-tract DNA influencing the global properties of DNA is the bending of the helix axis that occurs when A-tracks are phased with the helical repeat of the double helix, originally discovered from the anomalously slow migration in polyacrylamide gels displayed by such sequences (8, 9). The A-tracks must be at least four base pairs long and may contain a 5′-AT-3′ step but not a 5′-TA-3′ step to produce significant bending (10–12). Hence, A-tracks of the type 5′-A-nmTA-3′, n + m > 3, produce bending when repeated in phase with the helix screw (13, 14). Apart from the importance of A-tracks for the organization of DNA in chromatin it has become increasingly apparent that intrinsic bending of DNA is likely to play an important role in gene expression and replication (15–18).

Although the presence of DNA bending in these types of alternating sequences is undisputed the explanation of the phenomenon on the base pair level has been debated (19–24). Crystal structures reveal that the base pairs in A-tracks are highly propeller-twisted and that the minor groove is unusually narrow (25, 26). NMR measurements carried out in solution (27–29) as well as hydroxyl radical cleavage patterns (30), cyclobutane thymine-thymine dimerization, (31) and uranyl photo-probing (32) are compatible with this type of structure.

Another feature typical of A-tract DNA is unusually slow imino proton exchange rates measured by NMR spectroscopy, signifying anomalously slow base pair-opening kinetics (33–36). A striking correspondence with gel mobility data was found. The gel migration anomaly produced by repeating A-tracts is highly sensitive to changes in the base pair composition of the tract. Substitution of the central base pair in the tract by a GC base pair completely restores normal mobility whereas introduction of a single IC base pair has almost no effect (37, 38). These results were paralleled in the base pair-opening kinetics measurements where insertions of base pairs in the tracts known to restore normal mobility and consequently diminish the bending, always led to a decrease of the base pair lifetimes to more “normal” levels, whereas an inserted IC base pair only caused a small reduction in the lifetimes. No energetic or structural explanation has yet been given to the anomalously slow kinetics, although it has been assumed that the structural features that set A-tract DNA apart from general sequence DNA, e.g. high propeller twist and narrow minor groove, in some way also are responsible for the unusual kinetics (33).

Recently, we found that the base pair-opening kinetics in G-tracts, contrary to what has been observed for A-tracts, is unusually fast in particular with high opening rates (39). One of many distinctive features of A- and G-tract DNA (39) is C5-methylation, which is present on the thymine base but...
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EXPERIMENTAL PROCEDURES

Sample Preparations and Titrations—All oligonucleotides were purified on NAP-10 columns (Amersham Biosciences), dissolved in a 5 mM borate buffer (90% H3O 10% D2O) adjusted to pH 8.8 and containing 100 mM NaCl. The duplex concentrations were in the range of 0.2–2 mM. Base catalyst titrations of the duplexes were carried out at 15 °C with a 6.3 mM ammonia buffer adjusted to pH 8.8. The pH value of the buffer was measured with a double-junction high-salt Orion 8103 Ross electrode, and the [acid]/[base] fractions of the buffer were obtained from the amounts of salt and liquid base used to prepare the buffer. The thymidine and deoxyuridine mononucleosides were dissolved in a 10 mM phthalate buffer to a concentration of 1 mM. The samples were adjusted to pH 4.0 in the NMR tube with an Orion 9826 Micro-pH electrode with the NMR tube immersed in a thermostatically controlled water bath maintained at 15 °C before exchange measurements were carried out by titration of a 0.4 mM NH4Cl solution to the mononucleosides. The Henderson-Hasselbalch equation was used to calculate the pH value using a pHKs value of 5.6 for ammonia at 15 °C (50).

