The Peroxidase Activity of a Hemin−DNA Oligonucleotide Complex: Free Radical Damage to Specific Guanine Bases of the DNA†

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Abstract: A specific DNA oligonucleotide−hemin complex (PS2.M−hemin complex) that exhibits DNA-enhanced peroxidative activity was studied by EPR and UV−visible spectroscopy and by chemical probing analysis. EPR data obtained from low-temperature experiments on the PS2.M−hemin complex showed both a low-field $g \sim 6$ and a high-field $g \sim 2$ signal. These EPR signals are typical of high-spin ferric heme with axial symmetry as judged by the EPR spectrum of six-coordinate heme iron in acidic Fe(III)-myoglobin. This similarity is consistent with the presence of two axial ligands to the heme iron within the PS2.M−hemin complex, one of which is a water molecule. Optical analyses of the acid−base transition for the hemin complex yielded a pK_a value for the water ligand of 8.70 ± 0.03 (mean ± SD). Low-temperature EPR analysis coupled with parallel spin-trapping investigations following the reaction of the PS2.M−hemin complex and hydrogen peroxide (H_2O_2) indicated the formation of a carbon-centered radical, most likely on the PS2.M oligonucleotide. Chemical probing analysis identified specific guanine bases within the PS2.M sequence that underwent oxidative damage upon reaction with H_2O_2. These and other experimental findings support the hypothesis that the interaction of specific guanines of PS2.M with the bound hemin cofactor might contribute to the superior peroxidative activity of the PS2.M−hemin complex.

Introduction

A folded guanine-rich oligonucleotide and its corresponding RNA version ("PS2.M" and "rPS2.M", respectively)[1] have been shown to complex readily with hemin (dissociation constants $K_d \sim 27 \pm 2 \times 10^{-9}$ M and $0.9 \pm 0.2 \times 10^{-6}$ M, respectively).[2] Detailed kinetic studies have been reported recently[2,3] of accelerated peroxidation reactions catalyzed by hemin complexed to these specific single-stranded nucleic acid molecules ("aptamers"). For example, the PS2.M−hemin complex-catalyzed (per)oxidation of 2,2′-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid in the presence of hydrogen peroxide (H_2O_2) occurs with a rate constant ~2 orders of magnitude greater than that of disaggregated, monomeric hemin (in the presence of low concentrations of detergents and of control DNA oligomers).[3] In contrast, other complexes of hemin and single-strand DNA oligonucleotides, including one formed by an unrelated guanine-rich DNA oligomer, have shown no acceleration of peroxidation over that of monomeric hemin.[2,3] The kinetic behavior of the *catalytic* hemin−oligonucleotide complexes suggested that the nucleic acid component activated the iron for reaction with H_2O_2 and catalyzed the breakdown of the hemin−H_2O_2 complex.[3] rPS2.M− and PS2.M−hemin complexes, therefore, constitute a novel class of catalytic nucleic acids (a ribozyme and a deoxyribozyme, respectively). Although this nucleic acid-catalyzed activity was modest compared to that of an evolved protein enzyme, such as horseradish peroxidase (HRP), the PS2.M−hemin complexes showed UV−visible spectral changes remarkably similar to those occurring during the peroxidative cycle of the protein enzyme.[3] This finding raised the possibility that specific hemin complexes of oligonucleotides of DNA (and corresponding RNA) could act as classic peroxidases.

Peroxidations catalyzed by DNA oligonucleotide-complexed hemin were favored by the presence of nitrogenous buffers,[3] which appeared to function as general acid/general base catalysts for the peroxide O−O bond scission. The buffer contribution toward peroxidations was much greater for the PS2.M/rPS2.M−hemin complexes than for monomeric hemin,[3] suggesting that buffer catalysis acted in conjunction with specific interactions of hemin in the active sites of the folded PS2.M and rPS2.M to facilitate the decomposition of the iron-peroxide complex.

