Electrostatics Rather than Conformation Control the Oxidation of DNA by the Anionic Reagent Permanganate

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Abstract: Kinetic study was used to compare permanganate oxidation of oligonucleotides and evaluate the structural basis for its specificity of reaction. The rate of oligonucleotide consumption was consistently dominated by reaction of unpaired and solvent accessible thymine residues, the primary targets of modification. Formation of fully complementary duplex structures suppressed reaction of the single-stranded oligonucleotides by as much as 20-fold and revealed a basal rate of modification that no longer correlated to the presence of thymine. Insertion of a mispaired or unpaired thymine into a duplex structure modestly enhanced the overall degradation rate of DNA. While steric effects should not be discounted, electrostatic effects appeared to be the major determinant in permanganate selectivity. The oxidation rate of thymidine 5′-monophosphate and thymine residues in a single-stranded oligonucleotide approached the rate of thymidine only when high salt concentration was present to shield the repulsion of the anionic reactants. Oxidation of duplex structures was also activated by 10- to 25-fold when the sodium chloride concentration was increased from 0.1 to 4.0 M.

Introduction

Chemical and physical techniques provide complementary and often distinct information about nucleic acid structure. X-ray crystallography and nuclear magnetic resonance respectively characterize the solid-state and solution conformations of an oligonucleotide. In contrast, chemical modification studies describe the most reactive conformation of an oligo- or polynucleotide sequence. Accordingly, the data and interpretations drawn from one method can only be extended to the domain of another with much care. Our laboratory, along with others, is working to establish direct correlations between the physical and chemical properties of nucleic acids. In this report, critical parameters affecting permanganate oxidation of thymine residues have been characterized in an effort to relate DNA structure and reactivity in a detailed manner.

Permanganate has been adopted as a chemical probe with increasing frequency due to its target selectivity and ease of handling. Under the appropriate conditions, the reagent oxidizes thymine with great specificity[1,2] and produces thymine glycol and barbituric acid derivatives.[3] This activity has been used successfully for identifying the primary sequence of thymine in DNA.[4] In addition, residues held within partially denatured or distorted conformations can be modified under conditions that do not effect residues held within a B-helical duplex. Footprinting techniques based on permanganate have therefore become important for characterizing structural perturbations of DNA induced by protein and drug interactions.[5–8] Equivalent procedures have also been used to characterize DNA containing hinge, junction, and triplex regions,[9] telomeric sequences,[10] A tracts,[11,12] duplexes of parallel strands,[13] and single base mismatches.[14]

Selective oxidation of particular thymine residues has been variously attributed to unwinding, unpairing, or unstacking of the base that in all cases increase the solvent exposure of its reactive carbon–carbon (C5–C6) double bond.[1,2] To identify the essential parameters controlling this exposure, we have now measured and compared the oxidation rates of oligonucleotide duplexes containing complementary, mispaired, and unpaired bases. This data has been further related to the oxidation rates of thymidine, TMP and unpaired single-stranded oligonucleotides. Rate measurement was based on the consumption of starting material rather than the formation of product in order to avoid complications associated with propagated or secondary reactions.[14,15] This experimental approach contrasts the typical focus on reaction products and contributes an alternative method for determining the dominant reaction of thymine. Most notably, our results reveal a strong electrostatic component that controls the modification of polyanionic DNA by the anionic oxidant, permanganate.

