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ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · APRIL 2010

Impact Factor: 3.3 · DOI: 10.1021/jp100589w · Source: PubMed

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Evidence of Formation of Adenine Dimer Cation Radical in DNA: The Importance of Adenine Base Stacking

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Received: January 21, 2010; Revised Manuscript Received: February 25, 2010

Deprotonation of the adenine (A) base in both mononucleotide and oligonucleotide (ODN) was measured by nanosecond pulse radiolysis. The cation radical ($A^{+\bullet}$) of deoxyadenosine (dA), produced by oxidation with $SO_4^{-\bullet}$, rapidly deprotonated to form the neutral A radical ($A(-H)^{\bullet}$) with a rate constant of 2.0×10^7 s^{-1} and a pK_a value of 4.2, as determined by transient spectroscopy. A similar process was observed in experiments performed on a variety of double-stranded ODNs containing adenine•thymine (A•T) base pairs. The transient spectrum of $A^{+\bullet}$ in an ODN composed of alternating A•T pairs was essentially identical to that of free dA and differed from the spectra of ODNs containing AA and AAA. In contrast, the spectra of $A(-H)^{\bullet}$ were not affected by the sequence. These results suggest that the positive charge on $A^{+\bullet}$ in ODNs is delocalized as the dimer is stabilized by π-orbital stacking between adjacent A's. The rate constants for deprotonation of $A^{+\bullet}$ in ODNs containing AA and AAA ($0.9-1.1 \times 10^7$ s^{-1}) were a factor of 2 smaller than the rate constants for deprotonation of $A^{+\bullet}$ in ODNs containing alternating A•T and dA (2.0×10^7 s^{-1}). This suggests that the formation of a charge resonance stabilized dimer $AA^{+\bullet}$ in DNA produced a significant barrier to deprotonation.

Introduction

Long-range hole migration along the π stack of DNA currently receives much experimental[1] and theoretical attention.[2] Charges apparently are transported over long distances (200 Å) through a multistep hopping mechanism between guanine (G) bases (G-hopping),[3−6] which have the lowest ionization potential among DNA bases. In addition, it has been suggested that adenine (A) plays a hole carrier in long A−T stretches.[7−9] Once a hole resides on an A base, as an A cation radical ($A^{+\bullet}$), hole transfer can occur over long A sequences rapidly and efficiently (A-hopping) if G does not interrupt the A sequence. Takada et al.[8c] reported that the efficiency of the charge transfer dramatically decreases if consecutive A bases are replaced with an AT repeat sequence. This suggests that the direct stacking between adjacent same-strand A bases is important for the A-hopping mechanism. However, the mechanistic details of A hopping remain unclear.

Pulse radiolysis and 248 nm laser photolysis experiments showed that the one electron oxidized form of deoxyadenosine (dA) at a pH range of 0−6 produced the deprotonated species $A(-H)^{\bullet}$ only, as determined by both optical and conductance detection.[10−12] From these results, Steenken concluded that the pK_a of $A^{\bullet+}$ was <1 in reaction 1.

$$A^{+\bullet} \rightleftarrows A(-H)^{\bullet} + H^{+} \qquad (1)$$

However, a pK_a of <1 was puzzling and may have been inconsistent with the pH-dependent rate of migration from $A^{+\bullet}$ to G observed in dinucleoside phosphates ApG and GpA.[12] The rate of hole transfer from $A^{+\bullet}$ to G in these dinucleoside phosphate systems decreased as the pH was raised from pH 2.3 to 7. On the other hand, different values of the pK_a have been measured in different experiments.[13−16] In an earlier study, Scheek et al.[13] reported that a pK_a value of 4 ± 0.2 was determined by an NMR study of the one-electron-oxidized A produced via laser CIDNP (chemical induced dynamic nuclear polarization) in 5′-AMP. Theoretical calculations predicted the pK_a of $A^{+\bullet}$ as 2[14a] and 3.2.[14b] A recent ESR spectroscopy study of γ-irradiated frozen solutions combined with theoretical calculations showed that the experimental pK_a of $A^{+\bullet}$ in a DNA oligomer $(dA)_6$ at 150 K was 8, much higher than the predicted pK_a for monomeric $A^{+\bullet}$ (ca. −0.3).[15,16] From these results, Adhikary et al.[16] proposed that the change in experimental pK_a was due to the deprotonation of stacked $A^{+\bullet}$.

