JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

A Simple Fluorescence Method for pK_a Determination in RNA and DNA Reveals Highly Shifted pK_a's

Jennifer L. Wilcox and Philip C. Bevilacqua*

Department of Chemistry and Center for RNA Molecular Biology, Pennsylvania State University, University Park, Pennsylvania 16802, United States

Supporting Information

ABSTRACT: Charged nucleobases exist in RNA and DNA at neutral pH owing to pK_a shifting. These bases can affect polymerase fidelity and participate in ribozyme general acid–base catalysis. Protonated RNA bases further influence miRNA processing and viral frameshifting. It is therefore important to have a simple and rapid method for determining the pK_a of nucleobases in RNA and DNA. Here we describe the application of 2-aminopurine (2AP), a fluorescent isomer of adenine, to report on the pK_a of a nearby ionizing base both in DNA secondary structure and RNA tertiary structure. We observe large, up to 5-fold quenching in fluorescence upon protonation of a nearby base. Using this method, we identify highly shifted pK_a's of 7.6 for adenine in a DNA oligonucleotide and 8.15 for cytidine in a tertiary structure element from beet western yellows virus (BWYV) RNA. These pK_a values, which were corroborated by ^{31}P NMR measurements and comparison to literature, are shifted over 4 units from their standard values. This fluorescence method can be used to determine pK_a's for ionization of both A and C and reveals that shifted pK_a's are prevalent in DNA and RNA secondary and tertiary structures.

The unpaired nucleobases in RNA and DNA are typically uncharged, with their pK_a's far removed from neutrality. Both A and C are usually deprotonated at their imino nitrogen, with pK_a's in their unpaired states of 3.5 and 4.2, respectively, while G and U/T typically have protonated imino nitrogens with pK_a's of ~9.3.[1] Upon Watson–Crick base pair formation, the pK_a's shift even further away from neutrality, by two or more pK_a units, because of hydrogen bonding, which makes charged nucleobases even less probable.[2] In certain specialized cases, however, non-Watson–Crick base pairing or a highly negative potential can shift pK_a's toward neutrality, enabling the formation of structures with positively charged bases.[3] These cases are of interest because they allow nucleobases to influence the fidelity of replication, miRNA processing, and viral frameshifting; and they also facilitate the participation of the bases in general acid–base catalysis.[4] In particular, protonated A$^+$•C base pairs are found in both DNA and RNA, where they are important for structure and function in the leadzyme, spliceosome, and viral RNA.[3]

The nucleobase 2-aminopurine (2AP) is fluorescent and has been widely used to study the dynamics and folding of RNA and DNA.[5] It is structurally similar to adenine and readily forms a two-hydrogen bond base pair with thymine (Figure 1A). In this study, we used 2AP as a reporter of base pair formation

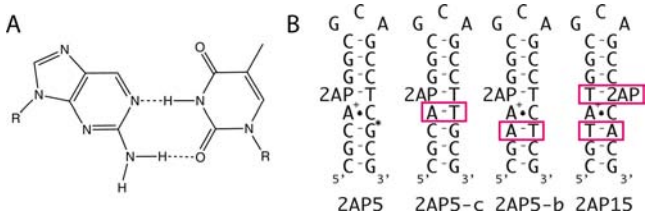

Figure 1. Fluorescent base pair and representative model DNA oligonucleotides. (A) 2AP base pairs with thymine in a two-hydrogen bond, nonwobble geometry. (B) DNA constructs used in fluorescence experiments. The naming corresponds to the position of 2AP in the helix. Position of phosphorothioate substitution, used in ^{31}P NMR constructs only, is denoted with an asterisk. '2AP5-c' is a control in which the A$^+$•C base pair is changed to A-T. In '2AP5-b' the base pair below the A$^+$•C base pair is changed from a C-G to A-T, while in '2AP15', the 2AP-T base pair is flipped.

upon protonation since the fluorescence of 2AP is quenched by stacking. Initially, pK_a shifting in DNA hairpins was examined, in which the 2AP was placed adjacent to an ionizing A$^+$•C wobble. We reasoned that at pH lower than the pK_a, the ionizing base would protonate and base pair with C. This interaction would restore native structure to the hairpin, which would lead to stacking and quenching of 2AP. Likewise, at pH higher than the pK_a, the ionizing base would deprotonate so its base pair with C would not form, which would lead to higher fluorescence.

