Nonnatural Nucleobases

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2-Aminopurine Incorporation Perturbs the Dynamics and Structure of DNA**

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2-Aminopurine (2AP) is a structural isomer of adenine (A), in which the amino group is at C2 instead of C6, and can form stable Watson–Crick (WC) type base pairs with thymine (T) (Figure 1).[1,2] While natural nucleobases do not emit at all,

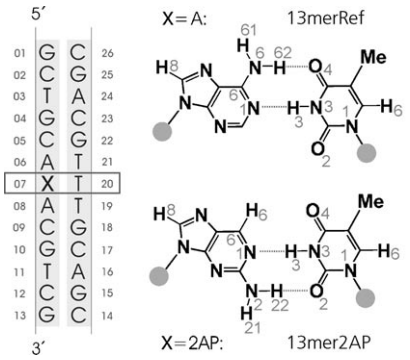

Figure 1. DNA duplex sequence with the chemical structure of the 2AP:T and A:T base pairs. A symmetric, nonpalindromic sequence of 13 base pairs was chosen to avoid mispairing or loop formation, to minimize fraying effects, and to have a single perturbation site.

2AP shows appreciable fluorescence. More importantly, its fluorescence quantum yield decreases 100-fold upon duplex formation.[3] Numerous studies exploit 2AP fluorescence to investigate problems in structural biology and biophysics: methyltransferase-induced base flipping,[4–6] conformational changes and enzymatic cleavage of the hammerhead ribozyme,[7,8] promoter binding and clearance of T7 RNA polymerase,[9,10] binding and strand separation of primer–template DNA by T4 DNA polymerase,[11–13] and charge-transfer mechanisms in DNA coupled to polar solvation.[14–16] Alternatively, structural changes can be monitored by a low-energy circular dichroism band observed with 2AP, as was demonstrated with RNA and DNA hairpin loops.[17,18] The high number of publications relating to 2AP reflects its importance in studies of biological macromolecules.

When structural transitions in biological systems are examined with a molecular probe, the assumption is that the modified system behaves like the natural one. Consequently the introduction of the probe must leave the structure and dynamics unchanged. Fluorophore-induced perturbations have been analyzed by solution NMR spectroscopy in many cases.[19–23] With 2AP, however, the changes are so small that the results in the original studies were inconclusive.[24,25] With high-field spectrometers and an expanded set of NMR parameters at hand we can now investigate 2AP-induced changes in detail.

We present herein the NMR solution structure and base-pair dynamics of two 13-mer DNA duplexes (Figure 1) with X = A in the reference sequence and X = 2AP in the modified sample (in the following called 13merRef and 13mer2AP, respectively). The only change introduced into the helix is the position of the amino group in A and 2AP. To what extent are structure and dynamics affected by this change? To answer this question we employed 2D NMR spectroscopy and measurements of residual dipolar couplings (RDC) in conjunction with simulated annealing calculations to determine the solution structure, selective NMR T_1 experiments to evaluate base-pair dynamics, and temperature-dependent absorption and fluorescence spectroscopy to characterize local melting. By combining information from these different approaches the effect of a single substitution A→2AP can be evaluated.

All NMR resonances except for the severely overlapped H5′/H5″ signals could be assigned by intra- and internucleotide NOEs.[26] WC base-pairing of 2AP is evidenced by the imino proton signal of T20 which is observed—though broadened—for 13mer2AP at 298 K.[26] In contrast to an earlier report,[24] all cross peaks expected for regular B-DNA are present in the NOESY spectra of both samples. However, for the diagonal imino proton signal for T20, fast decay with increasing mixing time is observed in the NOESY spectrum. To probe whether water exchange is the reason for this phenomenon, we performed water saturation-transfer experiments. The absence/reduction of the T20/adjacent imino proton signals upon presaturation of water (Figure 3) indicate very fast water exchange for 2AP:T and increased rates for the adjacent base pairs.

