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Electron and hydrogen transfer reactions of nucleotides: from Stern–Volmer quenching to nucleoprotein structure

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Contents

Abstract

The complex $Pt_2(pop)_4^{4-}$ abstracts hydrogen atoms and electrons from DNA upon photolysis into the $d\sigma^* \rightarrow p\sigma$ excited state ($pop = P_2O_5H_2^{2-}$). In duplex DNA, the hydrogen atoms are abstracted from the 4′- and 5′- positions of the deoxyribose functionality, and electrons are abstracted by tunneling from the guanine nucleobase. At high Mg^{2+} concentrations, the $Pt_2(pop)_4^{4-}$ tetraanion can associate more intimately with the duplex, and both hydrogen atom and electron transfer are efficient; however, at low Mg^{2+} concentrations, the complex is situated far from the duplex so that the only efficient pathway is electron tunneling. Therefore, the guanine/sugar ratio decreases with increasing Mg^{2+}. The electron transfer pathway can also be examined in the absence of the hydrogen transfer pathway in thermal reactions where the electron is abstracted from guanine by $Ru(bpy)_3^{3+}$ (bpy = 2,2′-bipyridine). These reactions can be initiated electrochemically by potentiation of the complex to the 3+ form, which produces catalytic enhancements in cyclic voltammograms. These enhancements show that guanine multiplets are more reactive than dispersed guanines. Finally, binding of

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DNA to the *Hha*I methyltransferase causes flipping of a cytosine into the active site of the enzyme, leaving behind an unpaired guanine residue that is more reactive towards electron transfer than the paired guanine, an effect visible in high-resolution electrophoresis gels after photolysis in the presence of $Pt_2(pop)_4^{4-}$. © 1998 Elsevier Science S.A.

Keywords: DNA; Electron transfer; Hydrogen transfer; Ru; Pt

1. Introduction

The interaction of two square-planar d^8 centers along the *z*-axis brings about a strong interaction between d_z2 and p_z orbitals that creates a $(d\sigma^*)^2$ ground state with no metal–metal bond [1]. The lowest energy absorption in these d^8–d^8 dimers therefore arises from a highly allowed $(d\sigma^*)^2 \rightarrow (d\sigma^*)^1(p\sigma)^1$ transition that formally produces a metal–metal bond. In the particular case of $Pt_2(pop)_4^{4-}$ ($pop = P_2O_5H_2^{2-}$), the short metal–metal separation produces a relatively high energy state with strong emission at $\lambda_{max,em} = 513$ nm and a 10 μs lifetime. The excited state is quenched by hydrogen donors with Stern–Volmer quenching constants of $\sim 10^5\ M^{-1}\ s^{-1}$ and electron donors with rate constants that are diffusion-controlled at sufficiently high driving force [1].

2. Results and discussion

2.1. Photoreactions of DNA with diplatinum(II)

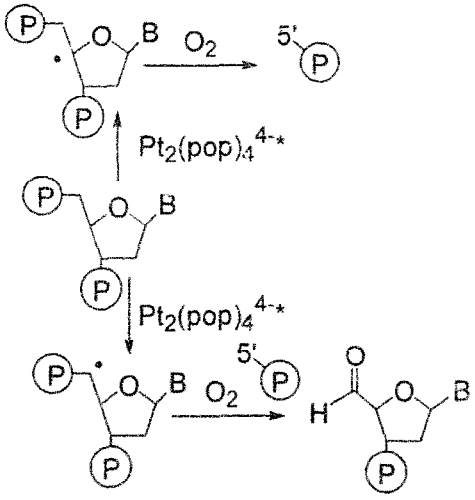
Generation of hydroxyl radical via the reaction of $Fe(EDTA)^{2-}$ with hydrogen peroxide is a powerful method for imaging structures of unusual DNAs and DNA–protein complexes [2]. We have developed a parallel approach to the $Fe(EDTA)^{2-}/H_2O_2$ system based on the tetraanionic complex $Pt_2(pop)_4^{4-}$ via the hydrogen transfer reactivity of nucleotide sugars [3,4]. We reasoned that DNA photocleavage by $Pt_2(pop)_4^{4-}$ would exhibit an unusual dependence on the charge properties of the nucleic acid, especially if direct abstraction of hydrogen from DNA by $Pt_2(pop)_4^{4-}*$ was responsible for strand scission [5]. Furthermore, the absence of binding of $Pt_2(pop)_4^{4-}*$ should provide a sequence-neutral ladder of bands in duplex DNA. Photolysis of $Pt_2(pop)_4^{4-}*$ in the presence of DNA leads to nicking as assayed by plasmid electrophoresis [5]. Conversion of form I to form II plasmid by photolysis of $Pt_2(pop)_4^{4-}$ in phosphate buffer is accelerated at high ionic strength, and sodium formate, a $\cdot OH$ scavenger, does not inhibit plasmid isomerization. These observations point to a mechanism where $Pt_2(pop)_4^{4-}*$ abstracts hydrogen atoms directly from DNA.