We, therefore, postulated that the decomposition of H_2O_2 by the hemin complexes likely occurred with a concomitant formation of a compound I-like intermediate, similar to that formed in the HRP peroxidation cycle.[4−6] Compound I is the key intermediate in the catalytic cycle of HRP and is known to

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(1) Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMPOX, 5,5-dimethyl-2-ketopyrrolidone-*N*-oxyl; DTPA, diethylenetriaminepentaacetic acid; ddH_2O, double distilled water; EPR, electron paramagnetic resonance spectroscopy; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; MES 2-[*N*-morpholino]ethanesulfonic acid; 2-methyl-2-nitrosopropane (MNP); PS2.M, a guanine-rich oligonucleotide (5′-GT GGT AGG GCG GGT TGG-3′); PS2.M−hemin complex, complex formed from hemin and the PS2.M oligonucleotide; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; Tris, tris[hydroxymethyl]aminomethane.

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be a porphyrin radical cation ($Fe^{IV} = O\ Por^{\bullet+}$).[4] Following two sequential one-electron transfers from an organic substrate, compound I is reduced back to the ferric state via the ferryl heme ($Fe^{IV} = O$ Por) compound II. Compound II, therefore, contains only one of the oxidizing equivalents of the peroxide.[4−6] This paper reports the use of electron paramagnetic (EPR) and UV−visible absorption spectroscopy, together with chemical probing analysis, to explore the active site afforded by the PS2.M oligonucleotide to the bound hemin cofactor.

Materials and Methods

Materials. 2-Methyl-2-nitrosopropane (MNP), 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), ethylenediaminetetraacetic acid (EDTA), di-*tert*-butyl nitroxide (DTBN), 2-[*N*-morpholino]ethanesulfonic acid (MES), tris[hydroxymethyl]aminomethane (Tris), Triton X-100, diethylenetriaminepentaacetic acid (DTPA), and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were obtained from Sigma (St. Louis, MO). DMPO was purified by stirring solutions (1 M in phosphate buffer, 50 mM pH 7.4) with activated charcoal (100 mg/mL) in the dark. After 30 min, the solution was filtered, and aliquots were stored at −80 °C prior to use. MNP was dissolved in acetonitrile (∼1 M) and diluted to the concentrations indicated in the figure legends: acetonitrile was maintained at <5% v/v to minimize effects of the organic solvent. Hydrogen peroxide (H_2O_2) was from BioRad (Richmond, CA). Hemin was obtained from Porphyrin Products (Logan, UT) and used without further purification. Buffers were prepared from either glass doubly distilled water (ddH_2O) or ddH_2O purified further by passage through a Barnstead Nanopure system and stored over Chelex-100 (BioRad) at 4 °C for ≥ 24 h to remove contaminating transition metals as verified by the ascorbate autoxidation analysis.[7] Organic solvents and all other chemicals employed were of the highest quality available.

Preparation of DNA Oligomer−Hemin Complexes. The DNA oligonucleotide PS2.M (5′-GTG GGT AGG GCG GGT TGG-3′) was synthesized at the University Core DNA Services (University of Calgary), while a control oligonucleotide (5′-GCC CCC CCT CGT CGT CGA CGG TA-3′) was synthesized at the Nucleic Acids and Protein Service Unit (University of British Columbia, Canada). The PS2.M- and control oligonucleotide−hemin complexes were prepared from corresponding stock solutions of oligonucleotides (2−4 mM) in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.8). Briefly, the stock of DNA oligonucleotide was diluted to 1 mM final concentration with 40KT buffer (100 mM MES, pH 6.2, 50 mM Tris, 40 mM KCl, 0.05% Triton X-100, and 1% DMSO): final concentration of DMSO <1% v/v. The oligonucleotide was next allowed to fold correctly at room temperature over ca. 30 min. Hemin (25 mM in 0.01 M NaOH) was then added to the solution (hemin:DNA oligonucleotide ∼1.5−2), and the complex was allowed to form for a further 30 min at room temperature.

