Effects of Base Stacking on Guanine Electron Transfer: Rate Constants for G and GG Sequences of Oligonucleotides from Catalytic Electrochemistry

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Abstract: The electron-transfer rate constants for oligonucleotides containing adjacent guanines were determined by digital simulation of cyclic voltammograms of $Ru(bpy)_3^{2+}$ in the presence of the oligonucleotides (bpy = 2,2′-bipyridine). These experiments showed that sequences containing an isolated guanine (included in a 5′-AGT segment) gave a rate constant of 1.4×10^5 M^{-1} s^{-1} (in terms of guanine concentration) while sequences containing a 5′-GG segment gave an overall rate constant of 7.5×10^5 M^{-1} s^{-1}. Both rate constants were independent of DNA concentration in the simulations. By assuming that the 3′-G of the GG doublet exhibits the same rate constant as the isolated guanine, we estimate the ratio of rate constants for the 5′-G of the GG doublet to the 3′-G to be $k_{GG}/k_G = 12 \pm 2$. This value was independent of DNA concentration and scan rate. Similar experiments using oligonucleotides containing inosine (I) in place of guanosine gave the same rate constant for a 5′-IG doublet as for isolated guanine ($k_{IG}/k_G = 1.0 \pm 0.2$) but gave significant enhancement for the 5′-GI sequence ($k_{GI}/k_G = 2.8 \pm 0.4$). These experiments show that it is in fact the 5′-G that is enhanced and support the assumption that the 3′-G of the GG doublet gives the same rate constant as isolated guanine. Stacking of guanines on the 5′ side of 7-deazaguanine did not produce current enhancements as large as those for the GG segments, strongly supporting the idea that favorable placement of the electronegative N7 atom of the 3′ base in the doublet is responsible for the increased electron donor reactivity.

Recent observations that the 5′-G of a GG sequence is more electron-rich than its 3′-neighbor[1,2] are of interest in understanding natural mutagenesis[3,4] and in probing the mechanisms of charge migration in DNA.[5−8] The selectivity for oxidation of the 5′-G of guanine doublets is easily observed on sequencing gels following treatment of labeled oligonucleotides with one-electron oxidants.[5−9] The increased nucleophilicity of the 5′-guanine is also revealed by the substitution kinetics of platinum amine complexes[10] and the covalent binding kinetics of chemical carcinogens,[11] although these reactions are more complicated than simple one-electron transfer. The observed site selectivity for the 5′-guanine cleavage upon irradiation in the presence of photosensitizers, which were expected a priori to react in a uniform fashion, prompted Sugiyama and Saito[2] to perform ab initio molecular orbital calculations of stacked nucleobase pairs. These calculations indicated that the ionization potential of a guanine located 5′ to another guanine is significantly lower than that of a single guanine and that the HOMO is located primarily on the 5′ guanine. Later calculations by Prat et al. supported this assertion.[12] This hypothesis is also supported by the observation that selectivity for the 5′-G is not apparent upon oxidation of GG doublets in DNA/RNA hybrids in the A form, where placement of the two guanines is significantly different.[13]

Because the 5′-guanine of a GG step has a lower ionization potential than any other DNA base, GG doublets have been used as a "hole trap" in the study of DNA-mediated electron transfer.[5−9] After chemical oxidation by a remote acceptor, cleavage at the 5′-guanine is monitored to determine the relative rate of electron transfer through DNA and to study interruption in the electron transfer upon introduction of structural deviations from the native B-form helix.[6] Hole migration is also thought to be a critical factor in mutagenesis caused by ionizing radiation,[14] so an understanding of the sequence-dependent guanine reactivity will therefore be helpful in the prediction of mutagenic sequences within the genome. Although there is considerable evidence supporting selective oxidation of the 5′-guanine from sequencing gels, there is no study of which we are aware that compares the relative reactivities of GG doublets and isolated guanines using a method that monitors the electron-transfer process in real time. Kinetic studies of guanine oxidation

(1) Saito, I.; Takayama, M.; Sugiyama, H.; Nakatani, K.; Tsuchida, A.; Yamamoto, M. *J. Am. Chem. Soc.* **1995**, *117*, 6406−6405.
(2) Sugiyama, H.; Saito, I. *J. Am. Chem. Soc.* **1996**, *118*, 7063−7068.
(3) Beckman, K. B.; Ames, B. N. *J. Biol. Chem.* **1997**, *272*, 19633−19636.
(4) Helbock, H. J.; Beckman, K. B.; Shigenaga, M. K.; Walter, P. B.; Woodall, A. A.; Yeo, H. C.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 288−293.
(5) Henderson, P. T.; Jones, D.; Hampikian, G.; Kan, Y.; Schuster, G. B. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8353−8358.
(6) Hall, D. B.; Holmlin, R. E.; Barton, J. K. *Nature* **1996**, *384*, 731−735.
(7) Meggers, E.; Michel-Beyerle, M. E.; Giese, B. *J. Am. Chem. Soc.* **1998**, *120*, 12950−12955.
(8) Nunez, M. E.; Hall, D. B.; Barton, J. K. *Chem. Biol.* **1999**, *6*, 85−97.
(9) Hickerson, R. P.; Prat, F.; Muller, J. G.; Foote, C. S.; Burrows, C. J. *J. Am. Chem. Soc.* **1999**, *121*, 9423−9428.
(10) Elmroth, S. K. C.; Lippard, S. J. *J. Am. Chem. Soc.* **1994**, *116*, 3633−3634.
(11) Said, B.; Shank, R. *Nucleic Acids Res.* **1991**, *19*, 1311−1316.
(12) Prat, F.; Houk, K. N.; Foote, C. S. *J. Am. Chem. Soc.* **1998**, *120*, 845−846.
(13) Sartor, V.; Henderson, P. T.; Schuster, G. B. *J. Am. Chem. Soc.* **1999**, *121*, 11027−11033.
(14) Grobelsek-Vracko, M.; Zaider, M. *Radiat. Res.* **1994**, *138*, 18−25.

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have been carried out by spectroscopic methods; [15−20] however, these experiments were not designed to determine the enhancement in reactivity in GG doublets. Studies on sequencing gels are always subject to caveats concerning the molecular events that occur between the initial electron transfer and the realization of strand scissions that can be visualized. We report here on a study of the absolute rate constants for GG oxidation that supports findings from sequencing gels and provides new quantitative information that should be helpful in interpreting studies of long-range electron transfer (or transport[18]) and in delineating the role of these processes in natural mutagenesis.