The deprotonation in reaction 1 is expected to be quite fast because the primary product of one-electron oxidation, $A^{+\bullet}$, has not been experimentally observed.[10−12] On the other hand, because the bases A and T are present in pairs in a double-stranded DNA, the equilibrium shown in reaction 1 may shift toward $A^{+\bullet}$. Less efficient deprotonation at N6 could enhance the lifetime of $A^{+\bullet}$. However, no previous reports have described direct observation of the deprotonation of $A^{+\bullet}$ in DNA at ambient temperature. If deprotonation were to occur prior to hole transfer, then the hole transfer would be inhibited. However, this assumption contradicts the experimental observations of A-hopping.[7−9,17] Hole transfer in A tracks has been experimentally characterized (rate constants of 10^8-10^{10} s^{-1}).[8,17]

Large quantities of the cation radical of nucleic acid bases can be efficiently formed in DNA and isolated nucleotides by pulse radiolysis in the presence of persulfate.[18−20] Our previous reports have used the pulse radiolysis technique to assess the deprotonation of $G^{+\bullet}$ in dG and double-stranded oligonucleotides (ODNs).[19,20] In addition, transient absorption measurements by pulse radiolysis have shown that $G^{+\bullet}$ is stabilized by base pairing with cytosine (C) and by stacking interactions with neighboring nucleobases.[20] This paper describes the use of this method to follow the deprotonation of $A^{+\bullet}$ both in dA and in ODNs containing A•T base pairs. We spectrophotometrically observed

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10.1021/jp100589w
Published on Web 04/06/2010

TABLE 1: Oligonucleotide Sequences

name	sequence (5′ → 3′)
AT	5′-ATATATATATAT-3′ 3′-TATATATATATA-5′
A_2T_2	5′-TTAATTAATT-3′ 3′-AATTAATTAA-5′
A_3T_3	5′-TTTAAATTTAAA-3′ 3′-AAATTTAAATTT-5′
$A_{12}T_{12}$	5′-AAAAAAAAAAAA-3′ 3′-TTTTTTTTTTTT-5′
ssA_{12}	AAAAAAAAAAAA

that $A^{+\bullet}$ was transiently present at neutral pH and deprotonated rapidly to form a neutral radical $A(-H)^{\bullet}$ with a pK_a of 4.2. We also analyzed the dynamics of a systematic series of ODNs using transient spectra to observe the kinetics of deprotonation of $A^{+\bullet}$ in these sequences.

Experimental Section

Materials. ODNs were synthesized and purified by HPLC at Sigma Genosis Biotech Co., Ltd., Japan. Table 1 shows the prepared double-stranded ODNs. The ODNs were dissolved in 20 mM sodium phosphate buffer (pH 7.0). The two strands were annealed by heating the samples to 90 °C for 5 min, then allowing the samples to cool slowly back to room temperature over a period of 1 h.

The formation of double-stranded ODNs was confirmed by monitoring the absorbance at 260 nm. All other reagents were of the highest purity available commercially.

Pulse Radiolysis. Samples for pulse radiolysis were prepared as follows. Aqueous solutions of either the double-stranded ODNs or dA contained 20 mM ammonium persulfate, 10 mM buffer, and 0.1 M NaCl, 0.1 M *tert*-butyl alcohol (for scavenging OH radicals) and were deoxygenated in sealed cells and flushed with argon. The buffers used were 10 mM potassium acetate buffer (pH 1−5.5), 10 mM potassium phosphate buffer (pH 5.5−8), or 10 mM potassium borate (pH 8−10). The pH was adjusted with NaOH or $HClO_4$. The quartz cells had an optical path length of 1 cm.