EPR Spectroscopy. X-band EPR (at 4 or 77 K) was performed using a Bruker ESP 300e spectrometer equipped with a Hewlett-Packard frequency counter. Where required, solutions of PS2.M oligomer−heme complex (∼1 mM in 40KT buffer) were treated with H_2O_2 (H_2O_2:PS2.M oligomer−heme complex ∼1 mol/mol) in both the presence and absence of a spin trap (spin trap:PS2.M−hemin complex ∼10−20 mol/mol). For low-temperature EPR, samples (250 μL) were placed into a 3 mm quartz cell (Wilmad, Buena, NJ), frozen in liquid nitrogen, and transferred to a liquid helium cryostat (Oxford Instruments, NJ) for analyses. Analyses of spin adducts were performed at 293 K on samples (50 μL) of the reaction mixture transferred into capillary tubes with a glass pipet: capillaries were placed into a quartz EPR tube and transferred to the cavity for EPR analysis. The limit of detection of a stable nitroxide (TEMPO) was ∼50 nM under identical conditions. Unless indicated otherwise, the time between removal of the reaction mixture, transfer to the cell, and tuning the spectrometer was consistently <30 s. In some instances reaction mixtures were incubated at 20 °C for the times indicated in the figure legend(s) prior to EPR analysis. Spectra were obtained as an average of 5 scans, with a modulation frequency of 100 kHz and sweep time 84 s. Microwave power, modulation amplitude, and scan range varied as indicated in the figure legends. Hyperfine couplings were obtained by simulation using the simplex algorithm[8] provided in WINSIM (available at the URL http://epr.niehs.nih.gov/). Hyperfine couplings are expressed in units of G (gauss). Simulations were considered acceptable at correlations $R > 0.85$. DTPA (100 mM) was included in all PS2.M oligonucleotide solutions prior to the addition of H_2O_2 to minimize the possibility of transition metal-mediated decomposition of peroxide by Fenton chemistry. Where required, the concentration of free radical was standardized against a solution of 5 mM TEMPO (in 50 mM phosphate buffer, pH 7.4) measured under identical spectrometer conditions.

Changes in $A_{300-800\ nm}$ upon Treatment of the PS2.M−Hemin Complex with H_2O_2. The reactions between the PS2.M−hemin complex (∼7 μM) and H_2O_2 were performed in 40KT buffer at 20 °C. The initiation of significant change to the absorbance envelope of solutions of PS2.M−hemin complex required a large excess of peroxide. Thus, the addition of 200−1000 μM of H_2O_2 resulted in rapid changes to the absorbance envelope of the PS2.M−hemin complex, as judged by recording the $A_{300-800nm}$ (Varian Cary 3E UV−visible spectrophotometer). Spectra were recorded ≤10 s after mixing and subsequently at 1 min intervals. In some studies, hydroquinone ($\epsilon_{295\ nm} = 2.5 \times 10^6\ M^{-1}\ cm^{-1}$) was also added (final concentration 1 mM). Both hydroquinone and its oxidized product (benzoquinone) showed negligible maxima above 300 nm.

DNA Chemical Probing Studies: Chemicals and Sample Preparation. The PS2.M oligonucleotide was 5′-end-labeled with polynucleotide kinase and [γ-^{32}P] ATP (3000 Ci/mol, Amersham) and purified by denaturing preparative gel-electrophoresis. Samples of the radiolabeled PS2.M oligonucleotide were then mixed with nonradiolabeled PS2.M (to give a final stock PS2.M oligonucleotide concentration of 2−4 mM) in either TE or MT (10 mM MES, 0.1 mM EDTA, pH 7.8) buffers. These radiolabeled stock solutions were then used to prepare the labeled PS2.M−hemin complex as described for the preparation of nonlabeled PS2.M (see above). Note, for the case of chemical probing studies, hemin was prepared in DMSO (not 0.01 M NaOH) and added to the DNA oligonucleotide(s) from a relatively high stock concentration (25 mM) to minimize residual organic solvent (<1% DMSO v/v). The complex was then allowed to stand for a further 30 min at 20 °C prior to use as before. Experiments performed with hemin dissolved in NaOH or DMSO yielded identical results (data not shown).