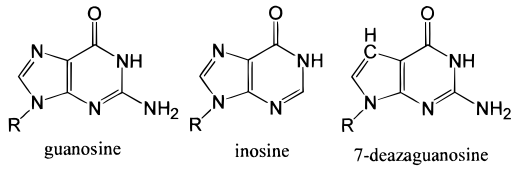
We have developed a system for measuring the rates of electron transfer from guanine in small quantities of oligonucleotides or polymers to weakly bound metal complexes.[21−24] This approach involves the acquisition of the cyclic voltammogram of $Ru(bpy)_3^{2+}$ at metal oxide electrodes with and without DNA (bpy = 2,2′-bipyridine). In the presence of DNA, the metal complex catalyzes the oxidation of guanine according to the cycle:

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^- \quad (1)$$

$$Ru(bpy)_3^{3+} + DNA \rightarrow Ru(bpy)_3^{2+} + DNA_{ox} \quad (2)$$

where DNA_{ox} contains guanine that has been oxidized by a single electron. The current due to direct electrochemistry of DNA at tin-doped indium oxide (ITO) electrodes is negligible, so enhancements in the oxidative current for $Ru(bpy)_3^{2+}$ in the presence of DNA can be attributed to catalytic cycling of Ru(III/II). We have developed methodology for determining the rate constants for eq 2 by digital simulation of cyclic voltammograms;[21−24] these rate constants have been confirmed by chronoamperometry,[23] square wave voltammetry,[22] and stopped-flow spectrophotometry.[22] The rate constants exhibit a dependence on driving force that is appropriate for outer-sphere electron transfer,[22] and our early estimates of the potential of the $G^{+/0}$ couple were in good agreement with later values determined by equilibrium titration (1.07 V at pH 7; all potentials versus Ag/AgCl).[25] Here, we use this approach to determine the rate constants for electron transfer from guanine to Ru(III) that is weakly bound to oligonucleotides that contain guanines stacked on the 5′ side of either another guanine or a derivative. Our results show that the rate of oxidation of the 5′-guanine in a GG doublet is enhanced by a factor of 12 compared to isolated guanine, which implies a potential for the stacked 5′-guanine of 0.95 V. Rate constants for guanine derivatives (Scheme 1) support the hypothesis that favorable placement of the N7 of the 3′ base, as suggested by Prat et al.,[12] is responsible for the stacking effect.

Scheme 1



Experimental Section

DNA. Synthetic oligonucleotides were obtained either from the UNC Department of Pathology or the Lineberger Comprehensive Cancer Center Nucleic Acids Core Facility. Phosphoramidites for modified bases were purchased from Glen Research. Purification was performed by HPLC, filtration on a Centricon 3 (Amicon, Bedford, MA) 3000 MW cutoff filter, or two successive ethanol precipitation steps. Oligonucleotide solution concentrations were determined spectrophotometrically in moles of strand.[26] For sequences containing modified bases, the contribution to the extinction coefficient from the modified base was estimated by substituting the value for guanine. Oligonucleotide strands were designed such that all guanines were located on a target strand; double-stranded samples were obtained by heating the guanine-containing DNA in the presence of a 2-fold excess of the complementary strand to 90 °C and cooling slowly over 2 h. All experiments were performed with double-stranded DNA, unless otherwise stated. Hybridization to form B-DNA was confirmed by native gel electrophoresis and circular dichroism spectroscopy; the procedures and results of these experiments are given in the Supporting Information.

Reagents. Deionized water was obtained by passing in-house distilled water though a Milli-Q (18 Ω) deionizing system (Millipore Corporation, Bedford, MA) and was used for the preparation of all aqueous solutions. Sodium phosphate salts and sodium chloride were obtained from Mallinckrodt (Paris, KY) and used as received. $Ru(bpy)_3Cl_2$ was obtained from Aldrich (Milwaukee, WI) and recrystallized once in acetonitrile prior to use. All other reagents were obtained from Aldrich and used as received.

Electrochemistry. ITO electrodes were obtained from Delta Technologies, Ltd (Stillwater, MN). All ITO electrodes were cleaned by sonication in Alconox, 2-propanol, and deionized water (twice), each step for 15 min, similar to the method introduced by Armstrong et al.[27] All electrochemical experiments were performed on either an EG&G PAR 273A or a BAS 100B/W potentiostat controlled by a Gateway PC. The reference electrode is a commercially available Ag/AgCl (3.0 M KCl) electrode obtained from Cypress Systems (Lawrence, KS). The counter electrode, a 22-gauge platinum wire (Alfa, Ward Hill, MA), is wrapped around the shaft of the reference electrode. Digital simulations were performed using the DigiSim software package (Bioanalytical Systems, West Lafayette, IN) as described elsewhere.[21−23] Further details of the fitting procedure are given in the Supporting Information.

Results

Guanine Multiplets. The relative reaction rates for guanine multiplets compared to that for guanine were investigated by cyclic voltammetry (CV) of oligonucleotides containing guanines in the sequences 5′-AGT (**G15**), 5′-AGGT (**GG16**), and 5′-AGGGT (**GGG17**). Previous studies have shown that the lowest reactivity is observed for a guanine with a T on the 3′-side,[2] so we have chosen AGT as the sequence for an "isolated guanine". The complete sequences of all of the oligonucleotides used in this study are given in Table 1. The voltammograms of

(15) Melvin, T.; Botchway, S. W.; Parker, A. W.; O'Neill, P. *J. Am. Chem. Soc.* **1996**, *118*, 10031−10036.
(16) Melvin, T.; Botchway, S.; Parker, A. W.; O'Neill, P. *Chem. Commun.* **1995**, 653−654.
(17) Lewis, F. D.; Wu, T.; Zhang, Y.; Letsinger, R. L.; Greenfield, S. R.; Wasielewski, M. R. *Science* **1997**, *277*, 673−676.
(18) Wan, C.; Fiebig, T.; Kelley, S. O.; Treadway, C. R.; Barton, J. K.; Zewail, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6014−6019.
(19) Manoharan, M.; Tivel, K. L.; Zhao, M.; Nafisi, K.; Netzel, T. L. *J. Phys. Chem.* **1995**, *99*, 17461−17472.
(20) Shafirovich, V.; Dourandin, A.; Huang, W.; Luneva, N. P.; Geacintov, N. E. *J. Phys. Chem. B* **1999**, *103*, 10924−10933.
(21) Johnston, D. H.; Thorp, H. H. *J. Phys. Chem.* **1996**, *100*, 13837−13843.
(22) Johnston, D. H.; Glasgow, K. C.; Thorp, H. H. *J. Am. Chem. Soc.* **1995**, *117*, 8933−8938.
(23) Sistare, M. F.; Holmberg, R. C.; Thorp, H. H. *J. Phys. Chem. B* **1999**, *103*, 10718−10728.
(24) Ropp, P. A.; Thorp, H. H. *Chem. Biol.* **1999**, *6*, 599−605.
(25) Steenken, S.; Jovanovic, S. V. *J. Am. Chem. Soc.* **1997**, *119*, 617−618.
(26) Fasman, G. D. *CRC Handbook of Biochemistry and Molecular Biology*; CRC Press: Boca Raton, FL, 1975; Vol. 1.
(27) Armstrong, N. R.; Lin, A. W. C.; Fujihira, M.; Kuwana, T. *Anal. Chem.* **1976**, *48*, 741−750.