Results

Reaction Kinetics. Oxidation conditions were chosen to maintain pseudo-first-order reaction kinetics. Permanganate concentration was therefore kept relatively low (300 μM) although well in excess of the oligonucleotides (5 μM). In every determination, consumption of the parent compound was fit to a first-order exponential decay (Figure 1). These measurements provided a reproducible set of rate constants for the comparisons

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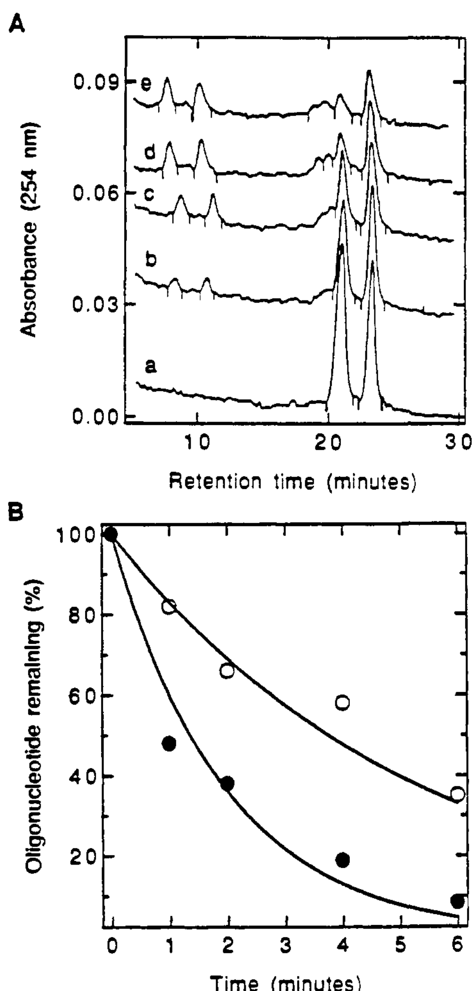

Figure 1. Sample rate analysis illustrated by the oxidation of duplex **3+5** (containing an unpaired thymine) in the presence of 4.0 M sodium chloride. (A) Anion exchange chromatography was used to separate the parent oligonucleotides **3** and **5** (retention times ca. 24 and 21 min, respectively) after exposure to permanganate for (a) 0.0, (b) 1.0, (c) 2.0, (d) 4.0, and (e) 6.0 min and subsequent treatment with piperidine as described in the Experimental Section. (B) The consumption of oligonucleotides was quantified by integrating the above elution profiles of **3** (○) and **5** (●). The theoretical loss of starting material (—) was calculated from the first-order rate constant determined from two independent sets of determinations.

Table I. Oligonucleotide Sequences and Their Single-Stranded Reactivity[a]

Oligonucleotide	Sequence	Number of Thymines	k x 10^2 (min^{-1})
1	5'd(CACGGGAACGCAGG)	0	0.56 ± 0.06
2	5'd(CACGGGAACGCATG)	1	8.1 ± 0.6
3	5'd(CACGGGTGCGCATG)	2	17 ± 2
4	5'd(CATGCGCACCCGTG)	2	17 ± 2
5	5'd(CATGCGCTACCCGTG)	3	17 ± 1
6	5'd(CATGCGTTCCCGTG)	4	25 ± 2

[a] Pseudo-first-order rate constants were determined by the loss of parent sequences in the presence of 0.1 M sodium chloride. See the Experimental Section for details.

in this report. For completeness, the oxidation of a representative oligonucleotide, **6** (Table I for nucleotide sequence), was also verified to be first order in permanganate[16] under concentrations proximal to the standard above (100–300 μM). Second-order rate constants (8.3 ± 2.1 and 88 ± 1 M^{-1} min^{-1} = $k_2 \times 10^{-2}$) were subsequently calculated for this reaction of **6** at 0.1 and 4.0 M sodium chloride, respectively.