Guanine-Specific DNA Cleavage and Methylation Protection. Freshly prepared dimethyl sulfate (DMS) solutions (2 and 3% v/v in ddH_2O) were added to the PS2.M oligonucleotide alone (to act as a control) and the PS2.M−hemin complex to yield final DMS concentrations of 0.2 and 0.3% v/v, respectively. Samples were incubated at 20 °C for 30 min and then precipitated with ethanol. The resulting pellets were dissolved in 10% piperidine (100 mL) and incubated for 30 min at 90 °C. Piperidine was removed by evaporation under vacuum followed by lyophilization (from solution in 50 mL of ddH_2O). Samples were finally dissolved in 5 mL of a 1:1 gel-loading dye:H_2O solution (gel-loading dye contained 95% formamide and the dyes xylene cyanole FF and bromophenol blue), heated at 90 °C for 3 min, and loaded into a 10% denaturing polyacrylamide gel. Autoradiography was carried out at −80 °C.

H_2O_2-Mediated DNA Oligonucletide Cleavage. Reactions of the PS2.M−hemin complex with H_2O_2 were carried out in 40KT buffer. Several control reactions were performed in the absence of potassium and/or in the absence of hemin. In a typical experiment, the reaction was initiated by addition of H_2O_2 (final concentration 1 mM) to either the PS2.M oligonucleotide alone (to act as a control) or to the PS2.M−hemin complex at mole ratios (r_0) of porphyrin to DNA oligonucleotide of $r_0 \leq 1$ (i.e., 0.8), 2, and 5 mol/mol. The reaction mixture was incubated for 30 min at 37 °C, and then the reaction was stopped by

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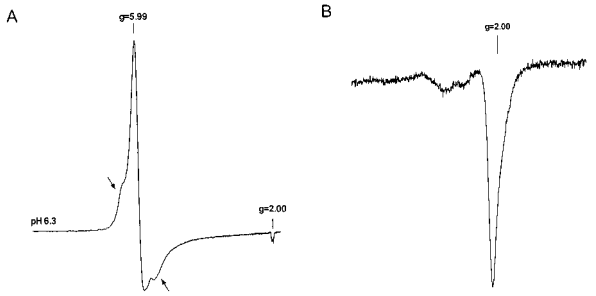

Figure 1. (A) EPR spectrum obtained from a frozen solution of 1 mM of the PS2.M−hemin complex at pH = 6.2 and at 4.2 K. Instrument parameters were as follows: microwave power 5 mW; modulation amplitude 5 G; and, scan range 3000 G. (B) The high-field peak ($g \sim 2$) expanded and enlarged.

ethanol precipitation. After centrifugation, the resulting pellet was redissolved in 10 μL of ddH_2O, and the solution was split into two aliquots. One sample was treated with piperidine (as described above), whereas the other was treated with an equal volume of the dye solution prior to loading onto a 10% denaturing polyacrylamide gel for electrophoresis.

Results

EPR of the Ferric PS2.M−Hemin Complex. It is well established that six-coordinate high-spin and low-spin ferric hemoproteins exhibit either axial or rhombic symmetry.[9] Moreover, several hemoproteins have been shown to contain a mixture of high- and intermediate-spin species.[10] To probe the coordination geometry of the PS2.M oligonucleotide's active site around the heme prosthetic group, we analyzed the resting ferric state of the PS2.M−hemin complex with EPR at 4 K, Figure 1. The low-temperature EPR spectrum obtained from the PS2.M−hemin complex showed both a low-field signal at $g \sim 6$, Figure 1A, and a high-field signal at $g \sim 2$ shown in expanded view in Figure 1B. These two EPR signals are typical of an axial high-spin ferric iron with the $g \sim 6$ and $g \sim 2$ signals assigned as the $g_{\perp(xy)}$ and $g_{\mathrm{II}(z)}$, respectively. Interestingly, the EPR spectrum of the PS2.M−hemin complex was markedly similar to that reported for the acid form of metmyoglobin,[11−13] as well as those of other ferric hemoproteins analyzed under similar conditions.[14] This result was consistent with previous findings that the PS2.M−hemin complex displayed an electronic spectrum similar to myoglobin-like hemoproteins.[2] Here, notably, the $g \sim 6$ signal of the PS2.M−hemin complex was significantly different from the distorted rhombic high-spin signals often observed for five-coordinate high-spin ferric proteins, such as HRP.[12,15] Together, these findings support the assignment of a six-coordinate high-spin ferric iron to the PS2.M−hemin complex.[2]

