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Molecular Dynamics of Hemiprotonated Intercalated Four-Stranded i-DNA: Stable Trajectories on a Nanosecond Scale

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Abstract: Molecular dynamics (MD) simulations are presented of hemiprotonated four-stranded intercalated d(CCCC)$_4$ and d(CCCA)$_4$, utilizing crystal coordinates as starting models. The central core region of these i-DNA molecules, consisting of consecutive layers of hemiprotonated cytosine•cytosine$^+$ (C•CH$^+$) base pairs, is exceptionally stable in all simulations, with root-mean-square deviations (RMSd) between theoretical and crystal structures around 1 Å. This result is surprising, as consecutive layers of hemiprotonated C•CH$^+$ base pairs are characterized by highly unfavorable base stacking interactions due to electrostatic repulsion between base pairs that carry a positive charge. In addition, MD simulations have been carried out of theoretical d(CCCC)$_4$ structures with alternating protonated C•CH$^+$ and neutral C•imC base pairs utilizing the imino cytosine tautomer to eliminate the electrostatic repulsion between consecutive protonated bases. These simulations yield again stable structures with only slightly higher deviations from the crystal data compared to the protonated structures, hinting at the possibility of some involvement of the imino cytosine tautomer in stabilizing i-DNA molecules. In the course of the simulation of d(CCCA)$_4$, the adenine•adenine (A•A) base pairs that extend the four-stranded intercalated cytosine motif are disrupted, providing further evidence that the stability of i-DNA originates primarily in the hemiprotonated C•CH$^+$ core region. All simulations were carried out with the AMBER4.1 force field, using the particle mesh-Ewald technique for electrostatic interactions, with total length close to 15 ns. The maintenance of a stable structure in the simulations challenges the traditional views on the role of base stacking in stabilizing nucleic acid conformation and illustrates the complexity of interactions in biomolecules.

Introduction

Nucleic acids show enormous conformational variability. Recent NMR and X-ray studies revealed an unusual four-stranded structure called i-DNA, which consists of two parallel duplexes stabilized by hemiprotonated cytosine•cytosine+ (C•CH$^+$) base pairs that intercalate into each other with opposite polarity (Figures 1 and 2).[1,2] All cytosines are equivalent, with a fast proton transfer within the hemiprotonated pairs.[1] Further characteristics are a stacking distance of 3.1 Å, a slow right-handed helical twist (12−18°), and stabilizing C−H···O sugar "zippers" in the narrow grooves of the molecule.[2d] Although there is some indication that base pairs other than C•CH$^+$ can be incorporated into the i-DNA motif, it is assumed that the stability originates in the "C•CH$^+$" core region.[1,2]

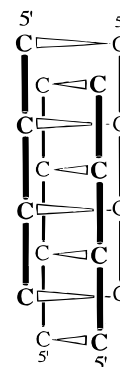

Figure 1. Schematic representation of hemiprotonated intercalated four-stranded i-DNA, d(CCCC)$_4$. Two parallel duplexes held together by C•CH$^+$ base pairs intercalate into each other with opposite direction. Hydrogen bond interactions are depicted by tapered bonds. 5′ termini of the strands are marked.

DNA sequences with stretches of cytosine residues occur frequently in the genome of cells, for example, at the ends of eucaryotic chromosomes, in the telomeres.[3] Telomeric sequences are thought to be important for the maintenance of chromosome integrity during replication. Structural investiga-

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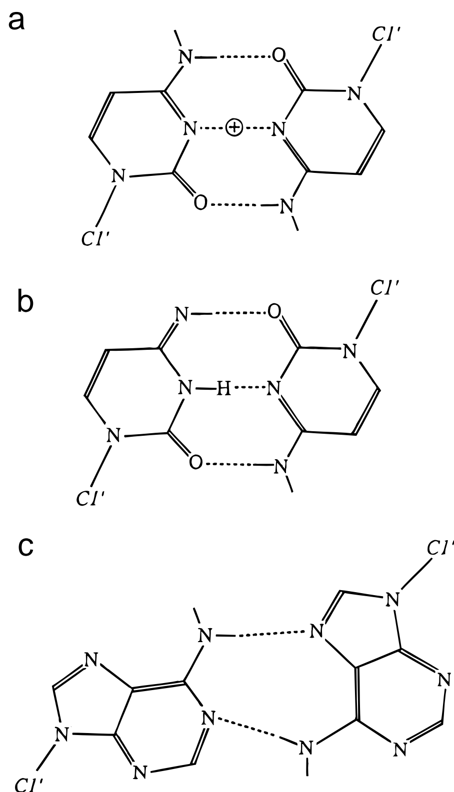

