

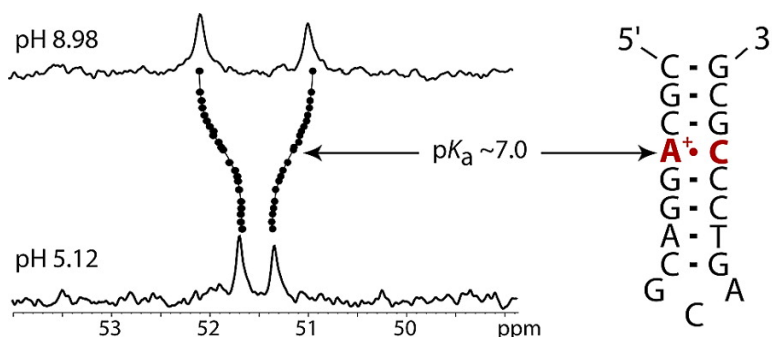
Communication

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

Ellen M. Moody, Trevor S. Brown, and Philip C. Bevilacqua

*J. Am. Chem. Soc.*, **2004**, 126 (33), 10200-10201 • DOI: 10.1021/ja047362h • Publication Date (Web): 28 July 2004

Downloaded from <http://pubs.acs.org> on April 1, 2009



### More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 1 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

[View the Full Text HTML](#)



**ACS Publications**  
 High quality. High impact.

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

Ellen M. Moody,<sup>†</sup> Trevor S. Brown,<sup>†,‡</sup> and Philip C. Bevilacqua<sup>\*,†,‡</sup>

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802, and The Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, Pennsylvania 16802

Received May 5, 2004; E-mail: pcb@chem.psu.edu

Nucleobases can have shifted  $pK_a$ 's, which have been separated into two classes.<sup>1</sup> Class I sites are those for which the loaded proton is involved in hydrogen bonding, such as a wobble A<sup>+</sup>•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.<sup>1</sup>

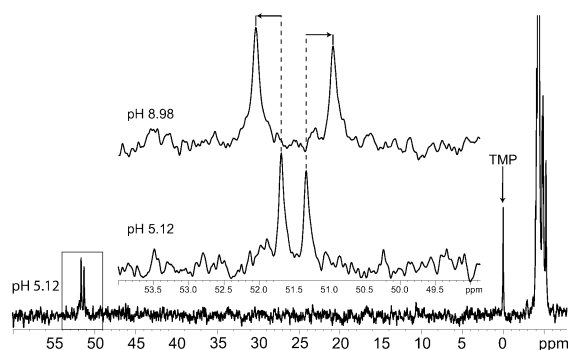
Free nucleobases have  $pK_a$ 's of 3.5 and 4.2 for N1 of adenine and N3 of cytosine, respectively, and 9.2 and 9.7 for N1 of guanine and N3 of thymine.<sup>2</sup> These values shift even *further* from neutrality in a typical Watson–Crick base pair due to the stability conferred by base pairing.<sup>1,3a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

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<sup>+</sup>•C basepairs by <sup>31</sup>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,<sup>5</sup> presumably caused by a change in local structure upon AC basepair formation.

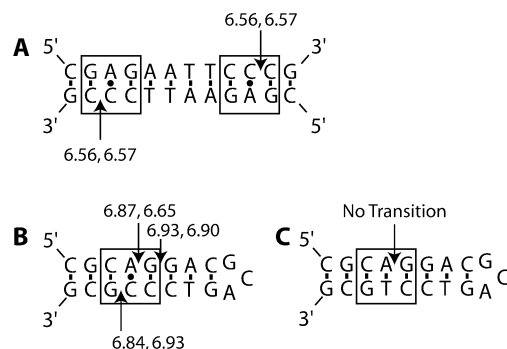
To test the method, we chose a self-complementary DNA duplex sequence (DNA1) with a published  $pK_a$  of 6.6, determined by <sup>1</sup>H and <sup>15</sup>N NMR.<sup>6a</sup> The phosphorothioate (\*) was incorporated 3' of C10 (DNA1 C\*, Figure 2A) or 5' of A3 (DNA1 \*A, Supporting Information). The chemical shifts of the R<sub>p</sub> and S<sub>p</sub> resonances were followed as a function of pH, with referencing to an internal TMP standard.<sup>7</sup> 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.<sup>6b</sup> Values of the Hill coefficient were 1.26 ± 0.13 and 1.32 ± 0.10, suggesting that the A<sup>+</sup>•C basepairs act independently, as expected.