Careful examination of the low-field $g \sim 6$ response, however, indicated that the EPR signal actually consisted of two components. First, a sharp $g \sim 5.99$ axial high-spin signal (with a peak-to-peak line width of ~50 G), and second, a smaller rhombic high-spin signal (marked by arrows in Figure 1A). Note that the precise g values of the rhombic species could not be determined owing to the significant overlap with the large axial high-spin signal. The relative intensities of these peaks suggested that the proportion of rhombic symmetry was minor compared to the population of complex that exhibited axial symmetry in this system. Together, these observations suggested that solutions of the PS2.M complex contained a mixture of five- and six-coordinate heme, with the latter being the dominant species.

Determining the pH-Dependent Acid−Base Transition for the PS2.M−Hemin Complex. Investigations on the identity of the sixth heme-ligand indicated a coordinated water molecule (likely acting as the sixth ligand) as judged by the pH-dependent changes in the visible region of the solution spectrum of the PS2.M−hemin complex, Figure 2A. For pH <8.0, the complex presented a sharp Soret peak at $A_{404\mathrm{nm}}$. In addition, sharp bands were observed in the visible region at $A_{505\mathrm{nm}}$ and $A_{632\mathrm{nm}}$. The peak intensity of the various maxima changed (as indicated by arrows) with increasing pH, with isosbestic points at $A_{380\mathrm{nm}}$, $A_{422\mathrm{nm}}$, $A_{498\mathrm{nm}}$, $A_{550\mathrm{nm}}$, and $A_{627\mathrm{nm}}$. Above pH 8.0, these maxima broadened, and the isosbestic points became less apparent. The pK_a for this transition was calculated from the pH-dependent changes at both the Soret peak ($A_{405\mathrm{nm}}$) and the maxima at $A_{355\mathrm{nm}}$ and was determined to be 8.70 ± 0.03 (mean ± SD), Figure 2B. This value is similar to that reported for hemoproteins where the acid−base transition is attributed to the ionization of a water molecule coordinated to the sixth (axial) position of the hemin (ferric) iron.[10] These data together with the low-temperature EPR analyses of the resting state complex support the argument that the heme-iron shows axial symmetry with water likely acting as a ligand.

EPR and Optical Spectra of the Reaction of PS2.M−Hemin Complex and H_2O_2. We next investigated the effect of H_2O_2 addition on the EPR spectrum of the PS2.M−hemin complex, Figure 3. Incubation of H_2O_2 with the complex (H_2O_2: PS2.M−hemin complex~1 mol/mol) resulted in a marked change of the EPR signal at $g \sim 2$, with a new asymmetric signal detected at $g \sim 1.995$ that has a peak line width of ~15 G (c.f., Figures 3 and 1B). This new EPR signal was characteristic of the presence of an organic radical species in that it was stable over 4−77 K (data not shown). The radical, however, could not be detected at room temperature (limit of detection ~50 nM, as assayed with TEMPO nitroxide). Moreover, incubation of the PS2.M−hemin complex with H_2O_2 resulted in a decrease of the major high-spin ferric EPR signal at $g \sim 6$ (data not shown), indicating that the iron was no longer in its initial high-spin ferric state after reaction with peroxide.