Figure 2. Base pairs studied in this contribution: (a) triply bonded hemiprotonated C·CH$^+$ base pair with two equivalent cytosines. (Note that the force field does not allow for inclusion of the proton transfer which makes both cytosines equivalent. The simulations were thus done with a base pair consisting of one protonated and one neutral base.) (b) Triply bonded C·imC base pair; neutral analogue of the C·CH$^+$ base pair and (c) asymmetric A·A base pair.

tion of cytosine-rich telomeric DNA and the unusual physiological properties associated with them stimulated speculation about the role of hairpin formation involving four-stranded intercalated i-DNA stem structures.[4,2a,b] A further example for the possible involvement of i-DNA structures in biological processes is provided by a recent study investigating the cytosine-rich strand of the human insulin-linked polymorphic region (ILPR). This study demonstrated the ability of this sequence to adopt hairpins with intercalated C·CH$^+$ pairs, and their occurrence is thought to enhance the possibility of slippage during replication of the ILPR thus causing polymorphism.[5]

From a chemical point of view, perhaps the most intriguing feature of i-DNA is the stacking of consecutive hemiprotonated base pairs, each carrying one positive charge, since this should be associated with a large charge−charge repulsion.[6] The base stacking energy between adjacent base pairs provides about −10 to −15 kcal/mol stabilizing energy in the case of classical A, B, and Z duplex DNA conformations.[7] In the case of hemiprotonated four-stranded intercalated i-DNA, in contrast, this contribution is repulsive and amounts to about +30 kcal/mol.[6a] Dipole−dipole interactions which were assumed to alleviate this repulsion were shown to be very weak, and quantum-mechanical calculations fully confirmed strong base stacking repulsion.[6a] Furthermore, electrostatic repulsion between consecutive protonated cytosines is well documented for DNA triplexes with pyrimidine·purine·pyrimidine base triplets. An important qualitative difference between i-DNA and triplexes with CH$^+$·GC base triplets is found in the fact that a sequence of two or more consecutive CH$^+$·GC trimers significantly destabilizes the triplex. Considerable effort has been devoted to obtaining neutral analogues of protonated cytosine capable of recognizing the G·C Watson−Crick base pair.[8] On the other hand, the stability of i-DNA appears to originate in the cytosine core region, consisting of stacked layers of presumably hemiprotonated C·CH$^+$ base pairs. To eliminate the charge−charge repulsion, a temporary formation of the imino cytosine (imC) tautomers replacing protonated cytosine was proposed, giving rise to neutral, triply hydrogen-bonded C·imC base pairs (Figure 2b).[6] A similar imC·GC model has also been proposed for triplexes with consecutive CH+·GC base triplets.[6c,9] In contrast to stacking of two adjacent hemiprotonated base pairs in i-DNA, stacking of adjacent C·CH$^+$ and C·imC base pairs would be attractive and provide for even higher stabilization energy than base stacking in B-DNA. Formation of the C·imC base pair is not unlikely considering the tautomeric equilibrium of cytosine[9,10] and base pairing properties.[6,9] It could in fact be promoted by the drastic improvement of stacking energy. However, there is no experimental evidence for the existence of imino tautomers to date.

With the aim to improve our understanding of the sources of stability of i-DNA, we carried out a set of unconstrained molecular dynamics simulations of fully solvated four-stranded i-DNA molecules. The reliability of molecular dynamics simulations of nucleic acids was recently significantly enhanced by introducing the particle mesh-Ewald technique, which allows a proper treatment of long-range electrostatic interactions.[11,12] Also, there have been significant improvements of the quality of the empirical force fields.[13] Current simulations provide, for the first time, stable trajectories of nucleic acid structures on a nanosecond scale.[12] Although the accuracy of these simulations is still limited by several factors such as the performance of the force fields, they represent a powerful theoretical tool

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capable of properly describing qualitative features of the dynamics of nucleic acid molecules. Analyses of novel nucleic acid structures of the kind described in this contribution provide further clues about the applicability and limitations of MD simulations.