Pulse radiolysis experiments were performed with a linear accelerator at the Institute of Scientific and Industrial Research, Osaka University.[19−21] The pulse width and energy were 8 ns and 27 MeV, respectively. The light source was a Xe flash lamp (a continuous spectrum from 300 to 1600 nm). The analyzing light was monitored with a Ritsu MC-10N monochromator and detected by PIN Si (Hamamatsu S1722) or InGaAs (Hamamatsu G3476) photodiodes. The signals were corrected by a Sony/Tektronics SCD transient digitizer. The concentration of $SO_4^{-\bullet}$ radicals generated by pulse radiolysis was determined by absorbance change at 450 nm using a millimolar extinction coefficient of 1600 M^{-1} cm^{-1}.[22]

Results

Acid−Base Properties of Doxyadenine. Pulse radiolysis experiments almost instantaneously generate $SO_4^{-\bullet}$, which, in turn, can oxidize A to the $A^{+\bullet}$ without deprotonation, as shown in reaction 2. Subsequently, deprotonation, as shown in reaction 1, occurs very rapidly.

$$SO_4^{-\bullet} + A \rightarrow SO_4^{2-} + A^{+\bullet} \quad (2)$$

At low concentration of dA (<1 mM), reaction 1 could not be observed. Under these conditions, the rate-determining step in these processes is the oxidation of dA by $SO_4^{-\bullet}$ ($k_1 > k_2[A]$). The equilibrium in reaction 1 is practically attained during the oxidation process. At above 3 mM dA, on the other hand, reactions 1 and 2 were kinetically resolved. A typical example is shown in Figure 1. Under that condition, reaction 1 was found to be complete within 20−30 ns. The initial rapid increases in the absorbance bands at 330 and 450 nm reflected the oxidation of dA by $SO_4^{-\bullet}$. At pH 7.2, as shown in Figure 1B, other spectral changes occurred on the time scale of nanoseconds. Slower absorption increases at 330 and 600 nm and a decrease at 450 nm were seen. In contrast, no corresponding absorbance changes were observed at pH 2.0 (Figure 1A). Figure 2 shows the transient spectra at 50 and 500 ns after pulse radiolysis of dA at pH 7.2. The spectrum corresponding to the faster phase has absorptions at 330 nm and around 480 nm, whereas the spectrum for the subsequent process has absorption increase at 330 nm and around 600 nm. The spectrum at 500 ns is similar to spectra reported previously[12,23,24] and was identified as corresponding to the deprotonated form of $A(-H)^{\bullet}$.[25] From these findings, it can be concluded that the slower spectral changes in Figure 1B arose from the deprotonation of $A^{+\bullet}$ to give $A(-H)^{\bullet}$. The rate constant for deprotonation was 2.0×10^7 s^{-1} at pH 7.2 and was independent, within experimental error (data not shown), of the concentration of dA in the range 2−6 mM. The deprotonation rate was similar to that of $dG^{+\bullet}$(1.8×10^7 s^{-1}) reported previously.[19] At a high concentration dA (>10 mM), on the other hand, stacking of dA occurs in aqueous solution.[26] Under that condition, the rate of deprotonation may be affected.[16]

To confirm the pH-dependence of the spectra, the spectra were measured over a range of pH values, from pH 1 to 8. The intensities of the slower absorbance changes, observed at neutral pH values, were found to decrease with decreasing pH and were lost below pH 2 in Figure 1A. To determine the pK_a for reaction 1, the ratios of total absorbance changes to the absorbance changes of the fast fraction ($\Delta A_b/\Delta A_a$), observed at 600 nm (Figure 3A), were plotted against the pH (Figure 3B). These changes fit well to a pK profile with an inflection point at 4.2. Therefore, it was concluded that the pK_a of $A^{+\bullet}$ was 4.2. This value was similar to the pK value (4 ± 0.2) reported by a CIDNP study of 5′-AMP.[13] When $A^{+\bullet}$ was produced at pH values below its pK_a, the spectrum observed was the same as the spectrum recorded initially at pH 7.2 (data not shown). The absence of additional spectral changes (Figure 1A) indicated that deprotonation did not take place at pH conditions below the pK_a.