Concomitant with the detection of the new $g \sim 2$ signal by EPR, the electronic absorption spectrum of the PS2.M−hemin complex also showed significant changes upon addition of H_2O_2, Figures 4A and 4B. Although 5−10 equiv of H_2O_2 was sufficient to cause an initial weak change, 40−200 equiv of peroxide was required to observe significant changes in the optical spectrum. This requirement for high H_2O_2 concentrations is, however, understandable in view of the low association constant of H_2O_2 (~3 mM) for the complex.[2] Thus, in the presence of excess H_2O_2, the PS2.M−hemin complex clearly showed a time-dependent decrease of the Soret ($A_{404\mathrm{nm}}$). Also, in the initial phase of reaction (≤1 min) a general time-dependent increase in absorbance was observed over $A_{450-750\mathrm{nm}}$, except for a decrease at ~$A_{500\mathrm{nm}}$ (e.g., note the sharp changes at the high-spin visible bands at $A_{500\mathrm{nm}}$ and $A_{600\mathrm{nm}}$), see the thick line in Figure 4B. Such changes in the electronic spectrum have

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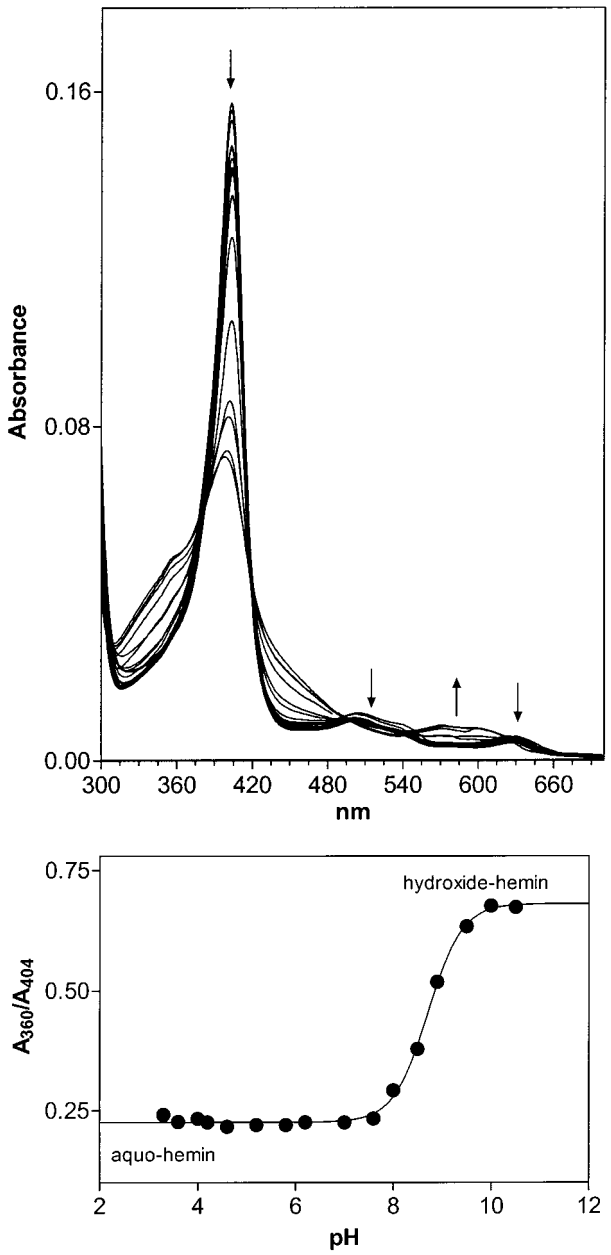

Figure 2. (A) pH-Dependent changes to the electronic spectra of the PS2.M−hemin complex in the pH range 3.5−10.5 measured at 20 °C. (B**)** Monitoring changes in A_{404nm} and A_{355nm} over the pH range 3.5−10.5 yields the pK_a for the acid−base transition of PS2.M−hemin complex. Filled circles represent the actual experimental data with curve fit to the $n = 1$ Henderson−Hasselbach equation.