Table II. Overall Reactivity of Duplex DNA in the Presence of Permanganate[a]

Duplex Structure	#	Oligonucleotides	k x 10^2 (min^{-1})
fully complementary	3	5'd(CACGGGTGCGCATG)	0.88 ± 0.11
	4	3'd(GTGCCCACGCGTAC)	0.81 ± 0.12
fully complementary	2	5'd(CACGGGAACGCATG)	1.2 ± 0.1
	6	3'd(GTGCCCTTGCGTAC)	1.1 ± 0.1
single T insert	3	5'd(CACGGGT--GCGCATG)	0.82 ± 0.12
	5	3'd(GTGCCCA$_T$CGCGTAC)	1.9 ± 0.2
TT · GT mismatch	3	5'd(CACGGG^{TG}CGCATG)	0.73 ± 0.10
	6	3'd(GTGCCC$_{TT}$GCGTAC)	1.6 ± 0.1
single G · A mismatch	1	5'd(CACGGGAACGCA^{G}G)	0.62 ± 0.09
	6	3'd(GTGCCCTTGCGT$_A$C)	0.99 ± 0.12
hairpin (T_4 loop)	7	5'd(CGCGCG^{T}T$_)$	5.3 ± 0.3
		3'd(GCGCGC$_T$T	

[a] Pseudo-first-order rate constants were determined by the loss of parent sequences in the presence of 0.1 M sodium chloride. See the Experimental Section for details.

Oxidation of Unpaired Oligonucleotides. The pseudo-first-order rate constants for oligonucleotide consumption reported here reflect the overall reactivity of the parent strands and represent the sum of microscopic rate constants for oxidation at each nucleotide.[17] As expected, thymine modification dominated the kinetic characterization throughout this analysis (Table I). Incorporation of a single thymine into a 14-nucleotide sequence (**2**) increased the strand's sensitivity to permanganate by over 14-fold. Incorporation of a second thymine (**3** and **4**) further doubled the sensitivity. The presence of a third thymine (**5**) had no detectable effect, but a fourth thymine (**6**) again increased the sensitivity by the same increment as the initial two thymines. Deviation from a strict correlation between thymine content and reactivity may reflect the experimental uncertainty or the persistence of distinct structures formed by these related single strands.[18]

Oxidation of Duplex Oligonucleotides Containing Complementary, Mismatched, and Unpaired Thymines. Hybridization of the oligonucleotides dramatically and specifically inhibited the thymine-dependent reactivity (Table II). Modification of **2–6** was suppressed 10- to 20-fold when annealed into duplex structures, whereas the reaction of **1**, a sequence without thymine residues, reacted equally slowly in a duplex or single-stranded structure. The rate constants for the fully complemented strands (**3+4** and **2+6**) were all reduced to the same background value exhibited by **1**. Consequently, this background is not dependent on nucleotide sequence or conformation.

The rate of the permanganate reaction was raised above the nonspecific background by the addition of a single unpaired thymine. When a thymine bulge was introduced into an otherwise complementary duplex (**3+5**), the sensitivity of the strand with the extra thymine was enhanced over background by more than 2-fold (Table II). However, the consequences of this bulge was very localized. The adjacent strand containing a thymine proximal to the unpaired nucleotide displayed no increase in reactivity. A single mispaired, rather than unpaired, thymine was also unable to induce any reaction above background for **3** of **3+6**. The modification rate for **6** in which two neighboring thymines were mispaired (**3+6**) was limited to a 1.5-fold increase above the rate

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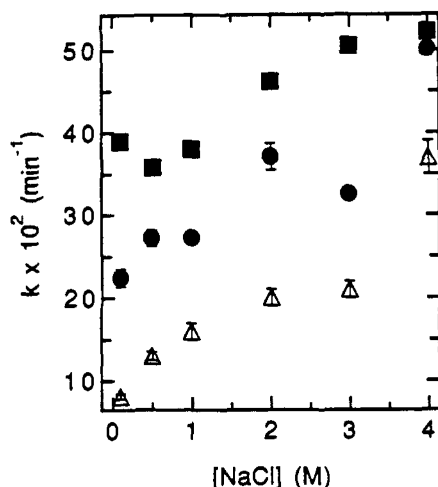

Figure 2. Pseudo-first-order rate constants for oxidation of thymidine (■), TMP (●), and **2** (△) at the indicated concentrations of sodium chloride. The initial concentration of the reaction target, the nucleobase thymine, was 5 μM in all determinations. The experimental uncertainty (standard deviation) is shown when it extended beyond the data symbols.