Oxidation of Oligonucleotides Containing A•T Base Pairs. It was of particular interest to study ODN sequences showing dependence on spectroscopy changes of the intermediates and the rate constants of the deprotonation of $A^{+\bullet}$. Figure 4 compares the transient spectra of ODNs A_3T_3, A_2T_2, and AT at 50 and 500 ns after pulse radiolysis. These spectra are indistinguishable from those of dA at pH 7.2. The spectra corresponding to the faster phase, which was characterized by absorption maxima at 330 and 450 nm, were consistent with the formation of $A^{+\bullet}$. The spectral changes corresponding to the slower phase had absorption bands at 330 and 600 nm and were characteristic of $A(-H)^{\bullet}$. From these findings, it can be concluded that in double-stranded ODN, $A^{+\bullet}$ was formed initially, followed by the deprotonation of $A^{+\bullet}$ to give $A(-H)^{\bullet}$.

It is worth noting that the characteristic absorption bands of $A^{+\bullet}$ differed according to the sequence. When the initial transient spectra in Figure 4B and D were compared, the transients ODNs A_3T_3 and A_2T_2, which were composed of AAA and AA residues, showed marked increases in the absorption intensities over the range 400−500 nm. Significant differences between the spectra

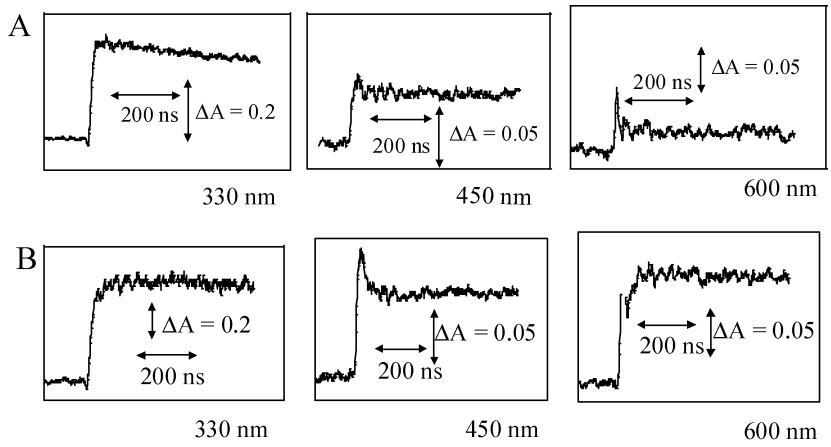


Figure 1. Absorbance changes at 330, 450, and 600 nm after pulse radiolysis of dA (5 mM) in the presence of ammonium persulfate (20 mM) and *tert*-butyl alcohol at pH 2.0 (10 mM acetate buffer) (A) and pH 7.2 (10 mM phosphate buffer) (B).

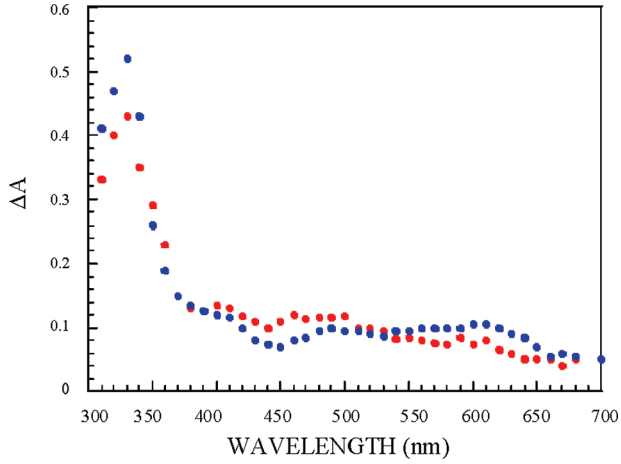


Figure 2. Kinetic difference spectra of pulse radiolysis of dA at 50 (red) and 500 ns (blue) after pulse radiolysis at pH 7.2. Experimental conditions were same as those described in the legend to Figure 1.