Imino Proton Exchange Theory—The connection between base pair opening and imino proton exchange is based on the assumption that exchange of the imino proton only occurs when the hydrogen bond to the acceptor of the complementary base in the base pair is shifted to some other proton acceptor present in the solvent, i.e. the base pair has opened (51). Several exchange pathways are possible. Direct exchange to water or exchange catalyzed by the complementary base always occurs, although rather inefficiently due to the low pHKs value of theses acceptors. By addition of a catalyst with higher pHKs values, e.g. ammonia, near opening-limited exchange can be reached. For a base pair with multiple open states, the exchange rate is given by

\[
\sum_{n=1}^{m} \frac{k_n}{k_{ex}} = \sum_{n=1}^{m} \frac{k_n}{k_{ex}} \frac{k_{ex}}{k_{ex}}
\]

where \( k_n \) is the imino proton exchange rate of the mononucleoside per mole of added base

\[
k_{ex} = \sum_{n=1}^{m} \frac{k_n}{k_{ex}}[B]
\]

and \( \alpha = \) a parameter taking into account the different accessibility of the imino proton in the open states and in the mononucleoside. For a base pair with a single opening mode (\( n = 1 \)) and with \( k_n \ll k_{ex} \), Equation 2 can be rewritten as Equation 4

\[
\tau_{ex} = \tau_{ex} + \frac{1}{K_{ex}k_{ex}[B]}
\]

where \( \tau_{ex} \) and \( \tau_{ex} \) are the inverse exchange and opening rates, respectively, and \( K_{ex} = k_{ex}/k_{ex} \) is the base pair dissociation constant. If Equation 4 is valid, a plot of \( \tau_{ex} versus 1/[B] \) yields a straight line where \( \tau_{ex} \) is obtained from the y-axis intercept and \( K_{ex} \) is obtained from the slope.

Imino Proton Resonance Assignments—Imino proton resonance assignments of the duplexes were obtained from NOESY1 experiments, run with a mixing time of 250 ms at 15 °C on a Varian Inova 600 MHz spectrometer. A jump-return observe pulse was used to avoid excitation of the solvent resonance (52). Linear prediction was employed in the indirect dimension to increase resolution. All two-dimensional data processing was carried out with Felix97 (Molecular Simulations Inc.).

Exchange Measurements—The NMR experiments on the duplexes and the mononucleosides were carried out on a Varian Inova 600 MHz spectrometer. The imino proton exchange times \( \tau_{ex} \) at different catalyst concentrations, were obtained from measurements of the inversion recovery times in presence (\( T_{1ex} \)) and in absence (\( T_{2ex} \)) of exchange catalyst according to Equation 5.

\[
\frac{1}{T_{1ex}} = \frac{1}{T_{1ex}} + \frac{1}{T_{2ex}}
\]

Apart from longitudinal dipolar relaxation, direct exchange to water as well as exchange catalyzed by OH ions and the acceptor nitrogen of the opposite base (53) contribute to the recovery rate of the imino protons in the absence of added catalyst (\( 1/T_{2ex} \)). However, these contributions remain constant when the catalyst is added and will be canceled in Equation 5. Consequently, the exchange time \( \tau_{ex} \) represents exchange only via the added catalyst.

The inversion recovery experiment utilized a 1–1.4- ms iBURP pulse for selective inversion (54) and a 0.7–1- ms Gaussian observe pulse for selective detection (55). Right shift and linear prediction of the free induction decay were employed to correct for magnetization evolution during the observe pulse. For the mononucleosides, exchange times were also obtained from the line widths of the imino proton resonances in presence \( (\tau_{ex}) \) and in absence \( (\tau_{ex}) \) of exchange catalyst according to Equation 6.

\[
\tau_{ex} = \frac{1}{\pi} \left( \frac{1}{T_{1ex}} - \frac{1}{T_{2ex}} \right)
\]

Line widths were measured in spectra acquired with a jump-return observe pulse (52).

Stacking Energy Calculations—Before the base pair stacking calculations were carried out the geometries of the isolated base pairs, with the sugar-phosphate backbone replaced by hydrogens, were optimized utilizing the Hartree-Fock (HF) approximation with the 6–31(d,p) basis set of atomic orbitals. The centers of mass of both base pairs in a base pair step were overlaid (implying no slide and shift), and a helical twist of 36 ° was introduced by a counterrotation of the base pairs around an axis passing through their center of mass (56, 57). When calculating the stacking energy of a base pair step the propeller twist was introduced as a counterrotation of the bases around the C-8(pur)-C-6(pyrim) base pair axes in the same way for both base pairs of the step. The vertical separation is between consecutive base pairs was optimized for each value of propeller twist. Note that optimization of vertical distance between base pairs is critically important to obtain a correct energy profile (58, 59).