To determine the optimal positioning of the 2AP in the helix, we tested DNA oligonucleotides with 2AP in the fifth (2AP5), sixth (2AP6), and seventh (2AP7) positions from the 5′-end (Figure 1b and Supporting Information [SI]). The hairpin constructs used were similar in structure and sequence to those from a previous study.[6] In that study, high cooperativity in secondary structure formation was observed when the protonation site was close to the terminus of the helix. Since we wished to avoid such complexity, only positions of 2AP 'above' the A$^+$•C base pair (i.e., closer to the hairpin loop) were investigated herein.

A fluorescence-detected pH titration of the 2AP5 construct, in which the 2AP was positioned adjacent to the protonated base, is provided in Figure 2. Fluorescence emission intensity

Received: December 22, 2012
Published: February 22, 2013

   dx.doi.org/10.1021/ja3125299 | J. Am. Chem. Soc. 2013, 135, 7390–7393

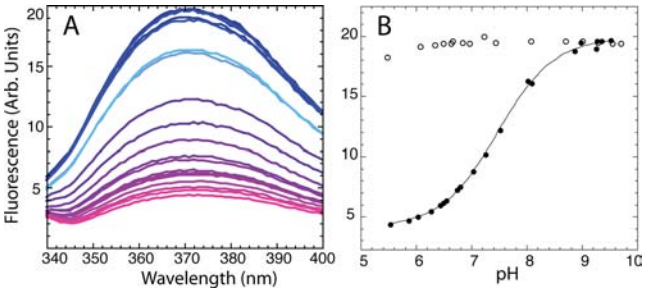

Figure 2. Fluorescence-detected pH titration of 2AP5 DNA. (A) Overlaid emission spectra. Fluorescence increased with increasing pH, going from pink to blue colored curves. (B) pH titration of 2AP5 (●) overlaid with 2AP5-c (○). Fluorescence intensities at 371 nm are plotted vs pH. The pK_a for this trial is 7.47 with a Hill coefficient of 0.85. The control construct 2AP5-c (○) showed no fluorescence change over the same pH range. For this plot, slit widths were adjusted separately for each construct.

increased between pH 5.5 and 9.5. Fitting to the Henderson–Hasselbalch (HH) equation gave an average pK_a of 7.58 and Hill coefficient of 0.87, which supports a two-state model. Remarkably, upon going from low to high pH, the fluorescence intensity increased almost 5-fold, indicating that formation of the protonated base pair significantly quenches 2AP's fluorescence.

Next we moved the 2AP further from the protonating base pair. This proved ineffective, however. Having the 2AP two (2AP6) or three (2AP7) base pairs from the site of protonation gave either a linear fluorescence response or no change in fluorescence (Figure S1 [SI]). The lack of change in fluorescence in these DNA constructs is likely due to stacking of the 2AP in the G-C rich stem in both the protonated and unprotonated states. From this, we conclude that positioning the 2AP immediately 3′ to the protonated base pair is optimal for reporting the pK_a in the A$^+$•C base pair in double-stranded DNA.

Next, we wished to confirm that the change in fluorescence observed in the pH titration was a result of protonation on the A$^+$•C base pair and not an artifact from quenching or a fluorescence impurity. A control oligonucleotide (2AP5-c) was derived from the pH-sensitive and highly fluorescent 2AP5 oligonucleotide, in which the A$^+$•C wobble pair was replaced with an A-T Watson–Crick pair. As expected, no change in fluorescence was observed over the same pH range of 5.5–9.5 (Figure 2B and Figure S2 [SI]).