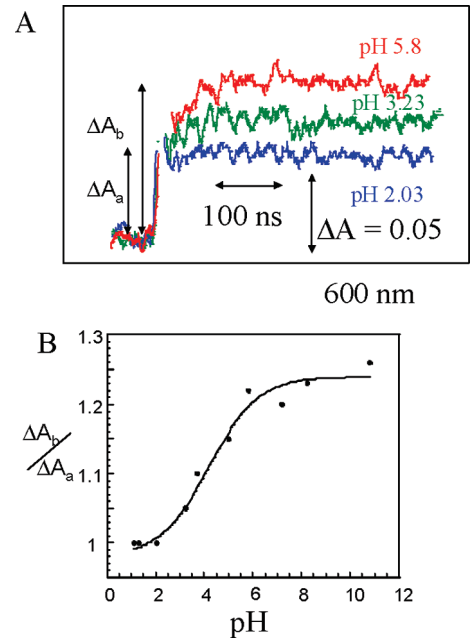


Figure 3. (A) pH-Dependent absorbance changes at 600 nm after pulse radiolysis of dA. (B) pH-Dependent changes of the ratio of the total absorbance change (ΔA_b) to the faster absorbance change (ΔA_a).

of the ODNs A_3T_3 and A_2T_2 were not observed. Similar spectra were obtained for the ODN $A_{12}T_{12}$ and single-stranded A_{12} (data not shown). Single-stranded A_{12} in aqueous solution is present in a stacked conformation, as demonstrated by CD and NMR studies.[27] In contrast, the ODN AT, composed of alternating A•T base pairs, had no distinct absorption bands over the range 400−500 nm, and the spectrum was similar to that of the isolated nucleotide dA. Therefore, it was concluded that the differences between the spectra shown in Figure 4 arose from the presence of stacking between two or three consecutive A bases. In contrast with the $A^{+\bullet}$ species, the spectral changes of $A(-H)^{\bullet}$ did not vary in these sequences, and the spectra were indistinguishable from the spectrum of free dA.

The increased absorbance at 600 nm due to the deprotonation of $A^{+\bullet}$ was affected by the adjacent sequences of ODNs, as shown in Figure 5. The rate constant for the deprotonation of AT (2.0×10^7 s^{-1}) was similar to the deprotonation rate constants of both dA and single-stranded A_{12}. In contrast, the rates of deprotonation in the ODNs A_3T_3 and A_2T_2($0.9-1.1 \times 10^7$ s^{-1}) were a factor of 2 smaller than those of the ODN AT (Table 2).

Discussion

Previous studies have suggested that the pK_a of $A^{+\bullet}$ is <1 and that the deprotonation into $A(-H)^{\bullet}$ in aqueous solution is quite fast.[10,11] Here, however, we describe 10 ns pulse radiolysis studies that demonstrate the transient formation of a free adenine cation radical, $A^{+\bullet}$ in neutral aqueous solutions, with subsequent deprotonation to form a neutral radical $A(-H)^{\bullet}$ with a half-life of 35 ns. Importantly, $A^{+\bullet}$ in a monomeric form was shown to have a pK_a of 4.2 at ambient temperature. The pK_a value obtained was much higher than the estimated ($pK_a < 1$)[10] and theoretical values ($pK_a = -0.3$),[16] but was similar to the pK_a measured previously in a CIDNP study of 5′-AMP ($pK_a = 4.0 \pm 0.2$).[13] The present data are also consistent with the pH-dependent rate of hole transfer from $A^{+\bullet}$ to G in the dinucleotide phosphate system.[12] The pH dependence can be explained by the difference between the reduction potential of $A^{+\bullet}$ and $A(-H)^{\bullet}$, which has a pK_a of 4.2. Correspondingly, the radical cation of 2-aminopurine deprotonates to form an N^2-centered neutral radical with pK_a of 2.7.[28] Thus, $A^{+\bullet}$ (6-aminopurine) is a weaker Brønstead acid than the radical cation of 2-aminopurine.