In double-stranded DNA, high-resolution electrophoresis reveals that $Pt_2(pop)_4^{4-}*$ induces frank scission to produce phosphate and phosphoglycolate termini in 5′-labeled oligomers, consistent with homolysis of the 4′ C-H bond by comparison with hydroxyl-mediated cleavage using $Fe(EDTA)^{2-}$ and H_2O_2 [6]. In

the 3′-labeled oligomer, phosphate termini are observed along with a modified terminus that is removed upon piperidine treatment and can be assigned to the aldehyde generated by homolysis of the 5′ C-H bond. Activation of the 1′ and 3′ C-H bonds has been ruled out in double-stranded DNA by related experiments. The quenching rate constants for mononucleotides fall in the $k = 10^4$–$10^5\ M^{-1}\ s^{-1}$ [7], also consistent with a hydrogen transfer mechanism. These findings are summarized in Scheme 1.

In addition to abstracting hydrogen atoms, the excited state of $Pt_2(pop)_4^{4-}$ is a competent one-electron oxidant [1]. Estimation of the excited-state oxidation potential from Stern–Volmer quenching suggests that guanine should be an effective quencher of $Pt_2(pop)_4^{4-}*$. Accordingly, piperidine workup following oxidation of end-labeled oligomers gives enhancement specifically at guanine. There is a modest, sequence-independent enhancement that results from a small number of piperidine-labile lesions generated by abstraction of the 4′-hydrogen; however, the guanine enhancement is easily detected against this small background [6]. The $Pt_2(pop)_4^{4-}$ assay, therefore, provides an opportunity to quantitate the accessibility of 4′ and 5′ sugar hydrogens and guanine bases in a single experiment.

The competition between hydrogen transfer and electron transfer reactions of $Pt_2(pop)_4^{4-}$ can be used to investigate the relative distance dependencies of the two pathways. At high Mg^{2+} concentration, the tetraanionic complex spends more time closer to the polyanionic DNA than at low Mg^{2+} concentration, and we have shown previously that the hydrogen transfer pathway is considerably more efficient at mM concentrations of Mg^{2+} [6]. If the electron transfer pathway exhibits a weaker distance dependence than the hydrogen transfer pathway, guanine oxidation should be less dependent on the Mg^{2+} concentration. Cleavage of double-stranded oligomers and quantitation of the extent of cleavage at each nucleotide shows that the ratio of guanine oxidation to T and C (sugar) oxidation is a maximum at zero added



Scheme 1. $Pt_2(pop)_4^{4-}*$ *induced sission.*

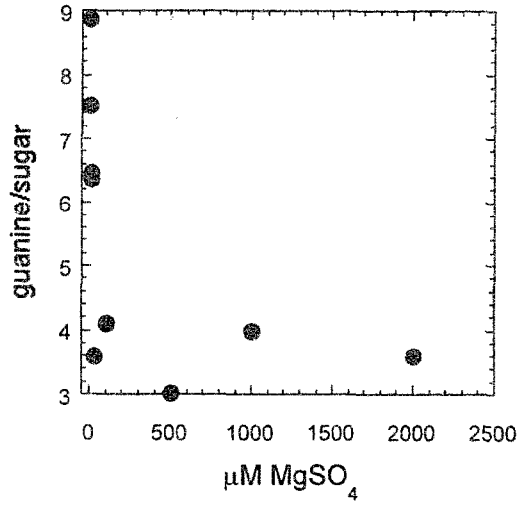


Fig. 1. Ratio of cleavage intensity at guanines divided by cleavage intensity at cytosine and thymine in a synthetic oligonucleotide in the duplex form following piperidine treatment.

Mg^{2+} and decreases with increasing Mg^{2+} concentration (Fig. 1), because the efficiency of sugar oxidation increases more steeply with Mg^{2+} concentration than the guanine oxidation. This experiment strongly supports the assignment of the sugar oxidation to excited state hydrogen atom transfer and the piperidine-labile guanine oxidation to guanine–metal electron transfer.