Base stacking energies were calculated using a standard empirical force field of the form (Equation 7)

\[
E^{S}(kcal/mol) = -\sum_{A} A_{Q} + \sum_{B} B_{Q} + 332 \sum_{Q} Q_{Q} /= \sum_{Q} Q_{Q}
\]

where \( A_{Q} \) and \( B_{Q} \) are inter-atomic distances, \( Q_{Q} \) and \( Q_{Q} \) are atom-centered point charges, and \( A_{Q} \) and \( B_{Q} \) are constants of the van der Waals term taken from the Cornell et al. force field (60) with one modification specified below. The atomic charges were derived by fitting to the molecular electrostatic potential around the bases obtained using the extended aug-cc-pVDZ basis set of atomic orbitals. The quality of the force field was tested against reference ab initio quantum-chemical calculations carried out at the MP2 level with diffuse-polarized 6–31G* (0.25) basis set of atomic orbitals and with correction for the basis set superposition error (56, 57, 61–63). We carried out 12 base pair step stacking ab initio calculations and about 20 adenine-thymine and thymine-thymine dimer stacking calculations. The empirical potential and ab initio calculations presented here are based on our extensive experience with base stacking calculations, and more details about the techniques and their accuracy can be found in the preceding studies (56, 57, 61–63). Based on these calculations, the van der Waals parameters of the force field were modified by reducing the van der Waals radius of the methyl group hydrogen atoms from 1.487 to 1.087 Å. Let us briefly justify this modification. The modification is due to a clear discrepancy between the original parameterization and the reference ab initio calculations leading to steric clashes, in common B-DNA geometries, between the methyl group and the adenine ring in the ApT steps. When relaxing the vertical separation of the base.

1 The abbreviation used is: NOESY, nuclear Overhauser effect spectroscopy.
pairs, the unmodified force field shows substantially earlier onset of the methyl-adenine repulsion, compared with the MP2/6-31G**(0.25) data. It should be noted that although the Cornell et al. (60) force field has been parameterized with the aid of Hartree-Fock quantum chemical calculations, no base stacking calculations were considered in the parameterization. Our modified parameters provide very close agreement with the reference ab initio data, and for the present special-purpose base stacking calculations such corrected parameters are superior to the original parameterization. It does not mean, however, that we suggest using the new parameters in condensed phase simulations unless their balance with other parameters of the force field is validated. Work is in progress in our laboratory to investigate the effects of the modification on explicit solvent simulations of nucleic acids, and the results together with the quantum chemical calculations will be presented in a forthcoming paper.

RESULTS

Mononucleoside Exchange—To utilize the exchange of the imino protons with the water protons to measure base pair-opening kinetics it is necessary to know the exchange expected for the imino proton in the free nucleoside where it is freely exposed to the solvent (see Eq. 2 and 4).

The exchange rates of the nucleoside imino protons were obtained from resonance broadening measured when increasing amounts of ammonia was added, using Equation 6. The imino proton transfer rates per mole of added base (k_r') values of (1.72 ± 0.03) × 10^8 and (2.12 ± 0.04) × 10^8 s^-1 mol^-1 for dT and dU, respectively. 

![Fig. 1. Ammonia catalysis of the imino proton exchange rates of thymidine and deoxyuridine at 15 °C and pH 4.0.](image)

The kinetic data were obtained by fitting to Equation 2 or 4 utilizing the mononucleoside transfer rates obtained from the fits in Fig. 1. The data for the T- and U-tracts are given above and below the sequence, respectively. Only one strand of each oligomer is shown with the 5’-end, from which the base pairs are numbered, indicated.

| Seq | 5°C A T A A | 5°C G C G C |
|-----|-------------|-------------|
| T   | Y Y A T G   | Y Y C G C   |
| U   | 0.19 104    | 0.2 0.7     |

---

**Table I**

Kinetic parameters for base pair opening of self-complementary T- and U-tracts at 15 °C

| Seq | 5°C A T A A | 5°C T G T G |
|-----|-------------|-------------|
| T   | Y Y C G C   | Y Y C G C   |
| U   | 0.1 1.7     | 0.1 1.7     |

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**Footnote:** The kinetic parameters for the fast mode of a fit to a two-state kinetic model (Equation 2) is given.