Table 1. Oligonucleotide Sequences

name	sequence (5′ → 3′)[a]
G15	AAATAT-A**G**T-ATAAAA
GG16	AAATAT-A**GG**T-ATAAAA
GGG17	AAATAT-A**GGG**T-ATAAAA
GxG18	AAA-TAT-A**G**T−A**G**T-ATA-AAA
GxGxG21	AAA-TAT-A**G**T−A**G**T−A**G**T-ATA-AAA
6G24	AAA-A**G**T−A**G**T−A**G**T−A**G**T−A**G**T−A**G**T-AAA
3GG24	AAATAT-A**GG**T−A**GG**T−A**GG**T-ATAAAA
2GGG24	AAATATT-A**GGG**T−A**GGG**T-AATAAAA
3G24	AAATAT-A**G**T−A**G**T−A**G**T-TATATAAAA
3GI24	AAATAT-A**GI**T−A**GI**T−A**GI**T-ATAAAA
3IG24	AAATAT-A**IG**T−A**IG**T−A**IG**T-ATAAAA
GI16	AAATAT-A**GI**T-ATAAAA
IG16	AAATAT-A**IG**T-ATAAAA
3GZ24	AAATAT-A**GZ**T−A**GZ**T−A**GZ**T-ATAAAA
3ZG24	AAATAT-AZ**G**T−AZ**G**T−AZ**G**T-ATAAAA

[a] All experiments were performed with double-stranded oligomers with the indicated sequences hybridized to their normal complements. For the inosine (I) and 7-deazaguanine (Z) sequences, the modified base was paired with cytosine in the complementary strand. Reactive bases are shown in bold.

$Ru(bpy)_3^{2+}$ in the presence of the sequences **G15**, **GG16**, and **GGG17** are shown in Figure 1A; the concentration of the DNA strand is the same in each experiment. We have shown previously that the rates of reaction for the guanines in a given sequence are related to the extent of catalytic enhancement observed in CVs such as those shown in Figure 1A.[21−23] The high currents and sigmoidal shapes of the CVs of the **GG16** and **GGG17** oligonucleotides are indicative of very rapid electron transfer from guanine to Ru(III).

Since the concentration of DNA strands is the same in each CV in Figure 1A, the **GG16** and **GGG17** sequences contain twice and three times the concentration of guanine as **G15**. Increasing the absolute concentration of guanine also increases the catalytic current, so we wanted to show that the enhancements seen for the guanine multiplets were not due simply to the increase in guanine concentration obtained by adding the guanines to the sequence. We therefore acquired the CVs of $Ru(bpy)_3^{2+}$ in the presence of oligonucleotides containing two and three isolated guanines, **GxG18** and **GxGxG21**, which contain 5′-AGTAGT and 5′-AGTAGTAGT sequences, respectively (Figure 1B). The increase in catalytic current is much greater for the guanine multiplet sequences in Figure 1A than for the addition of the same number of isolated guanines in Figure 1B, implying a much greater intrinsic oxidation rate for GG and GGG multiplets. All of the CVs in Figure 1 were taken at the same concentration of DNA duplex and also at high salt concentration, where there is no appreciable DNA binding of $Ru(bpy)_3^{2+}$.[21−23] Thus, the reactivity observed in Figure 1A is not a result of changes in the binding affinity of the metal complex for the different sequences.

In studies using sequencing gels, the increase in selectivity for the 5′-G is generally much greater in duplex DNA than in single-stranded DNA.[28] We therefore expect that the relative increase in current enhancement on going from G to GG to GGG should be much greater for duplex DNA than for single-stranded DNA. Results for the oligonucleotides from Figure 1A in the single-stranded form are shown in Figure 1C. As we have discussed elsewhere,[22] the absolute current enhancements are larger for single-stranded DNA than for duplex because the solvent accessibility is higher in the single-stranded form. Nevertheless, the relative enhancement upon guanine stacking in Figure 1C is much smaller than that in Figure 1A, supporting