With double-stranded ODNs, similar transient absorption spectra were obtained after oxidation of ODN with $SO_4^{\bullet-}$. Therefore, the conversion from $A^{+\bullet}$ to $A(-H)^{\bullet}$ in DNA was found to occur with the concomitant release of a proton on time scales comparable to the deprotonation time scales measured

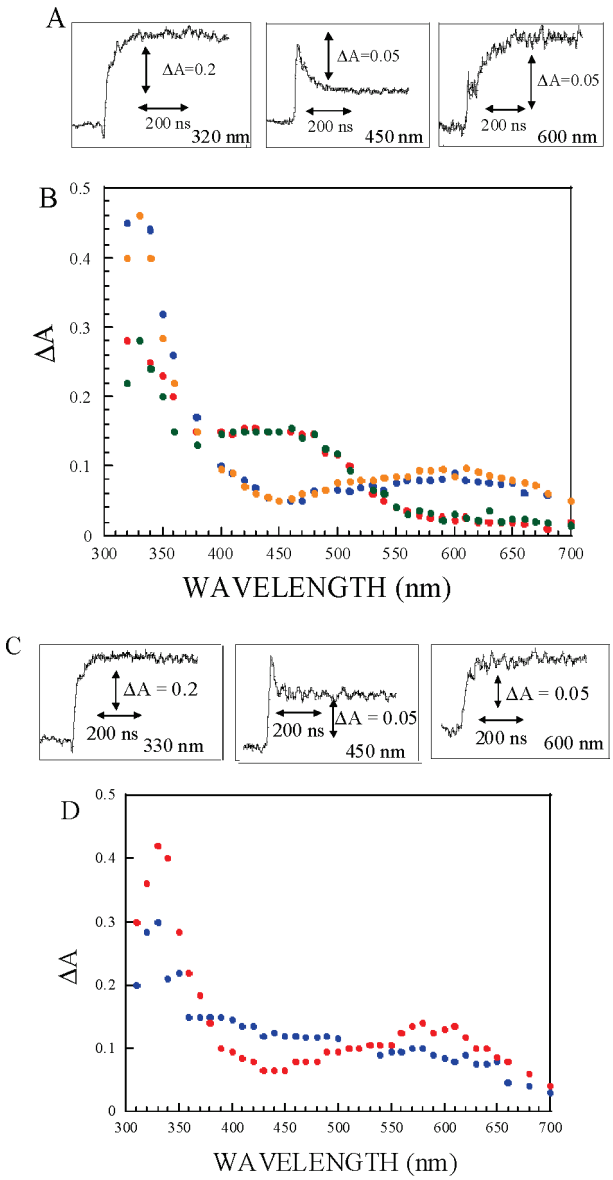


Figure 4. (A) Absorbance changes at 320, 450, and 600 nm after pulse radiolysis of ODN A_3T_3. Samples contained ODN (2.1 mM), ammonium persulfate (20 mM), NaCl (0.1 M), *tert*-butyl alcohol (0.1 M), and phosphate buffer (20 mM, pH 7.0). (B) Kinetic difference spectra of ODN A_3T_3 monitored at 50 (green) and 500 ns (blue) and ODN A_2T_2 at 50 (red) and 500 ns (gold). (C) Absorbance changes at 330, 450, and 600 nm after pulse radiolysis of ODN AT. Samples contained ODN (2.0 mM), ammonium persulfate (20 mM), NaCl (0.1 M), *tert*-butylalcohol (0.1 M), and phosphate buffer (20 mM, pH 7.0). (D) Kinetic difference spectra of ODN AT monitored at 50 (blue) and 500 ns (red).