Methods

Calculations were carried out using the AMBER4.1 program[14] with a Cornell et al. force field.[15] The nucleic acid molecules were surrounded by a periodic box of water molecules described by the TIP3P potential.[16] The periodic box was extended to a distance of 10 Å from any solvent atom. Around 2500 explicit water molecules were included in the simulations. Charges of modified bases were fitted to the electrostatic potential around the bases at the Hartree−Fock level with the 6-31G* basis set. The molecules were neutralized by Na^+ cations initially placed using Coulombic potential terms with the LEAP modul of AMBER4.1. All calculations were carried out using the Sander module of AMBER 4.1 with SHAKE on the hydrogen atoms with a tolerance of 0.0005 Å and a 2-fs time step. A 9 Å cutoff was applied to Lennard-Jones interactions. Simulations were performed using Berendsen temperature coupling algorithm (with a time constant of 0.2 ps). The nonbonded pair list was updated every 10 steps. Equilibration was started by 1000 steps of minimization with the positions of the nucleic acid fixed. After this initial equilibration, all subsequent simulations were performed using the particle mesh-Ewald method (PME) for inclusion of long-range electrostatic interactions into calculations without truncation. The PME charge grid spacing was approximately 1.0 Å, and the charge grid was interpolated using a cubic B-spline with the direct sum tolerance of 10^{-6} at the 9 Å direct space cutoff. To speed up the fast Fourier transform in the calculation of the reciprocal sum, the size of the PME charge grid was chosen to be a product of powers of 2, 3, and 5. Equilibration was continued by 25 ps of PME dynamics, with the position of the nucleic acid fixed. Subsequently, 1000 steps of minimization were carried out with 25 kcal/(mol·Å^2) restraints placed on all solvent atoms, continued by 3 ps MD simulation using the same restraint. This equilibration was followed by five rounds of 1000-step minimization with solute restraints reduced by 5 kcal/(mol·Å^2) in the course of each round. Then 20 ps of MD followed, with the system heated from 100 to 300 K over 2 ps. Equilibration was continued by 2−3 ns of production simulation. Coordinates were written to trajectory files after each picosecond. The results were analyzed using the Carnal module of AMBER 4.1. Starting coordinates were obtained from the crystal structures of d(CCCC)$_4$ (NDB code UDD024) and d(CCCAAT)$_4$ (NDB code UDF034).[2a,c]

Results

We first studied a structure with eight consecutive C·CH$^+$ base pairs [starting coordinates: molecule 1 in the d(CCCC)$_4$ crystal[2a]]. Two MD runs (2.5 ns and 3.0 ns long) were carried out. We expected, that the repulsive base stacking would lead to a fast destabilization and coming apart of the i-DNA structure. However, both runs provided a stable trajectory, yielding a molecule which is exceptionally close to that found in the crystal (Figure 3). We did not observe destabilization (even temporary) of the base pairing, and the sugar−sugar zipper[2d] was well conserved. Positions of bases and sugar rings are also maintained, with the RMS deviation of the whole structure being around 0.9−1.1 Å compared to the crystal coordinates. The RMS deviations of bases and sugar rings were below 1 Å throughout the simulations, while phosphate atoms exhibited highest mobility with RMS deviations of 1.3−1.6 Å. One phosphate group adopts a different geometry than in the crystal, this being the main difference identified. The positional shift of this phosphate atom is characterized by a sharp increase of its RMSd value from 1 to 4 Å. This shift occurred at 0.5 and 1.5 ns during the 2.5 and 3 ns simulations, respectively. The phosphate group remains in the new position until the end of the simulation without any further positional alterations. Detailed inspection of the RMSd values of all phosphate groups in the molecule revealed movement of another phosphate atom, exhibiting two-state ocsillations with intervals mostly around 100 ps. The observed mobility of phosphate groups does not propagate to the attached sugar rings, and these geometrical changes do not influence the structure as a whole. Interestingly, similar mobility of individual phosphates was observed at atomic resolution in crystal structures of DNA oligonucleotide fragments, where a coexistence of two distinct conformations of individual phosphate groups in the crystal lattice could be resolved, also not affecting the geometry of the adjacent sugar rings.[17]