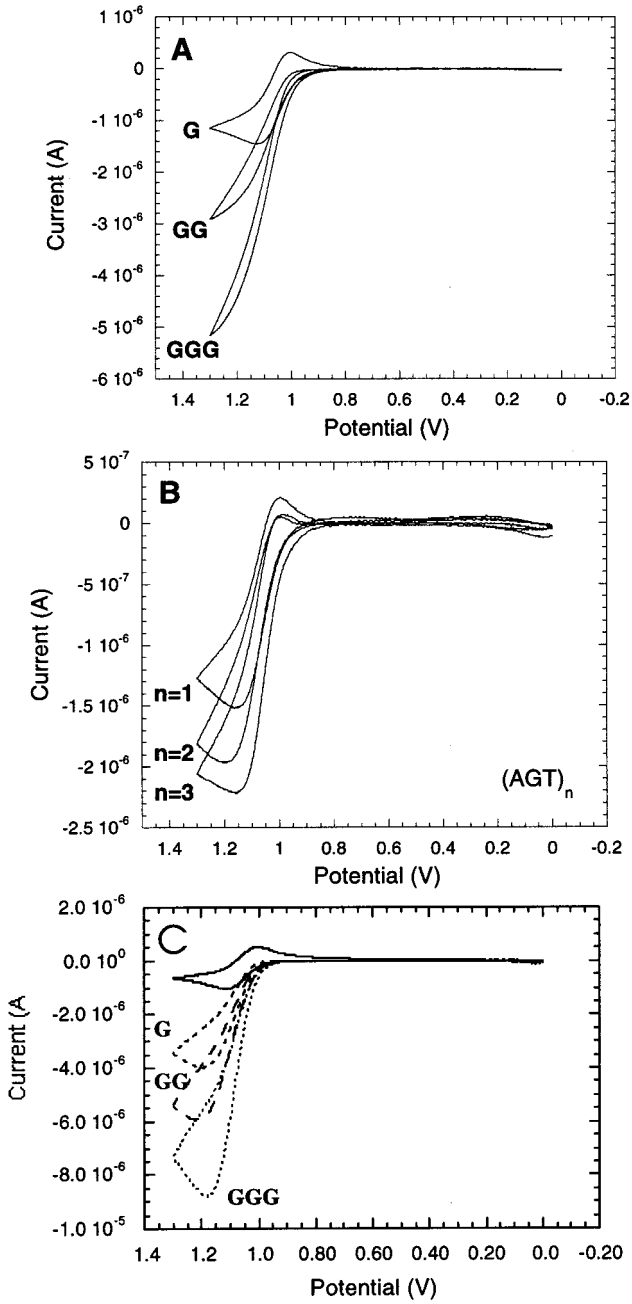


Figure 1. Cyclic voltammograms of $Ru(bpy)_3^{2+}$ (25 μM) in the presence of 75 μM DNA in 50 mM sodium phosphate buffer with 700 mM NaCl added. Scan rate = 25 mV/s. (A) Added sequences are the duplex forms of **G15** (G), **GG16** (GG), and **GGG17** (GGG). (B) Added sequences are the duplex forms of **G15** ($n = 1$), **GxG18** ($n = 2$), and **GxGxG21** ($n = 3$). (C) Added sequences are the single-stranded forms of **G15** (G, short dashed), **GG16** (GG, long dashed), and **GGG17** (GGG, dotted). The CV of $Ru(bpy)_3^{2+}$ alone is shown as the solid line.

the idea that the increase in electron density is greatest for duplex DNA.[28]

As we have discussed elsewhere,[21−24] the electron-transfer rate constants for the reaction of Ru(III) with guanine can be determined by digital simulation of the cyclic voltammograms. Because of the difference in the absolute concentrations of guanines in Figure 1, these oligonucleotides were not useful for digital simulation, because different DNA concentrations were required. We therefore designed oligonucleotides with either six isolated guanines (**6G24**), three GG doublets (**3GG24**), or two GGG triplets (**2GGG24**). These oligonucleotides could then be used for fitting at the same concentrations of DNA strand

(28) Kan, Y.; Schuster, G. B. *J. Am. Chem. Soc.* **1999**, *121*, 10857−10864.

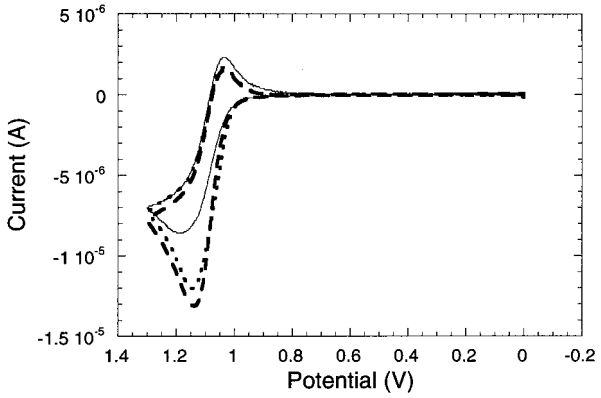


Figure 2. Cyclic voltammograms of $Ru(bpy)_3^{2+}$ (50 μM) in the presence of 4.17 μM (strand concentration) **6G24** (solid), **3GG24** (dashed), and **2GGG24** (dotted). Solutions were 50 mM sodium phosphate buffer with no added NaCl, and voltammograms were acquired at a scan rate of 250 mV/s.

and absolute guanine concentration. The CVs of $Ru(bpy)_3^{2+}$ in the presence of these oligonucleotides are shown in Figure 2. Even after correction in this manner for the absolute guanine concentration, the guanine multiplets clearly give enhanced reactivity compared to the isolated guanines. Since the results at high salt concentration given in Figure 1 demonstrate that the binding affinity is not responsible for the change in catalytic current, the CVs used for fitting were collected with no added salt (50 mM sodium phosphate only). The low salt conditions provide higher signals, which allowed the use of lower quantities of oligonucleotide and the collection of extensive data sets with varied DNA concentration and scan rate. The similarity in current for **3GG24** and **2GGG24** probably arises from the fact that in the GGG triplet, the middle G is generally more reactive on sequencing gels than the 5′-G.[7] Thus, the degree of enhancement may not be the same for each stacked G.

The fitting procedure has been described elsewhere,[21] and we summarize the main points here briefly; further details are given in the Supporting Information. The CVs under low salt conditions as shown in Figure 2 were fit using DigiSim to a model containing a series of square schemes that account for the binding of the oxidized and reduced forms of $Ru(bpy)_3^{2+}$ to the DNA. The parameters for the binding equilbria are taken from studies of $Os(bpy)_3^{2+}$, which does not oxidize guanine.[29] The electron-transfer rate constant is modeled as a first-order process for the bound $Ru(bpy)_3^{3+}$ form and is a linear function of the DNA concentration, as expected. The first-order rate constant can be converted to a second-order rate constant that is in terms of guanine concentration using the steady-state approximation. The fitting is improved by inclusion of a second electron-transfer reaction between Ru(III) and guanine that has been oxidized by one electron; however, the rates of this reaction are generally less than 1% of the rate of the first electron transfer. Thus, the second electron transfer does not complicate the steady-state analysis that produces the second-order rate constant, as we have shown elsewhere.[23] Finally, when oligonucleotides are used that contain a small number of guanines, binding of the mediator to sites that are not oxidized must be incorporated in the model.[23] For **6G24**, the voltammograms were fit well by a model where collision of $Ru(bpy)_3^{3+}$ with 36−48 of the nucleotides (out of 48 total in the duplex) led to productive electron transfer. For **3GG24**, simulations allowing electron transfer following collision with 30−36 of the 48 nucleotides accounted well for the data. The lower number of active nucleotides in **3GG24** is consistent with the longer AT stretches on the ends of the sequence compared to **6G24**.[23]

The CVs for **6G24** were simulated over a range of scan rates (25−250 mV/s) and DNA concentration (80−800 μM in nucleotide phosphate). For 200 μM DNA, there was a modest dependence on scan rate, and the rate constants determined ranged from $(0.82 \pm 0.05) \times 10^5$ M^{-1} s^{-1} at 25 mV/s to $(3.3 \pm 0.3) \times 10^5$ M^{-1} s^{-1} at 250 mV/s. The scan rate dependence results from biphasic kinetics that we have observed by chronoamperometry and is typical of reactions between bound metal complexes and DNA.[23] At a scan rate of 100 mV/s, there was no systematic dependence on the DNA concentration, and the rate constant was $(1.4 \pm 0.2) \times 10^5$ M^{-1} s^{-1} from 80 to 800 μM DNA concentration (nucleotide phosphate). For **3GG24**, the rate constant ranged from 6.6×10^5 to 24×10^5 M^{-1} s^{-1} over the same range of scan rates, and the similar range for **2GGG24** was 11×10^5 to 26×10^5 M^{-1} s^{-1}. Again, there was no DNA concentration dependence of the rate constants for either **3GG24** or **2GGG24**.