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This indication was tested with base-pair lifetime experiments. The spin–lattice relaxation time T_1 of a resonance is measured in the presence of a base B which catalyzes proton exchange [Eq. (1)].

$$\frac{1}{T_1} = \frac{1}{T_{10}} + \frac{1}{\tau_{ex}} \quad (1)$$

Here, T_{10} refers to the relaxation time T_1 of the proton in the absence of base. In the limit of the dominant catalyzed exchange, the imino proton exchange time τ_{ex} depends linearly on the inverse catalyst concentration [Eq. (2)].[27]

$$\tau_{ex} = \tau_{op} + \frac{1}{K_D}\frac{1}{[B]} \quad (2)$$

Here, K_D is the apparent dissociation constant. Extrapolation to infinite catalyst concentration provides the base-pair lifetime τ_{op}. Figure 2 shows the corresponding data and linear fits for the seven central base pairs and their extrapolated lifetimes for 13merRef and 13mer2AP. All of the determined lifetimes agree with previously published data.[25,28,29] Lifetimes in green refer to overlapped resonances in the imino proton region. As their overlap is complete and signal recovery identical, only averaged lifetimes can be given. The R^2 values for all linear fits are above 0.996. Confidence intervals for each lifetime were also determined from the fits.

The lifetimes of terminal base pairs could not be measured because of "base-pair fraying", which is commonly observed at the helix termini. Here base-pair lifetimes are considerably shortened; the signal from the terminal bases is broadened to the point of vanishing and the signals from neighboring groups are weakened.[29,30] Although influenced by the helix termini, lifetimes for the semiterminal G:C (0.5 ms) and A:T base pairs (1.1 ms) are found to be identical for 13merRef and 13mer2AP within the estimated error. Since base-pair fraying is sensitive to solution properties (pH, buffer, temperature), the agreement shows that lifetime measurements are reliable even below 1 ms and the solution properties comparable. In contrast to the outer base pairs, lifetimes for the inner seven pairs differ between 13merRef and 13mer2AP. The substitution A→2AP reduces τ_{op} severely for the central A:T pairs (by 45%, 80%, 30% for T21, T20, and T19, respectively) and less so for the more remote G:C pairs (by 20–30%).

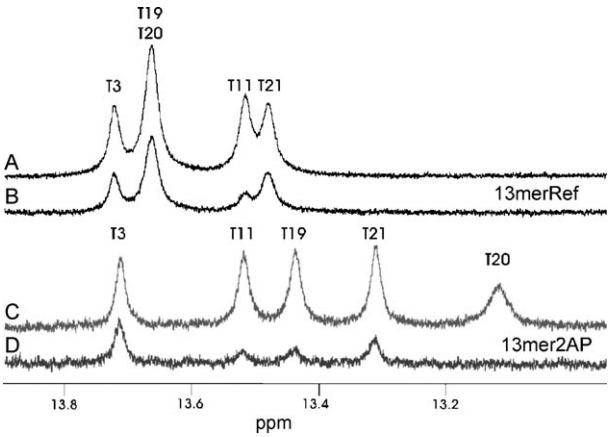

Figure 3. Saturation-transfer experiments in H_2O. Resonances from exchanging protons are reduced in the 1D spectra (B, D), reflecting the relative rates by which imino protons exchange with the solvent. Water saturation is avoided in 1D WATERGATE spectra, which are shown for comparison (A, C).

Let us return to the saturation transfer (Figure 3) which provides information on dynamics in the absence of added base catalyst. The observed reduction of signal is caused by the opening of a base pair, followed by exchange under intrinsic catalysis. For example, consider the T3 and T20 imino protons of 13mer2AP. Even though their τ_{op} is comparable, the reduction of signal upon water saturation is much larger for T20 than for T3, implying an increased rate constant of intrinsic catalysis for the 2AP:T base pair. The reduction of signals for the three central pairs (Figure 3) is consistent with the results from base-pair lifetime measurements. We have thus shown that the entire center portion is affected dynamically by a single substitution A→2AP.