2.2. *Guanine electron transfer reactions*

We were the first to show that one-electron guanine oxidation can be initiated by *ground-state* oxidants at room temperature in native DNA, and that this reaction could be detected in cyclic voltammograms and on sequencing gels [8]. Using $Ru(bpy)_3^{3+}$ as a ground-state oxidant, this reaction can be initiated electrochemically and detected as a catalytic enhancement in cyclic voltammograms where at high salt ($[Na^+] \sim 1$ M), a simple two-step mechanism can be used to fit the voltammograms by digital simulation [9]:

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

$$Ru(bpy)_3^{3+} + G \rightarrow Ru(bpy)_3^{2+} + G^+. \quad (2)$$

The rate constant for double-stranded DNA determined by digital simulation of cyclic voltammograms at high salt is $k_2 = 1.0 \times 10^4\ M^{-1}\ s^{-1}$ [10], which has been confirmed by independent stopped-flow experiments using authentic $Ru(bpy)_3^{3+}$ and calf-thymus DNA [9]. Variation of the driving force using substituted derivatives of $Ru(bpy)_3^{2+}$ gives the dependence of rate on ΔG° predicted by the Marcus theory. At lower salt concentrations, the voltammetry must be fit to a more complicated mechanism than that shown in Eq. (1) and 2 that simulates using established binding

models the binding of both the 2+ and 3+ forms to DNA [10]. The kinetics and thermodynamics of the binding steps were worked out independently for $Os(bpy)_3^{2+}$ [11], which is structurally indistinguishable from the Ru analog but which does not oxidize guanine. The analysis yields a net 10-fold increase in the effective second-order rate constant due to concentration of the metal complex on the DNA ($k_2 = 1.4 \times 10^5\ M^{-1}\ s^{-1}$).

Since calf thymus DNA has a large sampling of different sequences, the rate constants given above reflect an average sequence environment for guanine in double-stranded DNA. The high sensitivity of cyclic voltammetry allows studies on synthetic DNA and RNA sequences, which are obtained in limited quantities. We therefore determined the rate constant for the synthetic oligomer shown in Table 1, both as a single strand and hybridized to its complement [12]. Hybridization decreases the measured rate constant by a factor of 200 because the double helix protects the guanine from the oxidant, resulting in a larger electron-transfer distance. (The rate constant for the duplex oligonucleotide is lower than that observed in calf thymus DNA because the 5′-GT sequence is the least electron-rich sequence environment for guanine.) Imperfect pairing of guanine in a mismatch gives intermediate rate constants in the order of GC ⩽ GA. As expected, the GT is most similar to GC since packing of the pyrimidine should cause less distortion of the helix than the G-purine mismatches (Table 1). Assuming that the entire difference in rate constant is due to distance effects (and not driving force) and that the tunneling rate depends on distance with a β of 1.5 $Å^{-1}$ [13,14], the difference in electron transfer distance for each sequence compared with the single strand (Δr) can be calculated (Table 1).

To determine the kinetics of guanine oxidation in a variety of sequence and structural contexts, we need a means for detecting the oxidation of a *single* guanine residue, since sequences with multiple guanines will give an average rate constant for each guanine environment. We have therefore determined that both inosine 5′-monophosphate ($k_2 = 97\ M^{-1}\ s^{-1}$) and 7-deazaguanosine 5′-monophosphate are not oxidized by $Ru(bpy)_3^{3+}$ under the same conditions where the guanine mononucleotide ($k_2 = 1.4 \times 10^5\ M^{-1}\ s^{-1}$) is oxidized efficiently (Fig. 2) [15]. Likewise,

Table 1
Rate constants for oxidation of guanine in DNA oligomers by $Ru(bpy)_3^{3+}$.

k ($M^{-1}\ s^{-1}$)[a]	Oligomer sequence	Δr_{Ru-G} (Å)[b]
1.2×10^3	(5′-AAATATAGTATAAAA)·(3′-TTTATATCATATTTT)	3.4
5.1×10^3	(5′-AAATATAGTATAAAA)·(3′-TTTATATTATATTTT)	2.4
1.0×10^4[c]	(5′-AAATATAGTATAAAA)·(3′-TTTATATGATATTTT)	2.0
1.9×10^4	(5′-AAATATAGTATAAAA)·(3′-TTTATATAATATTTT)	1.4
1.8×10^5	(5′-AAATATAGTATAAAA) single strand	0

[a]DNA concentrations used to determine rate constants were based on the moles of guanine nucleotide. Diffusion coefficients for all oligomers were fixed at $1.0 \times 10^{-6}\ cm^2/s$.