The apparent rate constant obtained from the simulations of the mediated voltammetry is an indication of the total rate of reduction of $Ru(bpy)_3^{3+}$ by the DNA. For the **6G24** sequence, all of the guanine bases are in an identical primary sequence environment (an AGT sequence). The rate of this electron-transfer reaction is thus identical for all of the guanine bases in the strand, and the rate constant obtained from the simulation applies to the oxidation of the guanine base in all of the AGT sequences. In the **3GG24** sequence, however, there are two types of guanine present, one in a 5′-A*GG* sequence, and the other in a 5′-G*G*T sequence. The overall oxidation rate observed in the voltammetry is thus the sum of the oxidation rates for each kind of guanine in the strand. Because the multiple guanine types are not differentiated in the mechanism for the simulations, the rate constant obtained from the simulation is the concentration-weighted mean value of the rate constants. Thus, the observed rate constant for the **3GG24** strand is:

$$k_{\mathrm{obs},3GG24} = \frac{3k_{GG} + 3k_G}{6} \qquad (3)$$

where k_{GG} is the rate constant for the 5′-G of the GG doublets and k_G is the rate constant for the 3′-G. Now we make the assumption that the rate constant for the 3′-G in the guanine doublet is identical with the rate constant for the isolated guanine in **6G24**. This assumption is based on the observation of similar reactivities of the 3′-G in GG doublets on sequencing gels[1] and is further substantiated by studies on inosine-containing oligonucleotides described below. Using eq 3, we can then calculate the rate constant for the 5′-G if we know the observed rate constant for the entire oligonucleotide ($k_{\mathrm{obs},3GG24}$) and k_G. The rate constant for the 5′-G is approximately an order of magnitude higher than that for the 3′-G; these values are shown with error limits in Table 2.

The absolute rate constants from CV exhibit a modest scan rate dependence arising from a biphasic time course for the electron transfer, which we have discussed previously for isolated guanine sequences.[23] While the absolute rate constants for the guanine multiplets also show a systematic increase of a factor 2−5 with scan rate, the *ratio* of rate constants for the 5′-G and 3′-G of the guanine doublets in **3GG24** was not dependent on the scan rate in a systematic fashion. This ratio, k_{GG}/k_G, gave a value of 12 ± 2 across the complete range of scan rates (25−250 mV/s) and DNA concentrations (80−800 μM). The small, random variations in k_{GG}/k_G with scan rate and

(29) Welch, T. W.; Thorp, H. H. *J. Phys. Chem.* **1996**, *100*, 13829−13836.

Table 2. Rate Constants for Stacked Guanines

sequence	$k_{obs} \times 10^{-5}$ (M^{-1} s^{-1})[a]	$k_G \times 10^{-5}$ (M^{-1} s^{-1})	$k_{GX} \times 10^{-5}$ (M^{-1} s^{-1})	k_{GG}/k_G[b]
6G24	1.4 ± 0.2	1.4 ± 0.2		
3GG24	7.5 ± 0.2		14 ± 1[c]	12 ± 2
3G24	2.1 ± 0.2	2.1 ± 0.2		
3IG24	1.8 ± 0.1	1.8 ± 0.1		
3GI24	5.9 ± 0.1		5.9 ± 0.1[d]	2.8 ± 0.4

[a] Observed rate constants given for 100 mV/s scan rate and DNA concentrations from 80 to 800 μM. Error limits are standard deviations from multiple trials at the different concentrations. Absolute rate constants were determined by digital simulation of cyclic voltammograms as described in ref 21 and the Supporting Information. [b] At all scan rates (25−250 mV/s) and DNA concentrations (80−800 μM). [c] Based on the assumption that the rate constant for the 3′-G (i.e., k_G is the same as that for the isolated guanine, see text). X = G. [d] X = I.

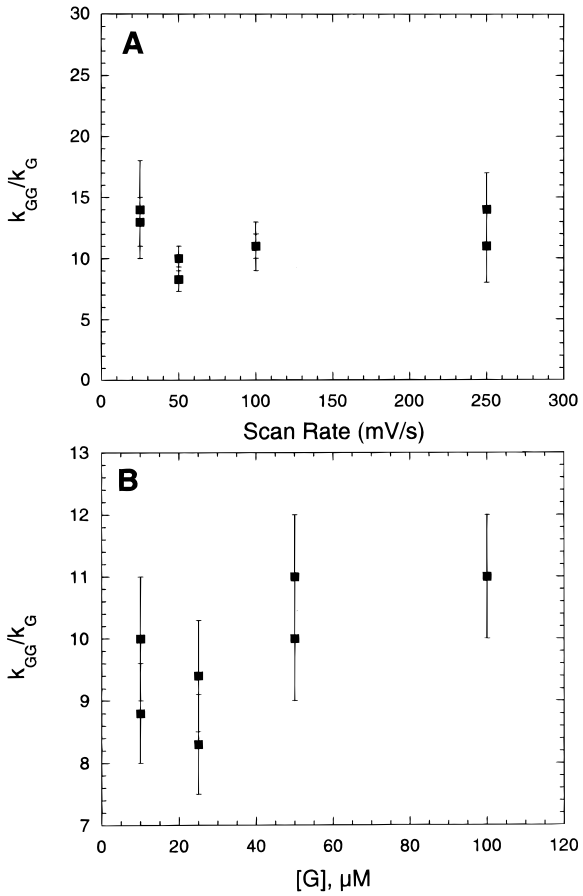


Figure 3. Ratios of the rate constant for the 5′-G of the GG doublet to the 3′-G (k_{GG}/k_G) (A) at 4.17 μM DNA strand concentration and (B) at 100 mV/s scan rate. Rate constants were determined on voltammograms collected under conditions shown in Figure 2 and fit by digital simulation as described in ref 16.

DNA concentration are shown explicitly in Figure 3. Thus, the two components that give the biphasic time course are both equally affected by the increased reactivity of the 5′-G in the doublet. Note that the absolute rate constants given in Table 2 are for 100 mV/s while the ratio k_{GG}/k_G is for all scan rates.