for **6** in a fully complementary duplex (**2+6**). Only hairpin[19] **7** was oxidized at a rate approaching that of a single-stranded oligonucleotide such as **2**. For this example then, the collective reactivity of the four thymines in the hairpin is best compared only to the reactivity of a single thymine within a fully unpaired structure. The significant inhibition of thymine oxidation within this loop conformation may confirm that strong base stacking persists in this non-classical structure.[20,21] However, a broader investigation was necessary to examine additional parameters that may dominate the access of permanganate to thymidine and its oligonucleotide derivatives.

Ionic Dependence of Thymidine, TMP, and Oligonucleotide Oxidation. Two additional determinations were used to augment the results above. First, the rates of thymidine and TMP oxidation by permanganate were investigated under the conditions of the oligonucleotide studies described above. The pseudo-first-order rate constants measured in this manner for thymidine (39 ± 1 $min^{-1} = k \times 10^2$) and TMP (22 ± 2 $min^{-1} = k \times 10^2$) were ca. 5- and 3-fold greater than that for the single thymine residue in oligonucleotide **2**. Therefore, oxidation of thymidine is suppressed by both its 5′-phosphorylation and its incorporation into single-stranded oligonucleotides. The difference in nucleoside and nucleotide reactivity is likely explained by the entropic barrier for encounter of the anionic nucleotide and the anionic oxidant. Thus, variation in the local concentration of negative charges could play an important role in selective modification of DNA.

Accordingly, the second determination examined the salt dependence of permanganate reaction in order to measure the response to shielding of the anionic charges on DNA.[22] Oxidation of neutral thymidine was found to be relatively unaffected by sodium chloride concentrations;[16] reaction rates increased only 1.3-fold when salt concentrations were varied from 0.1 to 4.0 M (Figure 2). The oxidation rate of TMP was accelerated 2.2-fold by an equivalent increase in salt, and at 4.0 M sodium chloride the reactivity of TMP and thymidine were nearly identical. The same increase in sodium chloride concentration also accelerated the reaction of single-stranded **2** (Figure 2) and **6** (data not shown) by 4.6- and 6.8-fold.

Permanganate oxidation of duplex structures tended to be even more sensitive to the ionic conditions. Rate measurements for the reaction of a fully complementary helix (**2+6**) and a helix containing a single thymidine insert (**3+5**) at high ionic strength revealed that both the specific oxidation of thymine residues and the nonspecific background reactions of DNA were enhanced. By increasing the sodium chloride concentration from 0.1 to 4.0 M, the rate constants for **2+6** increased 5- and 10-fold to 5.9 ± 0.2 and 11 ± 1 min^{-1} ($k \times 10^2$) and those for **3+5** increased 22- and 27-fold to 18 ± 3 and 51 ± 5 min^{-1} ($k \times 10^2$), respectively.

Discussion

Correlation between Oligonucleotide Sequence, Structure, and Reactivity. Permanganate has been used with repeated success in characterizing DNA and, in particular, identifying the perturbations of helical structure that are caused by DNA–protein interactions.[5,8] Selective modification of individual thymine residues has become diagnostic of their unusually high accessibility to permanganate.[1,5] If steric constraints were the only variable controlling this process, then the relative rate of oxidation might serve as a quantitative guide to DNA structure and dynamics. We chose a well-defined series of oligonucleotides to begin our correlation between oxidation rate and thymine conformation. Such a system minimized the heterogeneity of the target and allowed for a more detailed investigation of the steric and electrostatic properties that control permanganate reaction.