**Footnote:** The resonances of the two imino protons merge in the course of the catalyst titration.
higher for the central base pair if the pyrimidine bases are unmethylated (see above) and the base pair lifetime is also shorter (see above).

However, the dampening of the base pair fluctuations are largest in the interior of the tracts. For example, the central AT base pair of d(CATA2T2ATG)2 has a base pair lifetime of 18 ms and an apparent dissociation constant of 0.5 \( \times 10^{-6} \), whereas the base pair lifetime is 4.5 times shorter and the base pair dissociation constant more than 40 times higher for the corresponding central AU base pair of d(CATA2U2ATG)2. As a second example, the AT6 and AU6 base pair of the A4Y4 tracts (Fig. 2C) have base pair lifetimes of 149 and 26 ms and dissociation constants of 0.1 \( \times 10^{-6} \) and 0.8 \( \times 10^{-6} \), respectively (Table I). Hence, the dissociation constant for the AU base pair is a factor of 8 higher, whereas the base pair lifetime is a factor of 6 shorter (Table I).

It can also be seen that the kinetics in the A2Y2-tracts of the dodecamers d(CGCGA2Y2CGCG)2 is slower than in the decamer d(CATA2Y2ATG)2, in particular for the outer base pair of the A2T2-tracts, probably reflecting reduction of the end fraying by the longer flanking sequences in the dodecamer.

The second trend evident by inspection of Fig. 2, and common to both the U- and T-tracts, is an increased damping of the base pair-opening fluctuations when the tracts become longer. For example, the central AU base pair of the decamer d(CATA2U2ATG)2 has almost the same base pair-opening kinetics as the reference AT base pair. The central AU7 base pair of d(CAGA4U4CTG)2, on the other hand, exchanges much slower than the reference. As judged from the dissociation constant the base pair is 20 times more stable, whereas the lifetime shows that the base pair opens six times less frequently than the reference base pair (AT) in this case (Table I). As mentioned above, the stability of the T-tracts are even larger. The central AT7 base pair of d(CAGA4T4CTG)2 is 60 times more stable and opens 50 times less frequently than the central reference AT base pair of d(CATGATCATG)2. Another common feature for the exchange of the two types of A4Y4-tracts is a destabilization of the central AY-step compared with the neighboring AA-steps (Fig. 2, Table I).

It is shown in Fig. 2, D–F that the base pair flanking the central tracts, AT3 of d(CATR2Y2ATG)2 and GC3 of d(CGCGA2Y2CGCG)2 and d(CAGA2Y2CTG)2, displays a very similar kinetics independent of the state of methylation of the tracts. This shows that the faster base pair-opening dynamics in the U-tracts compared with the T-tracts is not due to an overall lower stability of the U-oligomers.
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Stacking Energies—Fig. 3 shows how the calculated intrinsic stacking energies for the central base pair of the trinucleotides AAA(TTT), AAA(UUU), AAT(ATT), and AUA(AUU) depend on the propeller twist. For all trinucleotides, propeller twisting is energetically favorable with an energy minimum found when the propeller twist is in the range of 12–20 °. The energy gain by propeller twisting is 3 kcal/mol for AAA(TTT), 2 kcal/mol for both AUA(UUU) and AAT(ATT), whereas the smallest improvement, 1 kcal/mol, is calculated for the propeller twisting of AAA(UUU). Furthermore, for all values of the propeller twist the U → T methylation improves the stacking energy. At the optimal propeller twist the U → T methylation provides 3 and 4 kcal/mol of stabilization for the central base pair of the AAA(UUU) and AUA(AUU) trinucleotides, respectively. From Fig. 3 it can also be seen that inclusion of a RY-steps leads to a less favorable stacking energy and that the energy penalty becomes more pronounced when the propeller twist increases. Examination of the different terms contributing to the energies in Fig. 3 reveals that stabilization mainly originates from the van der Waals energy, whereas the electrostatic term is weakly repulsive and insensitive to the propeller twist magnitude. The stacking energy is dominated by intra-strand base terms, whereas the interstrand cross-peaks connecting AH2 of the central AT/U base pair with the H-1' sugar proton of the 5' neighbor of the complementary T/U nucleotide.