In the course of our studies of the kinetics of guanine electron transfer to Ru(III), we have been able to confirm much of our analysis of CV using stopped-flow spectrophotometry on the direct reaction of Ru(III) with DNA.[22] However, this technique requires considerably more material than the electrochemistry measurements, and detailed analysis of stopped-flow data has been limited to calf thymus DNA and mononucleotides. Sufficient quantities of the guanine multiplet oligonucleotides were not available to permit a detailed kinetic analysis; however, to confirm the qualitative trend indicated by the electrochemistry, stopped-flow data were collected on the reaction of authentic Ru(III) with the multiplet oligonucleotides. At high salt concentration, the half-times for reduction of Ru(III) were 1.5 s for **6G24**, 80 ms for **3GG24**, and 50 ms for **2GGG24**, supporting the CV results shown at high salt concentration in Figure 1. At low salt concentration, the half-time was 40 ms for **6G24**, and the reaction was complete in the mixing time for **3GG24** and **2GGG24** under the same conditions, in agreement with the CV results shown in Figure 2. As seen in chronoamperometry, the decay curves were biphasic, which we have assigned as the origin for the modest scan rate dependence of the simulated rate constants from CV.[23] Thus, the scan-rate dependence and the enhancement in rate for guanine multiplets are not artifacts of the heterogeneous process in the electrochemical experiment.

Because the primary evidence for selectivity for the 5′-G to this point has been from sequencing gels, we sought to show that the same selectivity was similarly observed in oligonucleotides related to those used here with the same Ru(III) oxidant. In particular, since the oligonucleotides for electrochemical analysis must contain few guanines, we wanted to confirm that the selectivity for the 5′-G of the GG doublet was present in a oligonucleotide with mostly AT base pairs. Accordingly, oxidation of 5′-d[AAAATTT**GG**TATAT**G**TATAAA] was performed with $Ru(bpy)_3^{3+}$ generated via PbO_2 oxidation of $Ru(bpy)_3^{2+}$.[30] The chosen oligonucleotide contains a 5′-GGT and an isolated 5′-GT for comparison. Analysis of the oxidation products showed that the intensity of cleavage at the 3′-G of the GG doublet was within experimental error of that of the isolated guanine; the ratio of cleavage intensities for the 3′-G to the isolated guanine was 1.02 ± 0.04 over eight trials from 4 to 18 μM $Ru(bpy)_3^{3+}$. In contrast, the ratio of intensities for the 5′-G of the doublet and the isolated guanine was 1.8 ± 0.2 over the same concentration range. Further conversion of potential 8-oxo-guanine products to strand cleavages via reaction with Ir(IV)[31] increased the overall intensity of cleavage but did not change the selectivity for the 5′-G outside the quoted error limits. Kan and Schuster have observed a similar ratio of 3−5 for photolytic oxidation of a 5′-AGGT sequence, depending on the conditions.[28] The driving force in the photolytic oxidation is likely much greater than that in our $Ru(bpy)_3^{3+}$ reaction, which is close to $\Delta G^\circ = 0$.[22] Hickerson et al. have shown that the ratio of cleavage for doublets containing a 5′-8-oxo-guanine stacked on a 3′-guanine is dependent on the redox potential of the one-electron oxidant.[9]

Modified Bases. To our knowledge, our kinetic analysis above provides the first quantitative measure of the relative rate constants for electron transfer from a GG doublet compared to isolated guanine. However, the calculation of k_{GG}/k_G requires the assumption that the 3′-G gives the same rate constant as the isolated guanine. The mediated electrochemistry does not permit direct resolution of the rates for the two guanines in the doublet. Evidence that the 5′-G of the GG doublet is enhanced preferentially to the 3′-G comes from gel electrophoresis data and theoretical calculations,[1,2,6,9] but the direction of enhancement has not been directly measured in a real-time experiment. We have shown previously that inosine, which is identical to guanosine but does not contain the exocyclic amine (Scheme 1), does not donate electrons to Ru(III).[32] The redox inertness

(30) DeSimone, R. E.; Drago, R. S. *J. Am. Chem. Soc.* **1970**, *92*, 2343−2352.

(31) Muller, J. G.; Duarte, V.; Hickerson, R. P.; Burrows, C. J. *Nucleic Acids Res.* **1998**, *26*, 2247−2249.

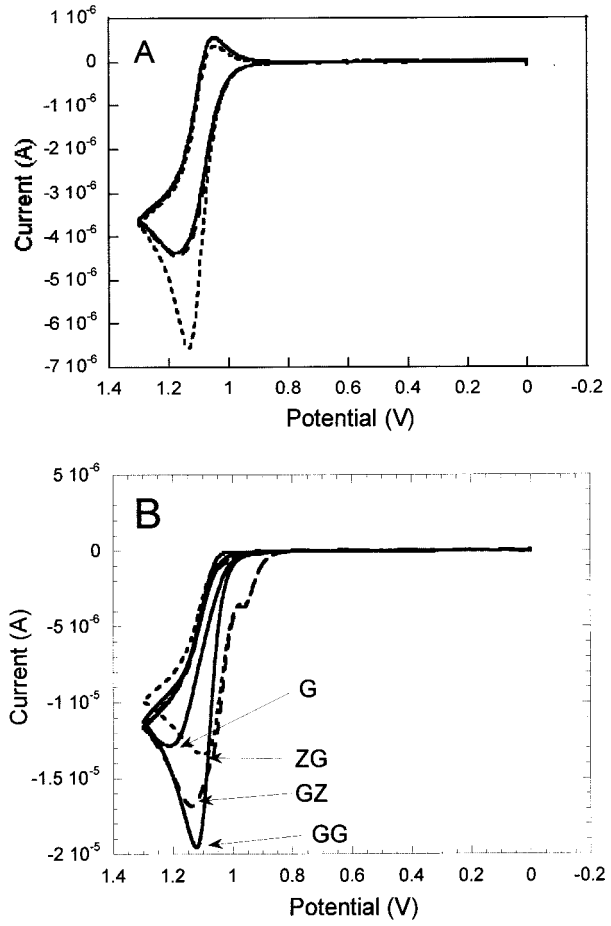


Figure 4. (A) Cyclic voltammograms of $Ru(bpy)_3^{2+}$ (50 μM) in the presence of 8.33 μM (strand concentration) **3G24** (solid), **3IG24** (dashed), and **3GI24** (dotted). Solutions were 50 mM sodium phosphate buffer with no added NaCl, and voltammograms were acquired at a scan rate of 50 mV/s. (B) Cyclic voltammograms of $Ru(bpy)_3^{2+}$ (50 μM) in the presence of 8.33 μM (strand concentration) **6G24**, **3ZG24**, **3GZ24**, and **3GG24**. Solutions were 50 mM sodium phosphate buffer with no added NaCl, and voltammograms were acquired at a scan rate of 100 mV/s.

of inosine has also been similarly exploited by Barton et al.[33] Theoretical calculations suggest that N7, which is present in inosine, is responsible for the stacking effect,[12] so we suspected that stacking of a 5′-G on inosine would provide an enhancement similar to that of the GG doublet but with only one redox-active base. We have therefore designed the sequences **3G24**, **3IG24**, and **3GI24**, which provide three guanines either isolated, in a 5′-GI doublet, or in a 5′-IG doublet.