To test whether addition of 2AP perturbs the pK_a of the A$^+$•C wobble, we measured the pK_a with and without 2AP using our previously developed ^{31}P NMR method.[7] Three sequences were studied, two having the above 2AP-T orientation but differing in base pairing strength (2AP5 and 2AP5-b), and another in which this base pair was flipped to T-2AP (2AP15) (Table 1). For each sequence, two different NMR constructs were studied; each construct had a phosphorothioate label (referred to as 'PS') whose position is provided in Figure 1, while one construct also had a 2AP (referred to as 'PS+2AP'). (The phosphorothioate label isolates the ^{31}P resonance downfield, as previously described.[7]) The pK_a's obtained by NMR with and without 2AP are in good agreement (Table 1 and Figures S3–S5 [SI]). In particular, for the 2AP5 sequence introduction of 2AP gave a difference of only 0.31 pK_a units (7.92 and 7.61 for PS and PS + 2AP, respectively), while in the background of 2AP5-b, a similarly

Table 1. pK_a's of DNA as Detected by NMR and Fluorescence

sequence[a]	label[b]	method[c]	pK_a[d]	n[d,e]
2AP5	PS	NMR[f]	7.92 ± 0.04	1.03 ± 0.08
	PS+2AP	NMR[f]	7.61 ± 0.03	0.99 ± 0.08
	2AP	Fl[g]	7.58 ± 0.02	0.87 ± 0.03
2AP5-b	PS	NMR[f]	7.34 ± 0.03	0.82 ± 0.05
	PS+2AP	NMR[f]	7.13 ± 0.02	0.98 ± 0.05
	2AP	Fl	7.20 ± 0.03	0.64 ± 0.03
2AP15	PS	NMR	7.41 ± 0.02	0.91 ± 0.03
	PS+2AP	NMR[f]	7.46 ± 0.01	0.95 ± 0.03
	2AP	Fl	7.20 ± 0.03	0.73 ± 0.04

[a]Secondary structures are provided in Figure 1B. [b]'PS' refers to phosphorothioate label, and '2AP' refers to 2-aminopurine. [c]NMR and fluorescence (Fl) methods are described in the SI. [d]Curves were fit to the HH equation to determine pK_a's and Hill coefficients. [e]Hill coefficients were approximately 1, supporting a two-state model. [f]Noted pK_a's and Hill coefficients determined by NMR are an average of the fits of the two diastereomer peaks. [g]Values are the average of three trials. Error is from the fits.

small difference of just 0.21 pK_a units was observed (7.34 and 7.13 for PS and PS + 2AP, respectively). Substitution of 2AP into 2AP15 had virtually no effect on the pK_a observed by NMR, with pK_a's of 7.41 versus 7.46 for PS and PS + 2AP, respectively. Overall, substitution of 2AP did not significantly shift the pK_a.

To assess the accuracy of the fluorescence-detected pK_a's, we then compared the pK_a's determined by NMR and fluorescence (PS + 2AP and 2AP labels in Table 1). The largest difference between the pK_a's determined by NMR and fluorescence was only 0.26 units, in the case of 2AP15 (7.46 versus 7.20). For 2AP5, the pK_a determined by NMR was just 0.03 units higher than by fluorescence (7.61 versus 7.58), while in 2AP5-b the pK_a by NMR was 0.07 units lower (7.13 versus 7.20). Overall, the pK_a's determined for these three sequences by fluorescence and NMR are in very good agreement, regardless of where the 2AP was substituted in the helix or the strength of base pairing.

When comparing differences in pK_a's among constructs, it is of interest to note that changes in nearest neighbor strength affect pK_a shifting. In 2AP5, C-G and 2AP-T base pairs flank the A$^+$•C, and the largest pK_a shift, to 7.58 as detected by fluorescence, is observed. Weakening the C-G base pair to A-T, in the case of 2AP5-b, shifts the pK_a to only 7.20. The lesser pK_a in the presence of AT is likely due to coupling of folding with pK_a change,[8] although other possibilities exist.

