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Simple Method for Determining Nucleobase pK_a Values by Indirect Labeling and Demonstration of a pK_a of Neutrality in dsDNA

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Nucleobases can have shifted pK_a's, which have been separated into two classes.[1] Class I sites are those for which the loaded proton is involved in hydrogen bonding, such as a wobble A^+·C basepair, and therefore not free to undergo proton transfer. Class II sites are those for which the loaded proton is not hydrogen bonded and thus free to partake in proton transfer including general acid/base catalysis.[1]

Free nucleobases have pK_a's of 3.5 and 4.2 for N1 of adenine and N3 of cytosine, respectively, and 9.2 and 9.7 for N1 of guanine and N3 of thymine.[2] These values shift even *further* from neutrality in a typical Watson−Crick base pair due to the stability conferred by base pairing.[1,3a] However, numerous folded state pK_a's are perturbed *toward* neutrality for RNA and DNA, with values ranging from 3.8 to 6.6.[3] pK_a's shifted toward neutrality can participate in RNA and DNA catalysis.[1,4]

We wanted to develop a simple approach for determining pK_a's in folded DNA and RNA molecules and investigate the extent to which basepairing can shift pK_a's. Phosphorothioates were substituted into dsDNA to study Class I A^+·C basepairs by ^{31}P NMR. This substitution shifts the phosphorus peak downfield by ~50 ppm (Figure 1). We found that these peaks had a pH-dependent change in chemical shift,[5] presumably caused by a change in local structure upon AC basepair formation.

To test the method, we chose a self-complementary DNA duplex sequence (DNA1) with a published pK_a of 6.6, determined by ^{1}H and ^{15}N NMR.[6a] The phosphorothioate (*) was incorporated 3′ of C10 (DNA1 C*, Figure 2A) or 5′ of A3 (DNA1 *A, Supporting Information). The chemical shifts of the R_p and S_p resonances were followed as a function of pH, with referencing to an internal TMP standard.[7] Fitting data for DNA1 C* to a Henderson−Hasselbalch equation adapted for fast chemical exchange on the NMR time scale (Supporting Information) gave pK_a values of 6.56 ± 0.04 and 6.57 ± 0.03 (Table 1, Figure 2), which are in good agreement with the published value of 6.6.[6b] Values of the Hill coefficient were 1.26 ± 0.13 and 1.32 ± 0.10, suggesting that the A^+·C basepairs act independently, as expected.

For DNA1 *A, peak 1 had a significan slope at pH values lower than 6, while peak 2 showed little change throughout the titration (Supporting Information). The origin of these effects is unclear at present, but phosphorothioates at other positions did not show this. Data for DNA1 *A peak 1 were fit with three different baseline methods, which gave pK_a's ranging from 6.46 to 6.89, in agreement with results from DNA1 C*.

The next sequence investigated (DNA2) was a DNA hairpin with a stable loop and closing basepair[8] that contains a single A^+·C basepair in the center of the stem. This sequence was also designed to have more stable nearest-neighbor interactions than DNA 1, which should favor the equilibrium constant for folding and a more

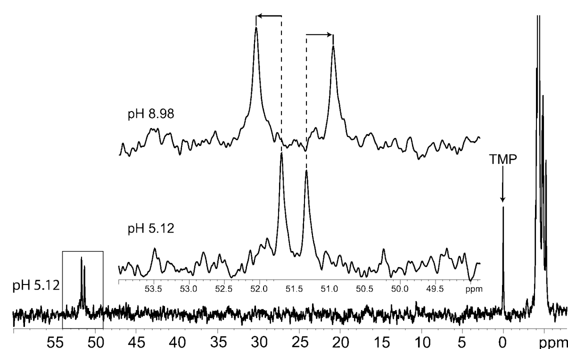

Figure 1. Representative NMR spectra highlighting downfield-shifted phosphorothioate resonances. Spectra are referenced to an internal TMP standard set to 0 ppm. (Inset) Blow-up of the boxed region and display of the lowest and highest pH spectra in the titration of DNA2 A* at 19 °C.

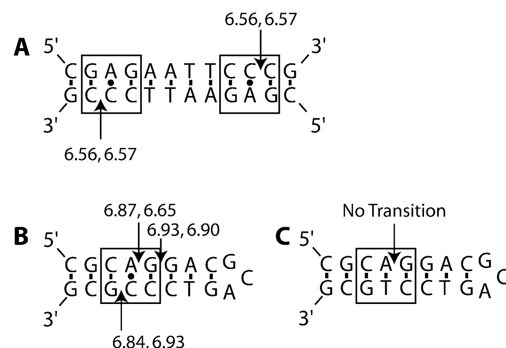

Figure 2. DNA oligonucleotides with arrows indicating locations of phosphorothioates. The R_p/S_p pK_a's are provided. Boxes enclose the A^+·C basepair and its nearest neighbors. (A) DNA1, (B) DNA2, (C) DNA2 ctrl.