[b]Estimated distance of tunneling through solvent. Distances calculated according to $k/k_{ss} = \exp[-\beta\Delta r]$, where $\beta = 1.5\ Å^{-1}$ and $k_{ss} = 1.8 \times 10^5\ M^{-1}\ s^{-1}$.

[c]Since the rate constants are relative to guanine concentrations, the observed rate for the GG mismatch has been normalized relative to the other oligomers containing a single guanine.

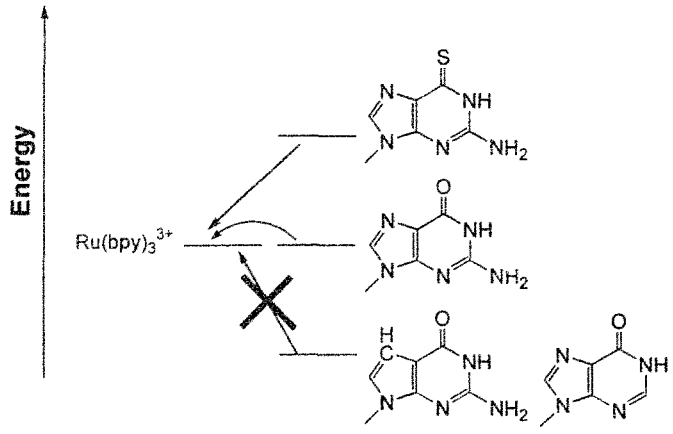


Fig. 2. Electron-transfer reactivity of modified guanines. Electron transfer from guanine (middle) to $Ru(bpy)_3^{3+}$ is efficient while electron transfer of hypoxanthine (bottom right) and 7-deazaguanine (bottom left) are not. Electron transfer from 6-mercaptoguanine (top) is even more efficient than from guanine. Hypoxanthine is the nucleobase of the inosine nucleoside.

6-mercaptoguanine is oxidized even more efficiently by $Ru(bpy)_3^{3+}$ and also by a less oxidizing mediator ($Ru(Me_2-bpy)_3^{3+}$), which does not oxidize guanine. Therefore, we can obtain a single oxidizable site either by substitution of the guanines that we want to avoid oxidizing by inosine or 7-deazaguanine or by substitution of the target guanine by 6-mercaptoguanine and using a less oxidizing mediator.

2.3. *Sequence effects on reactivity*

The effects of sequence context on the redox potential and reactivity of guanine in DNA has been of recent interest, primarily due to the repeated observation of enhanced reactivity of GG doublets on sequencing gels following piperidine treatment [16–18]. These gels show that the guanine on the 5′-side is preferentially oxidized in GG doublets; this effect is increased again in GGG triplets with the 5′-G most reactive, followed by the middle G, followed by the 3′-G. Despite the potential insight into base stacking available from studies of this effect, there have been no systematic studies of the *absolute* rate constants of these G multiplets. The only information available is from yields of cleavage on sequencing gels and gas-phase redox potentials calculated by ab initio methods [19].

Because the electrochemical methodology developed in our laboratory allows for the study of synthetic oligonucleotides on convenient quantities of material, the absolute reactivity of G can be evaluated in a variety of sequence contexts. Oligonucleotides were synthesized with (AG_nT) and $(AGT)_n$ inserts between a common flanking sequence (Table 2). At a fixed scan rate, the amount of catalytic current obtained is a function of the number of catalytic cycles that occur, which is a function of the rate constant for guanine oxidation [10]. As shown in Table 2, a greater increase in catalytic enhancement is observed upon adding adjacent guanine

Table 2
Ratios of catalytic current obtained for oxidation of G multiplets

Sequence[a]	i/i_1^b	Sequence[a]	i/i_1^b
5′-AGT	1	5′-AGT	1
5′-AGTAGT	1.15	5′-AGGT	1.89
5′-AGTAGTAGT	1.28	5′-AGGGT	3.38

[a]The complete sequence is 5′-AAA-TAT-(X)-ATA-AAA with the indicated sequence in the X position. The oligonucleotides were hybridized to complementary strands (which contained no guanine residues) under standard conditions. [b]Ratio of the peak current obtained for the indicated sequence divided by the peak current obtained for a sequence containing a single G. Conditions: 25 μM $Ru(bpy)_3^{2+}$, 100 μM oligonucleotide, scan rate = 25 mV/s, pH 7 phosphate buffer (50 mM) with 700 mM NaCl.

residues to the sequence, compared with adding interspersed guanine residues. This result provides direct experimental evidence that the *intrinsic* redox reactivity of the guanine multiplets is increased compared with isolated guanine.