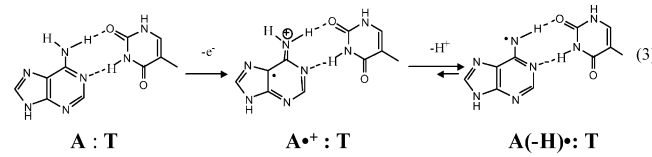
for free dA at neutral pH. Importantly, however, it was shown that the spectra of $A^{+\bullet}$ in ODNs differed from that of the isolated nucleotide dA and were affected by the ODN sequences. These results demonstrate that the transiently formed $A^{\bullet+}$ was stabilized by stacking interactions between adjacent A bases. The differences between the spectral and kinetic behavior of ODNs AT and A_2T_2 (or A_3T_3) were understood in terms of the stacking interactions (or absence thereof) between two or three consecutive A bases. In contrast, differences were not observed between ODNs A_3T_3 and A_2T_2. These results imply that the cation radical $(AA)^{+\bullet}$ apparently exists primarily in the stacked form as a dimer, not as a multimer $(A)_n^{+\bullet}$. The stacked dimer cation radicals are stabilized by the delocalization of the charge across two A bases. The $\pi-\pi$ dimer cation radicals show a characteristic charge resonance band in the near-IR region.[29−31] In our systems, however, a characteristic charge resonance band due to $\pi-\pi$ interactions was not observed in the spectral range from 800 to1200 nm. In contrast, the spectra of A(− H)• in ODNs were essentially identical to the spectrum of dA and were not affected by the ODN sequence. This suggests that the radical orbital of A(− H)• was essentially localized on an A base site, which is consistent with theoretical predictions.[15,16]

TABLE 2: Rate Constants of Deprotonation in Various Oligonucleotides

name	k (s^{-1})
dA[a]	$(2.0 \pm 0.2) \times 10^7$
AT	$(2.0 \pm 0.2) \times 10^7$
A_2T_2	$(1.1 \pm 0.1) \times 10^7$
A_3T_3	$(0.95 \pm 0.1) \times 10^7$
$A_{12}T_{12}$	$(1.3 \pm 0.1) \times 10^7$
A_{12}[b]	$(2.0 \pm 0.2) \times 10^7$

[a] pH 7.2. [b] Single-stranded.

The $A^{+\bullet}$ species is deprotonated at the N6 amino group, yielding A(N6′ − H)•, a highly reactive imine radical, which has been observed in the crystal of adenine at 10 K.[32,33] In the adenine cation/radical thymine base pair ($A^{+\bullet}-T$), however, it was not clear whether the N6 proton on A shifted to T, because T is a very poor proton receptor (pK_a of $T(H^+)$ is -5),[11a] and the equilibrium should strongly favor A in the radical cation state. In the present study, however, no significant differences were observed between the deprotonation rates of free dA and the ODN AT. This implied that the proton was released to the solvent, as in the case of free dA (reaction 3), suggesting that a water molecule can easily access the A•T moiety in an ODN. The N6 proton of A would yield a deprotonated A radical and a T base pair, which would account for the spectra observed in Figure 4B and D. In contrast, the rate constants for deprotonation in ODNs A_3T_3 and A_2T_2 ($0.9-1.1 \times 10^7$ s^{-1}) were a factor of 2 smaller than those for ODN AT (Table 2). The difference



(3)

was attributed to the stabilization of the A stacked dimer radical cation and was consistent with the spectral intermediates characteristic of the formation of $A^{+\bullet}$. This suggests that the charge resonance stabilized species $AA^{+\bullet}$ in DNA results in a significant barrier to deprotonation. A similar conclusion was