DISCUSSION

In the present work we have investigated the base pair-opening dynamics in a series of oligonucleotides with A-tract cores of the form $A_nT_n$, where the pyrimidine base Y either is thymidine or deoxyuridine. It is well known that the exocyclic N-2 amino group strongly influences the extent of bending as detected by polyacrylamide gel mobility, both when introduced inside the A-tracts and in the intervening sequences (32, 37, 38). However, the effects of the exocyclic C5 methyl group on polyacrylamide gel mobility is much smaller (46–49).

Particularly striking is the results by Hagerman on the polyacrylamide gel mobility of repeating DNA of the form $G\alpha T\beta C\gamma$, and $(G\delta U\epsilon C\zeta)$. Although the sequences formed by both repeating units display a similar and pronounced reduction of the electrophoretic mobility, the migration of the non-methylated $G\delta U\epsilon C$ is in fact the slowest (48). The small influence of C5 methylation is also supported by an earlier study by Koo and Crothers (37) on repeating DNA sequences where the tracts were $A_5T_5$ and $A_5U_5$. In this case only a slight increase of mobility of non-methylated tract was found. Hence, in view of the strong correlation between anomalous polyacrylamide gel mobility and base pair-opening kinetics previously observed (33) a large effect by methylation on the base pair-opening dynamics would not be expected.

Furthermore, only small differences are discernable when structures with and without the C5 methyl group are compared in crystals (40, 43) as well as in solution (41, 42, 44, 45). The NOESY spectra of the d(CATA$_2$T$_2$ATG)$_2$ and d(CATA$_2$U$_2$ATG)$_2$ oligomers that have been investigated in the present study are in agreement with these earlier studies. In Fig. 4 the H6/H8-H1' cross-peak regions are shown, and it is evident that the cross-peak intensities are virtually identical in the two oligomers, indicating that the base-sugar distances are independent of the C5 methylation. Most importantly, a cross peak connecting A5H2 with the sugar proton of the 5' neighbor of the complementary T/U nucleotide is present in both sequences with similar intensity. The presence of this type of cross-peaks is a
typical feature of A-tract DNA and arises from a high propeller twist that compresses the minor groove (27, 66). Moreover, chemical shift differences are mainly restricted to protons in the direct vicinity of the C5 positions (Fig. 4). These spectral features indicate that the C5 methyl group exerts only a minor influence on the helix structure in keeping with crystal (40, 43) as well as solution structure studies (41, 42, 44, 45).

In view of this lack of significant structural impact by the U → T C5 methylation, the very large effect on the base pair-opening dynamics seen in Fig. 2 is surprising. For example, in Fig. 2A both AU base pairs of d(CATAUUATG)₄ exhibit faster base pair-opening dynamics than the central AT base pair of d(CATGATCATG)₄ that is represented by a solid line in Fig. 2, A–C and used as a reference for non-A-tract kinetics. The central AT base pair of d(CATATTATG)₂ displays much slower kinetics with about a 40-times lower dissociation constant than the corresponding unmethylated base pair (Table I), whereas the outer base pair of the A₂T₂ tract exhibits kinetics closely similar to the reference base pair (Fig 2). Given that the helical structures of d(CATAUUATG)₄ and d(CATATCATG)₄ are closely similar, the anomalously strong damping of the base pair dynamics of the AT base pairs of the A₂T₂ tract can be entirely attributed to the presence of C5 methyl groups. The kinetic behavior of the decamers (dCGCGATY₆CGCG)₂ in Fig. 2B is similar to that of the decamers, although overall somewhat slower both in the U- and T-tract.