Next, we tested whether the fluorescence method could be applied to tertiary structure in RNA. Beet western yellows virus (BWYV) RNA belongs to a group of viral RNAs that utilize −1 ribosomal frameshifting to regulate translation and requires formation of a pseudoknot to initiate a change in the reading frame (Figure 3A).[10] Protonation of C8 in BWYV allows the pseudoknot structure to form at neutral pH. The pK_a of the protonated base has been calculated using thermodynamic linkage, but has not been measured directly.[11] The C8 residue participates in a base quartet with G12, C26, and A25,[9] as shown in Figure 3. An analogous base quadruple is found in the C41 quartet in the genomic hepatitis delta virus (HDV) ribozyme and in other viral frameshifting RNAs.[12] Mutational studies indicate that this quartet is critical for viral frameshifting.[13]

We tested whether the 2AP fluorescence method could be used to determine the pK_a for the BWYV RNA pseudoknot

dx.doi.org/10.1021/ja3125299 | J. Am. Chem. Soc. 2013, 135, 7390–7393

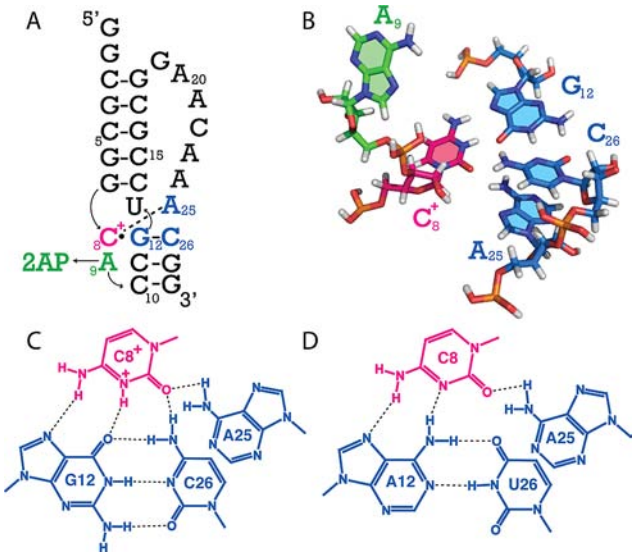

Figure 3. Beet western yellows virus (BWYV) RNA. (A) Secondary structure of BWYV. The base quartet is shown with $C8^+$ (pink)-G12-A25-C26 (blue). A9 (green) was replaced with 2AP. (B) Crystal structure of the bases of interest (PDB 437D)[9]. (C) Interactions in the base quartet with protonated C8. (D) Double mutant with G12A and C26U mutations to maintain structure with neutral C8.

motif. Residue A9 is in close proximity to the protonated base $C8^+$ and was therefore substituted with 2AP. A9 is not involved in base pairing to the protonated base quartet, as shown in Figure 3B, and is therefore an ideal choice for substitution. Titration of this RNA over a pH range of 6.8−9.8 led to an increase in fluorescence with pH, similar to that observed with the DNA oligonucleotides (Figure 4). Fitting of these data to

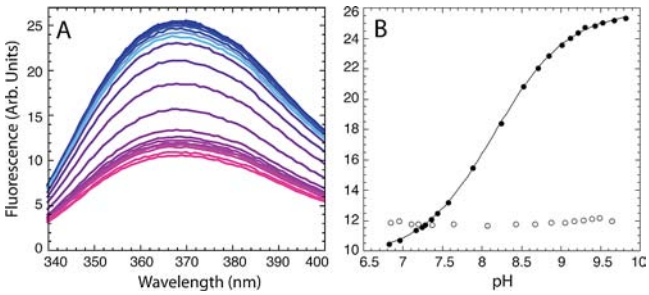

Figure 4. Fluorescence-detected pH titrations of BWYV and BWYV double mutant control RNAs. (A) Overlaid emission spectra. Fluorescence increased with increasing pH, going from pink to blue colored curves. (B) pH titration of BWYV RNA (●) overlaid with BWYV-dm RNA (○). Fluorescence intensities at 371 nm were plotted vs pH. The pK_a of $C8^+$ in the BWYV was 8.15 ± 0.01 with a Hill coefficient of 0.94 ± 0.01. The control construct showed no fluorescence change over the same pH range. For this plot, instrumental settings were the same for both constructs.