2.4. Footprint of M.HhaI

Base flipping, the rotation of a base 180° out of the DNA double helix into a hydrophobic enzyme pocket, plays a key role in DNA repair and processing [20–22]. High-resolution X-ray structures of protein–DNA complexes demonstrate base flipping for several enzymes, including the M.*Hha*I methyltransferase [23], which binds the sequence 5′-GCGC and flips cytosine into an active site for methylation (Fig. 3). The primary means for assigning base flipping has been from high-resolution X-ray crystal structures of protein–DNA complexes, so a convenient assay that can be performed on limited quantities of material would allow for the ubiquity of base flipping to be assessed. Based on the results in Table 1, we reasoned that the orphan guanine left behind after flipping of the cytosine into the protein active site would be more susceptible to electron transfer to $Pt_2(pop)_4^{4-}$.

A synthetic DNA 35-mer containing the recognition sequence for M.*Hha*I was

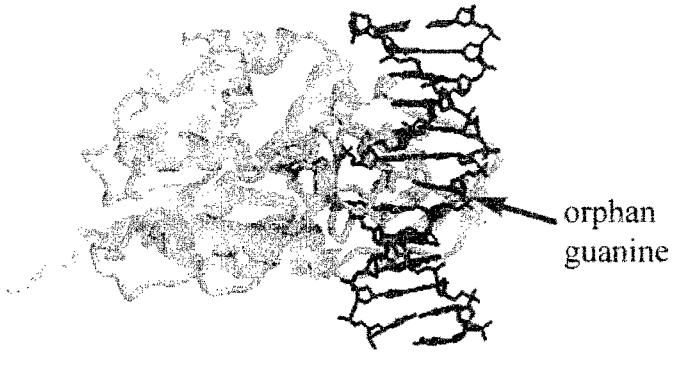


Fig. 3. X-ray crystal structure of M.*Hha*I methyltransferase with DNA showing the orphan guanine. Coordinates were taken from reference [23].

reacted with $Pt_2(pop)_4^{4-}$ in the presence and absence of the protein. Binding of the enzyme to a single side of the helix was ensured by methylating the target base on one strand and not the other. The strand containing the orphan guanine was 5′-^{32}P-labeled, and an even cleavage ladder was obtained with or without piperidine treatment. In the presence of the protein, two half-sites of protection were observed on either side of the recognition sequence prior to piperidine treatment, as expected from the X-ray structure. Use of other footprinting reagents with M.*Hha*I did not provide nearly the resolution for sugar protection seen with $Pt_2(pop)_4^{4-}$ [24], although the high-resolution pattern is fully predictable from the X-ray structure. Following piperidine treatment, a $\geq 50\%$ enhancement was observed at the orphan guanine in the presence of the protein. This enhancement was not observed without piperidine treatment. The result is particularly striking in light of the fact that the other guanine residues contacted by the protein were protected from cleavage. The enhancement likely arises from an increased rate of the one-electron oxidation of the orphan guanine, which is apparently more accessible to $Pt_2(pop)_4^{4-}$* in the presence of the protein.

That the enhancement in cleavage at the orphan guanine was due to electron transfer was confirmed by substitution of inosine for the orphan guanosine. This duplex still bound the M.*Hha*I protein, but no cleavage enhancement in the orphan inosine was observed. Likewise, the original guanine-containing duplex was bound to the *Hha*I endonuclease [25], which recognizes the same sequence as the methyltransferase but does not flip cytosine into the active site. No enhancement at the orphan guanine was observed for this enzyme. Therefore, cleavage enhancement at the orphan guanine was observed only when electron transfer was feasible and when the cytosine was flipped. This result, therefore, constitutes a potential approach to a general assay for base flipping proteins.

3. Conclusions

The efforts of the human genome project [26] and the combinatorial selection of synthetic protein-binding nucleic acids [27] are producing new nucleoprotein complexes rapidly. As a result, there is a strong need to detect special features of nucleoprotein complexes on limited quantities of material. We have described how the excited state of $Pt_2(pop)_4^{4-}$ can be used to probe hydrogen and electron transfer reactions of DNA and nucleoprotein complexes. The hydrogen transfer reaction is sequence-independent in duplex DNA and a sensitive probe of protein binding loci and DNA conformation. The electron transfer reaction of guanine is sensitive to changes in the neighboring bases and the paired base in well defined ways, leading to new assays for single base mismatches and base flipping.

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