From the results on the longer tracts in Fig. 2C it is, however, clear that the unmethylated A₂U₂-tract exhibits anomalously slow imino proton exchange kinetics as well, in fact, quite similar to the exchange kinetics of the central AT base pairs of the A₂T₂-tracts. Hence, methylation is not the only factor that contributes to the damping of base pair-opening fluctuations in A-tracks. Because the A-tract type of structure is known to cooperatively build up with the length of the tract (67) and if we assume that the structure roughly is independent of the state of methylation, we may conclude that the A-tract type structure also exerts a dampening effect on base pair-opening fluctuations by itself even in the absence of methylation. However, the largest contribution to the suppression of base pair opening in A-tracts clearly comes from the C5 methylation (Fig. 2, Table I).

It was recognized early that propeller twisting of the base pairs in many dinucleotide steps are energetically beneficial (68, 69). Notable exceptions are 5′-YR-3′ steps where a purine-purine cross-strand clash prevents propeller twisting and steps including the guanine base where the minor groove N-2 amino group sterically interferes with propeller twisting. The calculated stacking energies of Fig. 3 have been obtained by adding the two dinucleotide stacking energies of the central base pair of a trinucleotide. It is seen that propeller twisting lowers the stacking energy of all base pairs of the tracts, including those of the central 5′-AY-3′ step of the A₂Y₂-tracts. Furthermore, methylation improves the stacking energy, and this improvement becomes larger for larger propeller twists. Hence, favorable stacking energies contribute to the higher stability of the methylated tracts and may also favor higher propeller twist.

Other effects may also contribute to the reduction of base pair breathing by the C5 methyl groups. Besides improving the stacking energies between the base pairs the methyl group is also likely to alter the hydration pattern in the major groove, which potentially could affect the dynamics. Furthermore, the larger hydrophobicity of the thymine base should favor less solvent-exposed geometries, and this could possibly yield a reduced base pair breathing relative to that of the uracil base.

Another reason for the higher stability of AT versus AU base pairs could be stronger hydrogen bonds in the former. To investigate this possibility we calculated the base pair interaction energies on the MP2/6–31G**//HF-6–31G** level. For the AT and AU base pairs the interaction energies were −12.35 and −12.47 kcal/mol, respectively. The difference in base pair hydrogen bond strengths is consequently negligible.

In view of the structural similarity of tracts with and without C5 methylation, it is not likely that the propeller twist is much different in the T- and U-tracks on average. Rather the stability toward the flattening of the propeller twist of the base pairs should be higher in the T-tract. All base pair dissociation constants in Table I fall in the range 10⁻¹⁰⁻¹⁷. Hence, only a very small fraction of the ensemble of a particular base pair is opened at any time, whether being a part of a methylated tract or not. Consequently, this fraction does not contribute significantly to the average structure that is reflected in NMR resonance chemical shifts and nuclear Overhauser effect intensities (Fig. 4). The lack of structural impact of C5 methylation as evidenced by Fig. 4 and previous studies (41, 42, 44, 45) is therefore compatible with the large dynamical effects.

In summary, we have in the present study shown that the main factor contributing to the anomalously slow base pair-opening dynamics in A-tract DNA is the C5 methyl group of the thymine base, which provides a continuous methylation of the major groove in A-tract DNA. Potential energy calculations using the Cornell et al. force field (60) suggest that improved stacking energy contributes to the unexpected dynamic stabilization exerted by the methyl group. Structural alterations of the helix caused by the methylation are likely to play a minor role.

It is intriguing to speculate that a similar dampening of the base pair-opening fluctuations by methylation of the cytosine base contributes to reduction in gene expression commonly observed by this base modification (70, 71). In this case it is unlikely that the base pair will take on a high propeller twist. However, as seen from Fig. 3, for a flat base pair methylation is energetically favorable also.

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