the HH equation provided a Hill coefficient of 0.94 and a pK_a of 8.15, which agrees well with the previously reported pK_a of 8.4 ± 0.3, calculated using a linkage model under identical solution conditions.[11]

As a control, we conducted a similar pH titration on a double mutant (BWYV-dm) with G12A/C26U mutations (Figure 3D). This mutant, which swaps a GC Watson−Crick base pair for an AU, has the same tertiary structure as wild-type BWYV but lacks protonation at C8. A similar double mutant was used to characterize the HDV ribozyme.[14] Moreover, this mutant maintains A25, the fourth base of the quartet, which has been shown to be critical for BWYV activity.[13] Lack of change in fluorescence in the BWYV-dm (Figure 4B) confirms that the pK_a determined in wild-type BWYV is due to protonation at C8. In addition, the intensity of BWYV-dm emission is nearly equal to that of wild-type at low pH under identical instrumental settings, as expected from the folding depicted in Figures 3C and D.

In summary, we have developed a method for pK_a determination using 2AP fluorescence that is simple and effective. Not only does 2AP fluorescence provide an accurate pK_a value, as compared to NMR the titration can be achieved in a fraction of the time, with much lower sample concentration, and with RNAs of any length. In particular, a typical NMR titration takes 14 to 36 h of labor-intensive experiment time, depending on the strength of the spectrometer. Not only is this time-consuming, depending upon the conditions under which the experiment is conducted, it can be detrimental to the sample integrity, especially for RNA. Fluorescence is also much more sensitive than NMR. A standard NMR experiment requires millimolar concentrations of nucleic acid, whereas a fluorescence experiment only requires micromolar amounts. Last, NMR is limited to RNAs less than 100 nt, while fluorescence can be used on RNAs of any size if semisynthetic methods are used to introduce the modification.[15]

Addition of 2AP into a DNA hairpin affected the pK_a by small amounts, ranging from 0.05 to 0.31 pK_a units. In addition, introduction of 2AP into an RNA tertiary structure gave a pK_a that agrees with an earlier measurement from thermodynamic linkage. This lack of perturbation can be contrasted with a recent method for determining pK_a values of RNA through the introduction of 8-azaguanosine or 8-azaadenosine, where the pK_a of G or A is perturbed by 1.1 to 1.3 units.[16] While the 8-azapurines are valuable for monitoring the pK_a of specific residues, our method is versatile in that it can be applied to a variety of nucleic acid systems, under many conditions, and without the need for a pK_a correction. Future experiments will explore the applicability of 2AP to reporting pK_a shifts due to electrostatic effects.

Here, we demonstrated that both A- and C-protonated bases can be detected using a nearby 2AP; the 2AP itself can be in a double- or single-stranded segment; DNA or RNA can be used; and the pK_a can come from secondary or tertiary structure. In principle, this method should be applicable to determining shifted pK_a's in anionic deprotonated bases, as well. With the utilization of a fluorescence plate reader, the method also has the potential to be high-throughput, allowing for analysis of multiple constructs or conditions in a short time.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods, fluorescence and NMR titration data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

pcb5@psu.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Scott Showalter, Alan Benesi, and Wenbin Lu for assistance with NMR titrations, and Steve Benkovic and Michelle Spiering for assistance with fluorescence. Funded by the National Science Foundation (NSF CHE-1213667).