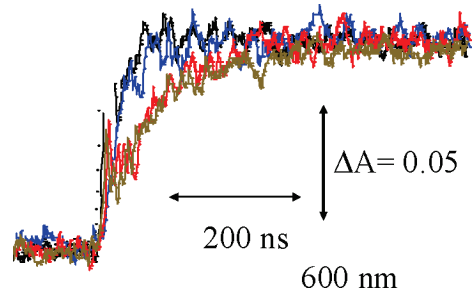


Figure 5. Kinetics of absorbance changes at 600 nm after pulse radiolysis of dA (black), ODN AT (blue), A_2T_2 (red), and A_3T_3(brown). The experimental conditions were the same as described in Figures 1 and 4.

obtained from a low-temperature aqueous glass ESR study of $A^{+\bullet}$ in the context of a DNA-oligomer $(dA)_6$ and from a complementary DFT calculation.[16] In this study, $A^{+\bullet}$ in DNA oligomer $(dA)_6$ had a pK_a value of 8 at 150 K, and deprotonation proceeded upon thermal annealing at 160 K. At ambient temperature, stacking of dA occurs even in aqueous solution.[27] Under that condition, the pK_a value of $A^{+\bullet}$ and the rate of deprotonation may be affected.[16] It will be important to carry out a similar experiment at a high concentration of dA (>10 mM). Some of these studies are now in progress in our laboratory.

In a quite different system, Okamoto et al.[31] reported that a rate of deprotonation of phenol derivatives in poly(4-hydroxystyrene) cation radical was 2.6−5 times slower than that of monomer cation. This effect was explained by a barrier to deprotonation due to the $\pi-\pi$ interaction between chromophores in the dimer cation radical.

The results presented here have several important implications for electron transport through DNA. First, the extremely rapid long-distance hole transfer over long A sequences may be explained by the direct stacking between A bases, leading to the hole delocalization between adjacent A's. This is consistent with the model that allows delocalized states through A tracts.[9] Hole transfer for A-hopping proceeds via a multistep mechanism between AA dimers, suggesting that the oxidation potential and surface stacking area of the AA dimer hole carriers are important factors. Second, our results show that the deprotonation of $A^{+\bullet}$ occurs on the time scale of 10 ns ($\sim 2 \times 10^7$ s^{-1}), which is much slower than the rate of the hole transfer via A-hopping (>10^{10} s^{-1}).[8] This suggests that the electron transfer for A-hopping along DNA occurs prior to deprotonation. If a G base is present within a few base pairs, hole transfer from $A^{+\bullet}$ to the nearby G would not compete with deprotonation. Deprotonation may not contribute to the rapid hole transfer between A bases. It is assumed that the reaction of the imine radical and subsequent formation of 8-oxo-7, 8-dihydroadenine derivatives is a minor process in the overall oxidation of A bases.[24] In contrast, the rate of G-hopping, from G to GG across one A base (5×10^6 s^{-1}),[34] is comparable to the rate of deprotonation of $G^{+\bullet}$ ($0.3-2 \times 10^7$ s^{-1}).[19,20] Therefore, the subsequent reaction of G(− H)$^\bullet$ may be an important step in DNA damage caused by ionizing radiation.

Conclusion

Employing pulse radiolysis techniques, the adenine radical cation ($A^{+\bullet}$) and its deprotonated radical (A(− H)$^\bullet$) were identified in both dA and double-stranded DNA. A systematic series of A•T-containing base pairs was studied, and the characteristic absorption of $A^{+\bullet}$ was found to be affected by the identity of the contextual ODN sequence, whereas the spectra of A(− H)$^\bullet$ were not affected. The adenine base stacking stabilized $A^{+\bullet}$ by hole delocalization to the adjacent adenine, presenting a significant barrier to deprotonation.

Acknowledgment. We thank members of the Radiation Laboratory of the Institute of Scientific and Industrial Research, Osaka University, for their assistance in operating the accelerator. We thank Prof. Seiichi Tagawa, Dr. Kiyohiko Kawai of the Institute of Scientific and Industrial Research, Osaka University, and Dr. Takeo Ito of the Faculty of Engineering, Kyoto University, for valuable advice and helpful discussion.

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JP100589W