The cyclic voltammograms of **3G24**, **3IG24**, and **3GI24** with $Ru(bpy)_3^{2+}$ are shown in Figure 4A with the same DNA strand concentration (and hence the same guanine concentration since all three oligonucleotides contain three guanines). The current enhancement is identical for **3G24** and **3IG24**, suggesting that the isolated guanine (5′-AGT) exhibits the same electron-transfer reactivity as the 5′-IG sequence. In contrast, the current enhancement for **3GI24** is much larger than that for the other two oligonucleotides, demonstrating a significantly greater reactivity for 5′-GI than for 5′-IG or isolated guanine. This result supports our assumption that the rate constant for the 3′-G is the same as for isolated guanine in the calculation of k_{GG}/k_G.

The rate constants for oxidation of the oligonucleotides from Figure 4A were determined by CV. The rate constants for **3G24** ranged from $(0.70 \pm 0.05) \times 10^5$ M^{-1} s^{-1} at 25 mV/s to $(3.7 \pm 0.3) \times 10^5$ M^{-1} s^{-1} at 250 mV/s, which was in good agreement with those for the other isolated guanine oligonucleotides.[21−23] The range for **3IG24** was nearly identical ($0.82-4.8 \times 10^5$ M^{-1} s^{-1}), as expected, while the range for **3GI24** was significantly enhanced at $2.2-12 \times 10^5$ M^{-1} s^{-1}. With these data, the ratio of the 5′-G and the 3′-G rate constants can be calculated directly to give a value of $k_{GI}/k_{IG} = 2.8 \pm 0.4$, which is also independent of both scan rate and DNA concentration. As expected, the ratio of rate constants for 5′-IG and isolated guanine was $k_{IG}/k_G = 1.0 \pm 0.2$ over the same range of scan rates and DNA concentrations. Thus, inosine is less effective at increasing the electron-transfer reactivity of a 5′-G, which is expected because the exocyclic amine has a large inductive effect. Nonetheless, the quantitative analysis further supports the assumption that the 3′-G of a doublet is identical in reactivity to an isolated guanine.

We have also studied the stacking effect of 7-deazaguanine (Z) in doublets with guanine. Numerous studies now show that 7-deazaguanine is an excellent electron donor and exhibits a redox potential lower than that of native guanine.[18,33,34] However, 7-deazaguanine is lacking N7, which is hypothesized to provide the doublet stacking effect.[12] Thus, we suspected that 7-deazaguanine would provide electrons to Ru(III) in a manner similar to guanine but might not give the same stacking effect in a 5′-GZ doublet. Shown in Figure 4B are the CVs of $Ru(bpy)_3^{2+}$ with **6G24**, **3GG24**, **3ZG24**, and **3GZ24**. As shown in the figure, the amount of catalytic current is similar for **6G24** and **6ZG24**, which is expected since both oligonucleotides contain six oxidizable bases. As with the inosine-containing sequences, the **3GZ24** oligonucleotide exhibits a larger catalytic current than **3ZG24**, but not as large as **3GG24**. The stacking of guanine on the 5′-side of a 7-deazaguanine enhances the electron-transfer reactivity somewhat, but not to the same extent as native guanine. This effect is attenuated compared to that of native guanine *even though 7-deazaguanine clearly has a lower redox potential*.[18,33] Thus, the stacking effect is not simply a measure of the overall electron density of the 3′-base, and the importance of N7 is implicated. Taken together with results from Schuster et al. showing that the GG stacking effect is not observed in DNA/RNA hybrids,[13] the results on 7-deazaguanine strongly support the idea that favorable orientation of the N7 of the 3′ base is a vital aspect of the stacking effect.[12]

Discussion

Quantitation of the Stacking Effect. The data given in Table 2 provide the first quantitation of the relative reactivities of the 5′-G and 3′-G in the GG doublet in an experiment that measures the rates of the primary electron transfer event. Because the electrochemical experiment provides information only on the sum of the rate constants for any oligonucleotide containing more than one type of guanine, we require the assumption that the 3′-G of the doublet exhibits the same rate constant as the isolated guanine, which we have defined here as lying in a 5′-AGT sequence. This assumption is supported by the fact that an oligonucleotide containing two 5′-AGT sequences gives much less catalytic enhancement than a GG doublet (Figure 1) and that the 5′-IGT sequence has an identical rate constant to

(32) Napier, M. E.; Loomis, C. R.; Sistare, M. F.; Kim, J.; Eckhardt, A. E.; Thorp, H. H. *Bioconj. Chem.* **1997**, *8*, 906−913.
(33) Kelley, S. O.; Barton, J. K. *Science* **1999**, *283*, 375−381.
(34) Original reports from our group (ref 32) that 7-deazaguanine was a poor electron donor were incorrect due to an artifact associated with the electrochemistry of the triphosphate. The results in Figure 4B and those in ref 18 demonstrate that 7-deazaguanine is a facile electron donor.

that of 5′-AGT while 5′-GIT is enhanced significantly (Table 2). Given this assumption, we find that the 5′-G of the GG doublet is 12 ± 2 times more reactive than the 3′-G. This ratio and associated error limit are taken from a large number of experiments over an order of magnitude in scan rate and concentration.

The relative rate constants for the 5′-G and 3′-G can be used to estimate the redox potential of the GG doublet. In an earlier paper, we suggested that the reaction of $Ru(bpy)_3^{3+}$ with guanine occurred with a potential very near that giving $\Delta G^\circ = 0$.[22] Later, the potential of guanosine oxidation was measured by absorption titration and showed that this estimate was correct to within 20 mV.[25] We have also estimated the reorganizational energy to be about 1 eV.[22] Lower reorganizational energies have been estimated for reaction of guanine with oxidants that are intercalated in DNA;[18] however, since the $Ru(bpy)_3^{3+}$ is weakly bound and highly exposed to water, we suspect a larger reorganizational energy for our reaction.[35] With a rate constant 12 times faster than that of the 3′-G, we can calculate that the potential of the 5′-G is 0.12 eV lower than that of the 3′-G (or isolated guanine). The redox potential for the 5′-G is therefore about 0.95 V based on a literature value for the guanosine potential of 1.07 V at pH 7.[25] This observation is consistent with our earlier observation that guanine doublets and triplets do not react with $Os(bpy)_3^{3+}$,[24] which has a redox potential of 0.62 V. Barton and co-workers have also shown that guanine doublets do not quench ethidium emission while 7-deazaguanine is an efficient donor for excited ethidium,[18] suggesting that the GG potential is significantly higher than that of 7-deazaguanine.