■ REFERENCES

(1) Izatt, R. M.; Christensen, J. J.; Rytting, J. H. *Chem. Rev.* **1971**, *71*, 439. Saenger, W., Ed.; *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984.
(2) Legault, P.; Pardi, A. *J. Am. Chem. Soc.* **1997**, *119*, 6621.
(3) Wilcox, J. L.; Ahluwalia, A. K.; Bevilacqua, P. C. *Acc. Chem. Res.* **2011**, *44*, 1270.
(4) Chin, K.; Sharp, K. A.; Honig, B.; Pyle, A. M. *Nat. Struct. Biol.* **1999**, *6*, 1055. Tang, C. L.; Alexov, E.; Pyle, A. M.; Honig, B. *J. Mol. Biol.* **2007**, *366*, 1475. Bevilacqua, P. C. Proton Transfer in Ribozyme Catalysis. In Ribozymes and RNA Catalysis, Lilley, D. M.; Eckstein, F., Eds.; Royal Society of Chemistry: Cambridge, 2008; pp 11−36. Veeraraghavan, N.; Ganguly, A.; Chen, J. H.; Bevilacqua, P. C.; Hammes-Schiffer, S.; Golden, B. L. *Biochemistry* **2011**, *50*, 2672.
(5) Ward, D. C.; Reich, E.; Stryer, L. *J. Biol. Chem.* **1969**, *244*, 1228. Nordlund, T. M.; Andersson, S.; Nilsson, L.; Rigler, R.; Graslund, A.; McLaughlin, L. W. *Biochemistry* **1989**, *28*, 9095. Jean, J. M.; Hall, K. B. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 37. Hall, K. B. *Methods Enzymol.* **2009**, *469*, 269.
(6) Siegfried, N. A.; O'Hare, B.; Bevilacqua, P. C. *Biochemistry* **2010**, *49*, 3225.
(7) Moody, E. M.; Brown, T. S.; Bevilacqua, P. C. *J. Am. Chem. Soc.* **2004**, *126*, 10200.
(8) Moody, E. M.; Lecomte, J. T.; Bevilacqua, P. C. *RNA* **2005**, *11*, 157.
(9) Su, L.; Chen, L.; Egli, M.; Berger, J. M.; Rich, A. *Nat. Struct. Biol.* **1999**, *6*, 285.
(10) Somogyi, P.; Jenner, A. J.; Brierley, I.; Inglis, S. C. *Mol. Cell. Biol.* **1993**, *13*, 6931.
(11) Cornish, P. V.; Giedroc, D. P. *Biochemistry* **2006**, *45*, 11162.
(12) Ferre-D'Amare, A. R.; Doudna, J. A. *J. Mol. Biol.* **2000**, *295*, 541. Nixon, P. L.; Cornish, P. V.; Suram, S. V.; Giedroc, D. P. *Biochemistry* **2002**, *41*, 10665. Nixon, P. L.; Rangan, A.; Kim, Y. G.; Rich, A.; Hoffman, D. W.; Hennig, M.; Giedroc, D. P. *J. Mol. Biol.* **2002**, *322*, 621.
(13) Kim, Y. G.; Su, L.; Maas, S.; O'Neill, A.; Rich, A. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14234.
(14) Wadkins, T. S.; Shih, I.; Perrotta, A. T.; Been, M. D. *J. Mol. Biol.* **2001**, *305*, 1045. Nakano, S.; Bevilacqua, P. C. *Biochemistry* **2007**, *46*, 3001. Veeraraghavan, N.; Bevilacqua, P. C.; Hammes-Schiffer, S. *J. Mol. Biol.* **2010**, *402*, 278.
(15) Moore, M. J.; Sharp, P. A. *Science* **1992**, *256*, 992.
(16) Liu, L.; Cottrell, J. W.; Scott, L. G.; Fedor, M. J. *Nat. Chem. Biol.* **2009**, *5*, 351. Cottrell, J. W.; Scott, L. G.; Fedor, M. J. *J. Biol. Chem.* **2011**, *286*, 17658.