Kinetic study indicated that the oxidation of thymine residues within a complementary duplex was very slow and not detectable above nonspecific background modification. The pseudo-first-order rate constant for each oligonucleotide of **3+4** and **2+6** was independent of thymine content and remained within 20% of an average value of 1.0 min^{-1} ($k \times 10^2$) (Table II). Strand oxidation was accelerated above background when thymine residues were unpaired and, in one example, mispaired in a duplex. However, oxidation of these non-canonical conformations proceeded at only a fraction of the rate observed for an unpaired strand of DNA containing even a single thymine residue (Table I). In general, the rate constants of Table II depict at most a mild dependence on structure. These values do not reflect the wide range of reactivity that has been observed during the mapping of polynucleotide–protein interactions.[5,8]

The contrasting data generated from the polynucleotide and oligonucleotide studies are not necessarily contradictory and may simply reflect differences in the extent of structural relaxation. The formation of specific protein–DNA complexes is often associated with major changes in conformation extending over a number of base pairs.[5,8] Duplex structures examined here contained perturbations that instead were highly localized. NMR spectroscopy has previously suggested that a sole extrahelical thymine could stack within a double helix without disrupting the flanking base pairs or helical character.[23] Analogous examination of T·T and G·T mismatches in duplex DNA has also demonstrated that these pairs could be easily accommodated in a standard B helix.[24] Thus, physical and chemical methods may provide comparable results. Thymine could still remain relatively inaccessible to permanganate when not hydrogen bonded to adenine.

Electrostatic Repulsion Greatly Affects the Oxidation of DNA by Permanganate. Steric considerations alone do not offer a satisfactory explanation for the observed rates of thymine oxidation. Osmium tetroxide, another oxidant acting on the C5–C6 double bond of thymine, is capable of modifying thymine

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mismatches with reasonable selectively.[25] Permanganate was reported to exhibit similar specificity only after the charge of DNA had been partially screened by counterions (tetraalkylammonium cations).[14] The extent to which permanganate activity is controlled by electrostatics was further examined in our laboratory. The influence of charge repulsion was illustrated most decisively by comparing the oxidation of thymidine and TMP. Conversion of neutral thymidine to anionic TMP inhibited reaction with anionic permanganate by 42% in the presence of 0.1 M sodium chloride (Figure 2). Incorporation of TMP into oligonucleotide **2** suppressed the oxidation even more effectively. This latter decrease, however, could have resulted from the greater steric hindrance and/or charge repulsion imposed by the oligonucleotide.

Steric and electrostatic contributions were distinguished by measuring the rate of thymine oxidation as a function of sodium chloride concentration. Added salt was expected to either (i) stimulate reaction by reducing charge repulsion and entropic barriers or (ii) inhibit reaction by promoting base stacking and general hydrophobic aggegration. In every example studied here, sodium chloride had the ability to accelerate reaction. High sodium chloride concentration completely offset the inhibition of oxidation created by the anionic phosphate on TMP (Figure 2). Moreover, the oxidation rate of thymine within the unpaired oligonucleotide **2** approached that of the uncharged nucleoside in the presence of 4.0 M sodium chloride. Duplex structures were even more sensitive to the ionic conditions. Oxidation of **5** (in **3+5**) containing an unpaired thymine was promoted 27-fold in the added presence of 4.0 M sodium chloride. In this case, the rate constant for reaction of **5** represents ca. 98% of the value of free thymidine. Such prominent effects of salt are not universal to all chemical modifications of DNA. Neutral reagents including hydroxyl radical and diethyl pyrocarbonate are not strongly activated, and may in fact be suppressed, in the presence of high salt.[13,26]

The rate constants for thymine oxidation measured in this report vary over two orders of magnitude. These constants represent the relative reactivity of permanganate with thymine derivatives that range from the free nucleoside to duplex DNA. Much of the variation can be explained by the electrostatic repulsion and entropic barrier of two approaching anionic reactants. Local neutralization of the backbone charge might then promote selective modification of DNA whether or not a helical structure is fully denatured. Electrostatic as well as steric factors must then be carefully evaluated when considering the details of DNA modification and its applications for footprinting DNA–ligand interactions.