For DNA1 \*A, peak 1 had a significant 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<sup>8</sup> that contains a single A<sup>+</sup>•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<sub>p</sub>/S<sub>p</sub>  $pK_a$ 's are provided. Boxes enclose the A<sup>+</sup>•C basepair and its nearest neighbors. (A) DNA1, (B) DNA2, (C) DNA2 ctrl.

**Table 1.** Parameters from Fits to pH Titrations

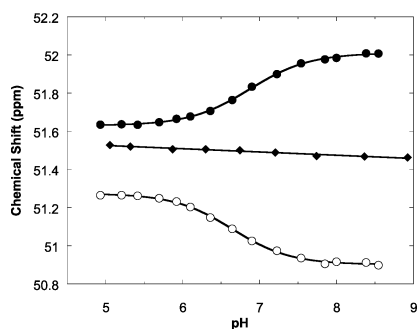
sequence	peak <sup>a</sup>	$\Delta\delta$ (ppm) <sup>b,c</sup>	$n^c$	$pK_a^c$
DNA1 C*	1	0.29 ± 0.01	1.26 ± 0.13	6.56 ± 0.04
	2	0.43 ± 0.01	1.32 ± 0.10	6.57 ± 0.03
DNA2 C*	1	0.197 ± 0.005	1.25 ± 0.09	6.84 ± 0.03
	2	0.196 ± 0.005	1.37 ± 0.10	6.93 ± 0.03
DNA2 A*	1	0.381 ± 0.004	1.11 ± 0.04	6.87 ± 0.02
	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

<sup>a</sup> Peak 1 is the more downfield shifted phosphorothioate peak. <sup>b</sup> Calculated as the difference between high and low pH. <sup>c</sup> Values of  $\Delta\delta$ ,  $n$ , 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  $\Delta\Delta G_{37}^{\circ}$  of -0.33 (GC) and -0.15 kcal/mol (AT).<sup>9</sup> These differences correspond to  $\Delta pK_a$ 's of 0.24 and 0.11 with respect to DNA1. In addition, SantaLucia

<sup>†</sup>Department of Chemistry.

<sup>‡</sup>The Huck Institutes of the Life Sciences.



**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.<sup>10</sup> They reported a  $\Delta G_{37}^{\circ}$  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  $\Delta pK_a$  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<sup>+</sup>C basepair, in DNA2 C\*. Fitting the pH dependence of the two DNA2 C\* resonances gave  $n$  values of  $1.25 \pm 0.09$  and  $1.37 \pm 0.10$ , respectively, and  $pK_a$ 's of  $6.84 \pm 0.03$  and  $6.93 \pm 0.03$ , 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<sup>+</sup>C basepair, DNA2 A\* (Table 1, Figures 2 and 3), gave  $n$  values of  $1.11 \pm 0.04$  and  $1.15 \pm 0.06$  and  $pK_a$  values of  $6.87 \pm 0.02$  and  $6.65 \pm 0.02$ , similar to those determined for DNA2 C\*. Second, the label was moved an additional basepair away from the A<sup>+</sup>C, in DNA2 AG\*, which gave  $n$  values of  $0.88 \pm 0.15$  and  $0.98 \pm 0.23$  and  $pK_a$  values of  $6.93 \pm 0.08$  and  $6.90 \pm 0.10$ , 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),  $|\delta_{\text{ave}}|$  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  $\Delta pK_a$  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 <sup>31</sup>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,<sup>11</sup> and  $pK_a$ 's of 7 could confer several catalytic strategies upon these enzymes, including electrostatic catalysis.<sup>1</sup> 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.