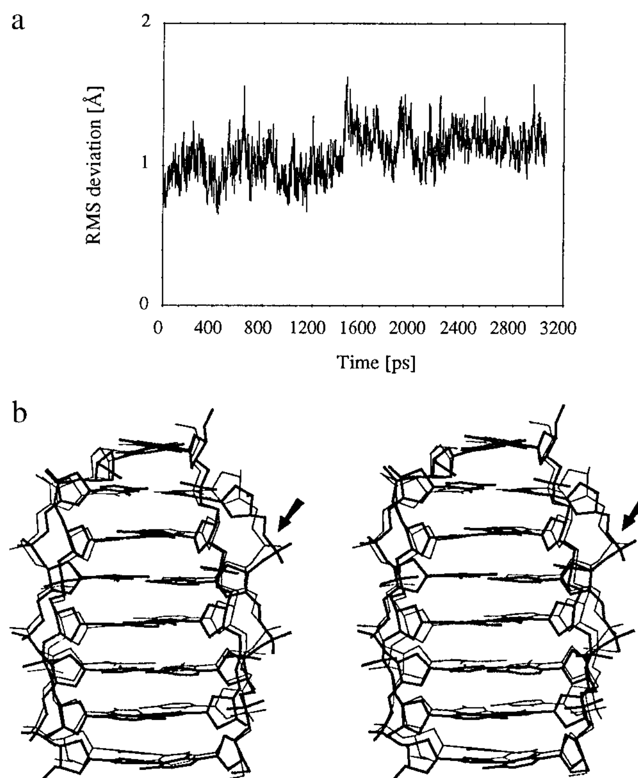

Figure 3. (a) RMS deviation along 3 ns MD trajectory of hemiprotonated d(CCCC)$_4$ after 20 ps equilibration period. Average RMSd is around 1.0 Å. (b) Stereo overlay plot of hemiprotonated d(CCCC)$_4$ crystal (thin lines) and averaged (2.5−3.0 ns) MD coordinates (thick lines). The arrow indicates a deviating phosphate position (see text).

In the course of our simulation, we observed further a slight vertical spread of the bases in the theoretical structure, mainly

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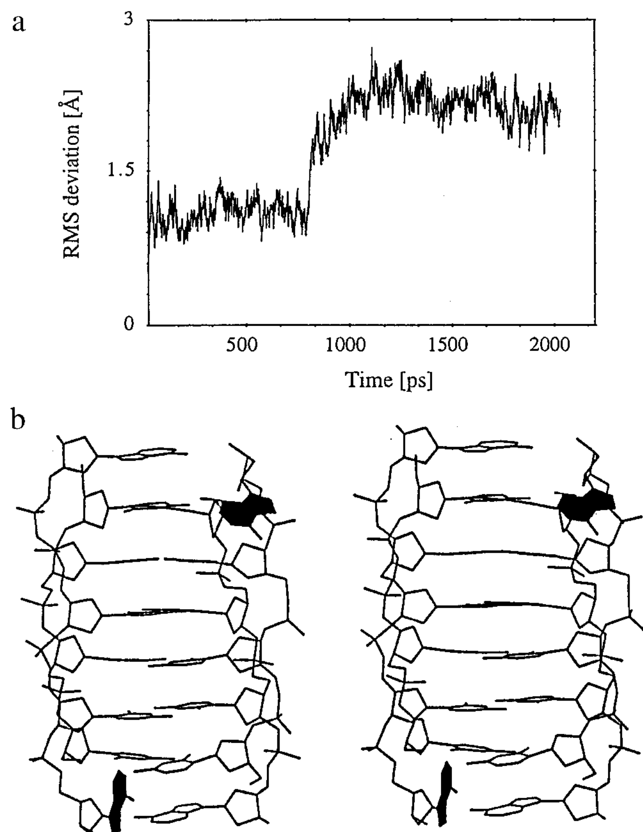

Figure 4. (a) RMS deviation along 2 ns MD trajectory of hemiprotonated d(CCCA)$_4$ after 20 ps equilibration period. The large RMSd values are solely due to the disruption of both A•A base pairs. (b) Stereo plot of the d(CCCA)$_4$ structure after simulation. Flanking A•A base pairs are disrupted. One adenine (filled in black) at each end is looped away from the helical axis and stacks on the molecule from the outside.

localized to the terminal base pair steps. The difference is less than 0.1 Å per step compared to the X-ray structure, which is energetically entirely acceptable.[18] It can be due to the effect of the charge−charge repulsion, some artifact of the force field, or the absence of the crystal packing forces in the calculations. While we obviously cannot rule out that our structure is locked in a local energetic minimum at the present stage, it is clear that this conformational state is a very stable one (cf. with other studies,[12] e.g., the "A to B" DNA transition takes ca. 0.5 ns to completion[12b]).