Experimental Section

Materials. All oligonucleotides were synthesized via standard solid-phase cyanoethyl phosphoramidite chemistry and purified by anion exchange chromatography (Mono Q, Pharmacia) under strongly denaturing conditions as described below. DNA concentrations were calculated per mole of oligonucleotide from their ϵ_{260} values estimated from the sum of nucleotide absorptivity as affected by adjacent bases.[27] Thymidine, thymidine 5′-monophosphate and other reagents were obtained at the highest grade commercially available and were used without further purification.

Potassium Permanganate Reaction. Oligonucleotides (5 μM per strand) were combined with 10 mM potassium phosphate at pH 7.0 and 0.1–4.0 M sodium chloride. For duplex annealing, these solutions (ca. 0.5 mL) were placed in a heated water bath (90 °C). This bath was immediately turned off and the samples were allowed to cool along with the bath under ambient conditions overnight. Oxidation was performed at 22($\pm$1) °C and was initiated by addition of a freshly prepared solution of potassium permanganate (300 μM). Aliquots (100 μL) of the reaction mixtures were removed at the indicated times and quenched with 20 mM sodium sulfite.[1] These samples were then individually dialyzed against water overnight and dried under high vacuum. Strand fragmentation at sites of oxidation was induced by standard treatment with 0.2 M piperidine at 90 °C for 30 min.[4] Finally, piperidine was removed under high vacuum and the resulting DNA was resuspended in water (1 mL) for the chromatographic analysis described below.

Oxidation of thymidine and TMP was examined under experimental conditions equivalent to those described for the oligonucleotides and included 5 μM of either the nucleoside or nucleotide. Reaction was again quenched with 20 mM sodium sulfite, but in this case, the side product manganese dioxide was removed by filtration through Celite 545 (Fisher Scientific). The eluant was then analyzed directly by reverse phase HPLC as described below.

Rate Determination. The parent oligonucleotides and their fragmentation products were individually separated by anion exchange chromatography (Mono Q, Pharmacia) under denaturing conditions using a gradient of 375–750 mM sodium chloride in 11.5 mM sodium hydroxide over 30 min (1 mL/min). Products of strand scission eluted according to sequence length and composition but always prior to the parent strands.[19,28] Consumption of the starting materials was quantified by integrating their remaining absorbance (254 nm) in each elution profile.[29]

The oxidative loss of thymidine and TMP was monitored by reverse phase HPLC (Spherex 5 μm C18, 250 × 4.6 mm, Phenomenex). Thymidine was separated from its products using a 0–6% gradient of acetonitrile in 50 mM triethylammonium acetate (pH 6.0) over 18 min (1 mL/min). TMP analysis employed an isocratic elution with triethylammonium acetate (50 mM, pH 3.0, 1 mL/min). In both procedures, starting material was quantified by integrating the elution profiles at 260 nm.

Rate constants were determined for each reaction by nonlinear regression of remaining starting material (%) as a function of time with the integrated area at zero time set at 100%. Reaction progress was monitored over at least the initial 20–30% of conversion in accord with the conditions commonly applied in footprinting of polynucleotides. A minimum of two sets of data were collected for each rate determination, and when necessary additional sets were collected to increase precision. Data were fit to a single exponential constrained to 100% at t_0 and uncertainties were assigned by the standard deviations of the regression.

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Supplementary Material Available: Kinetic data on the permanganate oxidation of single-strand oligonucleotides **1–6** and the duplexes **3+4**, **2+6**, **3+5**, **3+6**, **1+6**, and **7** at 0.1 M sodium chloride, single-strand **6**, duplexes **2+6** and **3+5** at 4.0 M sodium chloride, and thymidine, TMP, and **2** at 0.1–4.0 M sodium chloride (12 pages). Ordering information is given on any current masthead page.

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