Melting curves of 13mer2AP (Figure 4) provide independent, thermodynamic evidence for cooperativity among the central base pairs. UV absorption can be used to monitor

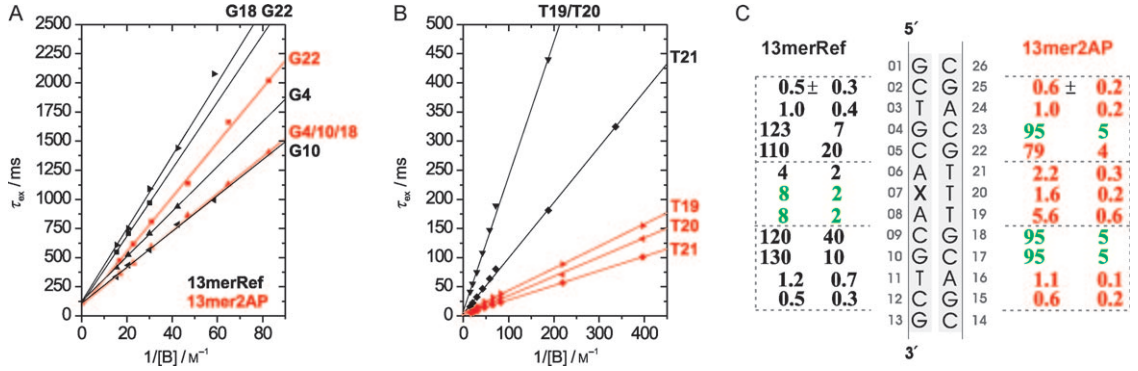

Figure 2. Determination of base-pair lifetimes. The inversion recovery of imino proton signals is affected by TRIS (tris(hydroxymethyl)aminomethane) base, which catalyzes exchange with water. The exchange time τ_{ex} depends on the inverse base concentration 1/[B], with different ranges for G:C and A:T pairs. Extrapolation to infinite concentration gives the lifetimes, which are collected in the right panel. Lifetimes in green could not be determined separately, but they should be similar because of identical recovery behavior.

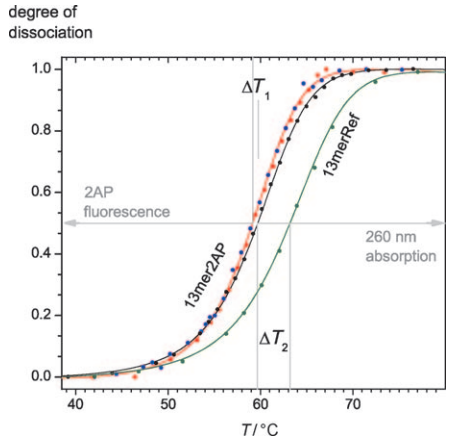

Figure 4. Melting curves determined from 13mer2AP absorbance (black) and 2AP fluorescence yield (blue: after heating; red: after cooling). A melting curve for 13merRef is also given (green). The total single-strand concentration in each case was 23.7 μM.

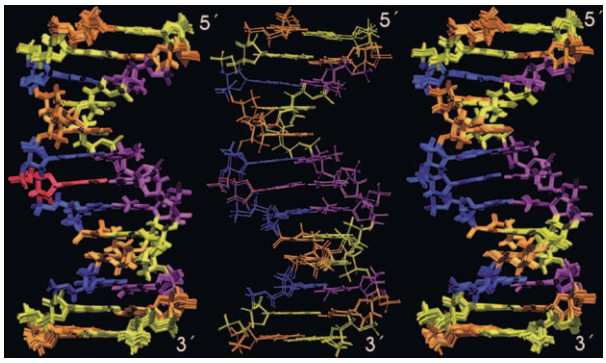

Figure 5. Overlay of the ten lowest-energy, minimum-violation structures for 13merRef and 13mer2AP. The root-mean-square deviations (RMSD) among each set of structures is 0.30 Å and 0.33 Å, respectively. The average structures for the two duplexes are compared in the middle (RMSD 0.46 Å).