Table 1. Parameters from Fits to pH Titrations

sequence	peak[a]	Δδ (ppm)[b,c]	n[c]	pK_a[c]
DNA1 C*	1	0.29 ± 0.01	1.26 ± 0.13	6.56 ± 0.04
	2	0.43 ± 0.01	1.32 ± 0.10	6.57 ± 0.03
DNA2 C*	1	0.197 ± 0.005	1.25 ± 0.09	6.84 ± 0.03
	2	0.196 ± 0.005	1.37 ± 0.10	6.93 ± 0.03
DNA2 A*	1	0.381 ± 0.004	1.11 ± 0.04	6.87 ± 0.02
	2	−0.372 ± 0.006	1.15 ± 0.06	6.65 ± 0.02
DNA2 AG*	1	−0.082 ± 0.005	0.88 ± 0.15	6.93 ± 0.08
	2	−0.049 ± 0.004	0.98 ± 0.23	6.90 ± 0.10

[a] Peak 1 is the more downfield shifted phosphorothioate peak. [b] Calculated as the difference between high and low pH. [c] Values of Δδ, n, and pK_a were obtained from nonlinear curve fitting to eq 1 (Supporting Information). NMR conditions were 31 °C for DNA1 and 30 °C for DNA2 in 100 mM KCl. pH values were determined at 30−31 °C. Data at these and additional temperatures are given in Supporting Information.

shifted pK_a. Nearest neighbor calculations using GC or AT in place of the AC favored DNA2 over DNA1 by a $\Delta\Delta G^\circ_{37}$ of −0.33 (GC) and −0.15 kcal/mol (AT).[9] These differences correspond to ΔpK_a's of 0.24 and 0.11 with respect to DNA1. In addition, SantaLucia

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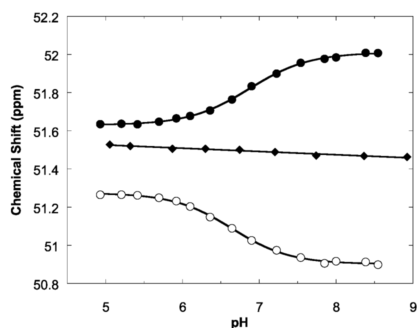

Figure 3. Titration curves tracking the change in chemical shift with pH for DNA2 A* peak 1 (●), DNA2 A* peak 2 (○), and DNA2 A* ctrl peak 1 (◆). Chemical shift values are with respect to TMP. Data for DNA2 A* were fit to eq 1, while data for DNA2 A* ctrl were fit to a linear equation ($R = 0.98$).

and co-workers calculated nearest-neighbor parameters of internal AC mismatches in DNA at pH 5 and 7 and found that the contribution of an AC was strongly dependent on its nearest-neighbors.[10] They reported a ΔG°_{37} difference for the nearest neighbors in DNA2 and DNA1 of −0.56 kcal/mol (1 M NaCl at pH 5.0), which corresponds to a ΔpK_a of 0.40 at 37 °C. Overall, these three calculations predict that DNA2 should have a higher pK_a than DNA1.

As in DNA1 C*, the phosphorothioate was first incorporated 3′ of the C of the A^+•C basepair, in DNA2 C*. Fitting the pH dependence of the two DNA2 C* resonances gave n values of 1.25 ± 0.09 and 1.37 ± 0.10, respectively, and pK_a's of 6.84 ± 0.03 and 6.93 ± 0.03, suggesting that nearest-neighbor interactions can drive a pK_a in dsDNA to neutrality (Table 1).

To ensure that placement of the phosphorothioate was not responsible for shifting the pK_a, the experiment was repeated with two other label placements. First, incorporation 3′ of A4 of the A^+•C basepair, DNA2 A* (Table 1, Figures 2 and 3), gave n values of 1.11 ± 0.04 and 1.15 ± 0.06 and pK_a values of 6.87 ± 0.02 and 6.65 ± 0.02, similar to those determined for DNA2 C*. Second, the label was moved an additional basepair away from the A^+•C, in DNA2 AG*, which gave n values of 0.88 ± 0.15 and 0.98 ± 0.23 and pK_a values of 6.93 ± 0.08 and 6.90 ± 0.10, similar to those for DNA2 A* and DNA2 C*. The change in chemical shift for DNA2 AG* was considerably smaller than that for DNA2 A* and C* (Table 1), $|\delta_{ave}|$ of 0.07 versus 0.38 and 0.20, consistent with a smaller structure perturbation at this remote position upon protonated basepair formation. Similarity among the six pK_a values for DNA2 C*, A* and AG* strongly supports the label not causing the pK_a shift.

As a last control, the AC was changed to an AT, in DNA2 A* ctrl. As expected, the plot of the downfield-shifted resonances versus pH no longer had a sigmoidal shape and could be fit with a straight line (Figure 3 and Supporting Information).

At 30−31 °C, the average pK_a's for DNA1 and DNA2 were 6.56 and 6.85, respectively, giving a ΔpK_a of 0.29, which is in agreement with that predicted from nearest-neighbor parameters. Lowering the temperature to 14−19 °C gave a higher average pK_a value for DNA2 of 7.06 (Supporting Information), which further supports folding providing a critical driving force for pK_a shifting.

In summary, phosphorothioate incorporation combined with ^{31}P NMR provides a simple method for determining pK_a values in structured DNA molecules. pK_a values of neutrality were promoted by favorable nearest-neighbor partners and lower temperature. Numerous DNA enzymes have been prepared in vitro,[11] and pK_a's of 7 could confer several catalytic strategies upon these enzymes, including electrostatic catalysis.[1] In the future, indirect labeling with a phosphorothioate could be used to measure shifted pK_a's in RNA and DNA molecules with complex structures, including Class II sites that involve a structural change upon protonation.

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Supporting Information Available: Protocols for sample preparation, ^{31}P NMR experiments, equation for fitting, and chemical shift vs pH plots for each sample. This material is available free of charge via the Internet at http://pubs.acs.org.

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