In addition, we carried out two 2 ns MD runs of the d(CCCA)$_4$ structure [starting coordinates − d(CCCA)$_4$ fragment of d(CCCAAT)$_4$ crystal[2c]]. In the d(CCCA)$_4$ structure, the cytosine-rich core region is enveloped by asymmetric adenine•adenine (A•A) base pairs at both ends (Figure 2c). In the simulations, we observed destabilization and even irreversible disruption of the flanking A•A pairs, with one adenine projecting away from the helix and stacked on the helix stem from the outside. This arrangement resembles the geometry of extrahelical thymines observed in another i-DNA structure, d(CCCT)$_4$.[2b] Interestingly, there was no propagation of the instability toward the cytosine region, even though the adjacent C•CH$^+$ base pairs showed some conformational flexibility during the simulations. The RMS deviations for the averaged structures of the cytosine region did not exceed 1.0 Å. The calculations with A•A base pairs illustrate that the simulations are long enough to detect instabilities if i-DNA is not favored by base−base interactions. The final geometry of the d(CCCA)$_4$ structure and the evolution of the RMS deviations during simulation are depicted in Figure 4. The A•A base pairs were observed in a crystal structure,[2c] and their destabilization during our simulations can be both due to the force field and also the absence of further stabilization elements found in the crystal such as additional base pairing and lattice packing interactions. Further investigations are necessary to address this point. The calculations presented here clearly demonstrate that the C•CH$^+$ base pairs are significantly more stable than the A•A pairs within the framework of i-DNA, providing further evidence that the stability of this four-stranded intercalated DNA motif originates in the cytosine core region.

Finally, we performed MD simulations on theoretical d(CCCC)$_4$ structures with alternating C•CH$^+$ and C•imC base pairs (Figure 2b). A first simulation (2 ns) was carried out with a structure where one strand consisted of imino cytosine residues only, a second (2.5 ns) with a structure with the four imino cytosine residues distributed among all four strands. Both structures remained stable and again very close to the crystal coordinates.[2a] Nevertheless, the RMSd values for averaged structures were slightly higher (1.1−1.4 Å), and more local deviations were observed than for the molecule with eight C•CH$^+$ base pairs, mostly in the sugar puckers. The slight vertical spread of bases was again noticed, thus we assume that this effect is probably not due to the charge−charge repulsion, as there is no charge−charge repulsion between C•CH$^+$ and C•imC base pairs.

Discussion and Conclusions

The most important lesson we learn from our analysis is that solvated DNA is indeed capable of adopting a three-dimensional structure with repulsive base stacking interaction. This raises important questions about the actual sources of stability of nucleic acids and about the validity of base-stacking calculations carried out with a neglect of solvent interactions in the rationalization of DNA structure and stability. The existence of i-DNA shows clearly that the situation might be much more complicated than generally assumed; let us also mention the ability of nucleic acids to incorporate nonpolar base pairs.[19] Although we do not need to involve the neutral imino cytosine into the current model of i-DNA, our calculations give some support for a participation of such tautomers. In this context, we wish to emphasize that a recent MD simulation of triple helices demonstrates the instability of a structure with three consecutive protonated cytosines, while the structure was stable when replacing the central CH$^+$ by imC.[9b] One qualitative difference between i-DNA and triplexes is illustrated by experiments showing that the stability of i-DNA originates in the C•CH$^+$ region, while consecutive CH$^+$•GC triplets are always destabilizing.[1,8,9] In this respect, the different MD results for i-DNA and triplex structures show agreement with the experimental data.

A further interesting point is the close agreement between the crystal structure and the averaged MD structure for d(CCCC)$_4$. This result is somewhat surprising, as the AMBER force field has not been parametrized for the geometry of unusual i-DNA, and we could not include either the proton transfer within the hemiprotonated pairs[1] or the polarization effects.[6a] Notwithstanding, the agreement between theoretical and experimental structures can be interpreted as being indicative of the actual conformational rigidity of this four-stranded, fully intercalated DNA motif.

The present results clearly illustrate the capability of current molecular dynamics simulations to properly reveal qualitative

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features of established and also novel nucleic acid structures. Unusual nucleic acids are currently the focus of intensive research efforts as modular building blocks for novel biocatalysts and aptamers with physiological functions.[20] G-DNA, the guanine-rich quadruplex counterpart of i-DNA, has clearly proven its aptitude in this respect. We anticipate that novel DNA motifs such as i-DNA will continue to be of particular importance toward the rational design of nucleic acid molecules with particular functions. Approaches of the kind described in this paper complement the experimental techniques and will be instrumental in deepening our understanding of the forces governing the conformational stability of these novel nucleic acid structures at the molecular level.

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