the behavior of the entire duplex, whereas the 2AP fluorescence yield is sensitive to the local environment only. In a different dodecamer[31] the melting temperatures determined by absorption (duplex) and fluorescence (only 2AP) differ by 1.6 K, indicating "premelting" of the 2AP-modified site. We observe melting temperatures of 59.2 and 59.7 °C, respectively, suggesting a negligible degree of premelting of the 2AP-modified site. The entire center portion of the duplex melts as a whole, lowering the global melting temperature by $\Delta T_2 = 3.5$ K relative to that of 13merRef. The effect indicates a decrease of melting entropy by approximately 3.4 cal mol^{-1} K^{-1} as the amino group is relocated from the major to the minor groove. The move liberates the O4 atom of T20 from WC hydrogen bonding and thus decreases its hydrophobicity. As a consequence, changes in hydration upon melting would be reduced and the entropic cost smaller, in agreement with our observations for base-pair dynamics. But enthalpic changes are also involved, as shown next.

After generating an NOE-based structure, we included the RDC restraints (measurement standard deviation 0.6 Hz) in the refinement with a single floating alignment tensor. From a family of 100 calculated structures the ten lowest-energy, violation-free structures are shown in Figure 5 for 13merRef and 13mer2AP. These were used to calculate an average structure for each. The accuracy of the calculations was checked by back-calculating the NOESY spectrum and the RDCs from the average structure, and then comparing these with the experimental spectrum and RDCs ($R = 1.000$, for details see the Supporting Information). The precision of the calculations can be assessed by the root-mean-square deviations (RMSDs) of the ten best structures from their average, 0.30 Å for 13merRef and 0.33 Å for 13mer2AP. An overlay of the averaged structures is shown in Figure 5 (middle part); their RMSD is 0.46 Å. The smaller RMSDs among the ten best structures support the idea that, while the overall conformation is identical, minor but significant differences exist. The question arises whether these differences can be localized. Helical parameters were therefore calculated for each of the ten best structures with the program 3DNA.[32] Small but significant perturbations are observed throughout the helix (for details see Figures S8–S13 and Tables S14–S26 in the Supporting Information). The "propeller twist" and "opening" of base pair X7:T20 exhibit the largest deviations, of 7.0° and 3.3°, respectively.

We have examined two DNA duplexes with a central …AXA… motif that differs in one nucleobase (X = A or 2AP; Figure 1). Intrinsic exchange in 2AP:T is accelerated, as revealed by rapid water exchange even in the absence of external exchange catalyst. The HNH···O=C hydrogen bond is moved from the major to the minor groove by the substitution A→2AP. The move has a cooperative effect primarily on dynamics: lifetimes of three adjacent base pairs in either direction are reduced. Cooperativity is also implied by thermodynamics, since 2AP does not show significant premelting but instead causes a lowering of the global melting temperature. The overall helical structure is not affected by the substitution. At the modification site we find small but significant changes of the "propeller twist" and "opening" values. Smaller changes are observed for base pairs throughout the helix, mainly in "shear", "shift", and "slide". All perturbations are weak compared to those caused by other base or base-pair analogues.[19–22] Note, however, that different sequence contexts may lead to different structural as well as dynamic results, as strong excitonic coupling with flanking A is known to affect the fluorescence of 2AP.[33,34] A key application of 2AP is as a fluorescence probe for large-scale motions such as the stacking–unstacking of nucleobases.[4–13] In this case structural analogies to the unmodified sample can be drawn unambiguously, but the rates of the stacking–unstacking motions are significantly affected as a result of the decreased base-pair lifetimes around the site of 2AP incorporation. The latter also affects the binding of restriction enzymes and methyltransferases, where the primary event is base flipping.[35,36] These observations may complicate the analogous interpretation of dynamic data.

Experimental Section

All NMR measurements were carried out at 298 K on a Bruker Avance 600 at a duplex concentration of 3 mM. Structure calculations were performed with Xplor-NIH v2.20. Duplex melting was monitored by optical absorption at 260 nm or fluorescence quantum yield from 310 nm excitation, at total single-strand concentration of 23.7 μM. Coordinate and input files are deposited at the protein databank with PDB ID codes 2kuz (13merRef) and 2kv0 (13mer2AP). Full experimental details are presented in the Supporting Information.

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