**Acknowledgment.** We thank Alan Benesi, Juliette Lecomte, and Bernard O'Hare for assistance with NMR experiments. We thank Vicki DeRose for helpful comments. We also thank J. Lecomte and David Proctor for comments on the manuscript. Supported by NSF Grant MCB-9984129, a NRSA Individual Fellowship from the NIH and an Alfred P. Sloan Scholarship to T.S.B and a Paul Berg Award to E.M.M.

**Supporting Information Available:** Protocols for sample preparation, <sup>31</sup>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>.

## References

- (1) (a) Narlikar, G. J.; Herschlag, D. *Annu. Rev. Biochem.* **1997**, *66*, 19–59. (b) Bevilacqua, P. C.; Brown, T. S.; Nakano, S.; Yajima, R. *Biopolymers* **2004**, *73*, 90–109.
- (2) Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984.
- (3) (a) Legault, P.; Pardi, A. *J. Am. Chem. Soc.* **1997**, *119*, 6621–6628. (b) Cai, Z.; Tinoco, I., Jr. *Biochemistry*. **1996**, *35*, 6026–6036 (c) Ravindranathan, S.; Butcher, S. E.; Feigon, J. *Biochemistry* **2000**, *39*, 16026–16032 (d) Blanchard, S. C.; Puglisi, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 3720–3725 (e) Huppler, A.; Nikstad, L. J.; Allmann, A. M.; Brow, D. A.; Butcher, S. E. *Nat. Struct. Biol.* **2002**, *9*, 431–435.
- (4) Bevilacqua, P. C. *Biochemistry* **2003**, *42*, 2259–2265.
- (5) Phosphorothioate substitutions have also been used to assign <sup>31</sup>P spectra. Eckstein, F.; Jovin, T. M. *Biochemistry* **1983**, *22*, 4546–4550. Phosphorothioates have been used to monitor ion binding in the hammerhead ribozyme and have been found to yield a higher field shift upon addition of Cd<sup>2+</sup> ions. Pecoraro, V. L.; Hermes, J. D.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5262–5271 and Maderia, M.; Hunsicker, L. M.; DeRose, V. J. *Biochemistry* **2000**, *39*, 12113–12120. They have also been used to identify inner-sphere metal ion coordination in a tetraloop. Maderia, M.; Horton, T. E.; DeRose, V. J. *Biochemistry* **2000**, *39*, 8193–8200.
- (6) (a) Wang, C.; Gao, H.; Gaffney, B. L.; Jones, R. A. *J. Am. Chem. Soc.* **1991**, *113*, 5486–5488. (b) Jones and co-workers reported a  $pK_a$  in D<sub>2</sub>O based on an uncorrected pH meter reading. A correction of a pH meter reading and a back correction for a pH titration on cytidine (Luptak, A.; Ferre-D'Amare, A. R.; Zhou, K.; Zilm, K. W.; Doudna, J. A. *J. Am. Chem. Soc.* **2001**, *123*, 8447–8452) yields a similar  $pK_a$ . Because of this, we make a direct comparison of our  $pK_a$  with that reported by Jones and co-workers.
- (7) The R<sub>p</sub> and S<sub>p</sub> diastereomers were not separated since their resonances did not overlap. This provided  $pK_a$ 's from two substitutions in a single experiment. Within error, these peaks gave the same  $pK_a$  values.
- (8) (a) Hirao, I.; Kawai, G.; Yoshizawa, S.; Nishimura, Y.; Ishido, Y.; Watanabe, K.; Miura, K. *Nucleic Acids Res.* **1994**, *22*, 576–582 (b) Yoshizawa, S.; Kawai, G.; Watanabe, K.; Miura, K.; Hirao, I. *Biochemistry* **1997**, *36*, 4761–4767.
- (9) Allawi, H. T.; SantaLucia, J., Jr. *Biochemistry* **1997**, *36*, 10581–10594.
- (10) Allawi, H. T.; SantaLucia, J., Jr. *Biochemistry* **1998**, *37*, 9435–9444.
- (11) Breaker, R. R.; Joyce, G. F. *Chem. Biol.* **1994**, *1*, 223–229.

JA047362H