Comparison of the ratio of 12 determined from cyclic voltammetry with the results from sequencing gels is warranted. As we report here, cleavage of a GG doublet with $Ru(bpy)_3^{3+}$, the same oxidant used in our electrochemistry experiments, gives a ratio of 1.8 ± 0.2 for 5′-GGT, considerably lower than that observed by electrochemistry. A recent study has provided similar ratios of the extents of cleavage for 8-oxo-guanine stacked on another guanine on the 3′-side.[9] Treatment with one-electron oxidants gave ratios of 3−4 times more piperidine-labile scission at the 5′-G of a G doublet compared to isolated guanine or the 3′-G of the doublet. Schuster et al. have observed a ratio of 3−5 in a similar sequence for normal GG doublets,[28] and many other studies show ratios that are similar.[5−9,36] We suspect the difference is due to the requirement for trapping of the oxidized guanine and formation of the piperidine-labile lesion. If these steps are less sequence-selective, the ratio of rate constants for the initial one-electron transfer measured here provides an upper limit of what might be observed on a sequencing gel. Consistent with this hypothesis, Schuster et al. have observed a higher ratio upon treatment with formamido-pyrimidine glycosylase (Fpg),[28] which cleaves 8-oxo-guanine residues potentially formed by electron transfer.[31,37,38] In fact, the scenario most consistent with our results is an *inverse* sequence selectivity for trapping of the guanine radical that counteracts the selectivity for the 5′-G apparent in the initial electron-transfer rates shown here. Greater stabilization of 5′-$G^{+\bullet}$G compared to 5′-$GG^{+\bullet}$ might in fact lead to more efficient trapping of the 3′-G.[39] Further experimentation with additional sequences, oxidants, and protocols for realizing strand scission will be required to clarify this point.

Another important comparison is that of our estimate of the redox potential with theoretical calculations, which give the qualitative trend but much larger values (~0.6 eV) for the difference in energies for GG and isolated guanine.[2,12] The calculations performed do not explicitly include charges and solvent and would not be expected to give absolute energy differences that are in agreement with those estimated by Marcus theory from the experimental rate constants in duplex DNA in neutral solution. Hickerson et al. have also commented that the calculations likely overestimate the effect of guanine stacking on the redox potential, since the calculations predict that 8-oxo-guanine would have a higher redox potential than GG.[9] Experimentally, 8-oxo-guanine is more readily oxidized than GG as observed both by Hickerson et al.[9] using gel electrophoresis and by our group using cyclic voltammetry.[24]

Origin of the Stacking Effect. Prat et al. have suggested that the origin of the GG stacking effect lies in the favorable orientation of N7 of the 3′-G compared to the site of oxidation in the 5′-G.[12] In particular, the N7 is aligned in B-DNA beneath the carbonyl group of the 5′-G. The results shown here support three aspects of this hypothesis. First, the fact that 5′-GI is also enhanced while 5′-IG is not (Figure 4A) shows that it is in fact the 5′-G that is reactive in the GG doublet. Previous evidence for specific enhancement at the 5′-G was from sequencing gels, which are analyzed following additional reactions that produce the strand scission needed for visualization. The results in Figure 4 strongly support the idea that the primary electron transfer is the step that is selective for the 5′-G. The fact that inosine provides a stacking effect but not one as large as that for guanine is also consistent with the present hypothesis, since inosine does possess an N7 but one that is less electronegative than that in native guanine. Next, we find that 7-deazaguanine, which is a better donor than guanine when isolated, does not provide a stacking effect as a 3′-base, presumably because the N7 is not present. This effect is apparent in Figure 4B where the 5′-GZ oligonucleotide behaves essentially the same as an oligonucleotide that contains one isolated guanine and one isolated 7-deazaguanine.

Electron Transfer versus Transport. Ultrafast kinetic experiments on guanine redox chemistry have defined two regimes for the oxidation reaction.[5,7,17,18,20,40−42] The first regime corresponds to electron *transfer* where the redox chemistry of intervening bases does not contribute to the overall process, and states involving the oxidized forms of other nucleobases need not be considered. The second regime involves electron *transport* through oxidized bridge states. We consider our reactions of electrostatically bound $Ru(bpy)_3^{2+}$ with guanine to occur in the electron-transfer regime, which is well supported by the driving force dependence of the reaction.[22] Further, we have observed that even with the condensed metal complex, the reaction occurs over no more than 2.5 base pairs.[23] The adherence of the reaction to the normal electron transfer model probably results from the relatively weak electronic coupling of the $Ru(bpy)_3^{3+}$ and the oxidized guanine. This difference must be considered when using these results to analyze reactions that occur with strong electronic coupling on much faster time scales in the electron transport regime.[5,18] Nonetheless, the

(35) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265−322.
(36) Arkin, M. R.; Stemp, E. D. A.; Pulver, S. C.; Barton, J. K. *Chem. Biol.* **1997**, *4*, 389−400.
(37) Cullis, P. M.; Malone, M. E.; Merson-Davies, L. A. *J. Am. Chem. Soc.* **1996**, *118*, 2775−2781.
(38) Spassky, A.; Angelov, D. *Biochemistry* **1997**, *36*, 6571−6576.

(39) We are grateful to a reviewer for this suggestion. We represent the guanine radical as $G^{+\bullet}$, although it may likely be deprotonated to form the neutral radical prior to trapping.[20]

(40) Ratner, M. *Nature* **1999**, *397*, 480−481. Priyadarshy, S.; Risser, S. M.; Beratan, D. N. *J. Phys. Chem.* **1996**, *100*, 17678−17682.
(41) Jortner, J.; Bixon, M.; Langenbacher, T.; Michel-Beyerle, M. E. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12759−12765.
(42) Fukui, K.; Tanaka, K. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 158−161.

results presented here are consistent with all of the studies of which we are aware on both intramolecular and intermolecular oxidation of GG doublets, where the 5′-G is generally more reactive.

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Supporting Information Available: Results and experimental details are given for native gel electrophoresis of the analyzed oligonucleotides, CD spectroscopy of the guanine multiplet oligonucleotides, analysis of cleavage by $Ru(bpy)_3^{3+}$ by high-resolution electrophoresis, and raw rate data from voltammetry of **6G24** and **3GG24** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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