previously been taken as evidence supporting the formation of compound I (i.e., a ferryl porphyrin π-cation radical, Fe^{IV}═O $Por^{+\bullet}$) in some hemoproteins and, in particular, HRP.[4,16] Thus, despite the PS2.M−hemin complex showing lower catalytic activity than HRP, we hypothesized that the complex was able to form an activated species that showed optical characteristics similar to compound I of HRP. However, the ongoing time-dependent decrease of the Soret absorption in solutions of the PS2.M−hemin complex and peroxide and the overall decay over $A_{450-750nm}$ at reaction times >1 min are also consistent with the significant heme degradation occurring under these conditions, e.g., Figure 4A. Importantly, heme degradation (and associated degeneration of the stable complex) may also contribute to the changes detected at $A_{450-750nm}$, thereby

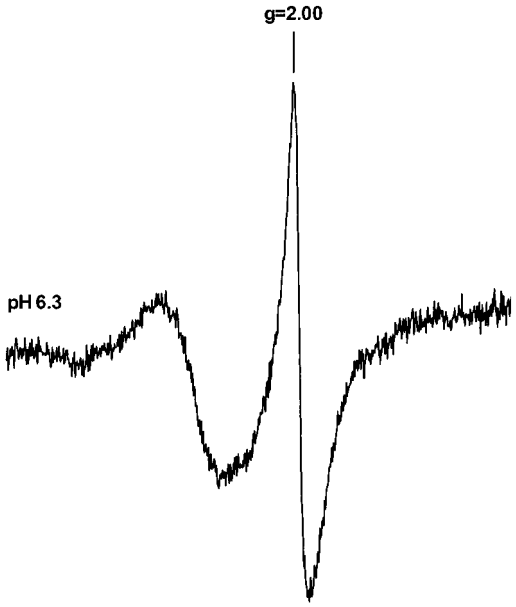

Figure 3. EPR spectrum of 1 mM PS2.M−hemin complex at pH = 6.2 and at 4.2 K, immediately after addition of 200 mM H_2O_2. Instrument parameters were as follows: microwave power 5 mW; modulation amlitude 1 G; and, scan range 450 G.

conflicting with the conclusion that the initial changes in $A_{450-750nm}$ result from the formation of a compound I-like species. That the organic radical produced in the reaction of PS2.M−hemin complex with peroxide was only detected at temperatures ≤77 K, while the changes to the electronic spectra were measured at significantly higher temperature (20 °C), also conflicts with the proposal that a compound I-like species exists under these conditions.

To probe further the possibility that a compound I-like species forms in the reaction of PS2.M−hemin complex and H_2O_2, we next assessed the effect of adding a reducing agent to the peroxidation reaction, Figure 5. Thus, H_2O_2 (200 μM or 1 mM) was added to both the PS2.M−hemin complex and to uncomplexed hemin (each ∼7 μM in heme), and the reactions were allowed to proceed for varying times before the addition of hydroquinone (H_2Q). H_2Q (final concentration 1 mM) and its oxidized product had negligible absorbance coefficients above 300 nm and thus did not interfere with these analyses. Addition of H_2Q ca. 5 min after the activation of hemin with H_2O_2 effectively reversed the initial decrease of the Soret band at A_{398nm}, nearly restoring the original Soret intensity, Figure 5A. However, after longer reaction times, only partial regeneration of A_{398nm} was observed, indicating that hemin was irreversibly modified to products that could not be rescued simply by adding H_2Q, Figures 5B and C. These latter observations are also consistent with similar studies reported for deuterohemin.[17]

Next we assessed the effect of H_2Q addition on the reaction of PS2.M−hemin complex with H_2O_2, Figures 5D−F. In the absence H_2Q, the initial rates of Soret decay were greater than those observed for uncomplexed hemin, consistent with the increased peroxidative activity of the PS2.M−hemin complex. In contrast to the result obtained from hemin alone, addition of H_2Q to the reaction of PS2.M−hemin complex and H_2O_2 after short reaction times (<1 min) did not result in the regeneration of the Soret band (data not shown). Although the addition of H_2Q at various times inhibited the time-dependent decrease of the Soret, no recovery of the original intensity was detected under any conditions, Figures 5D−F. Indeed, the A_{404nm} in

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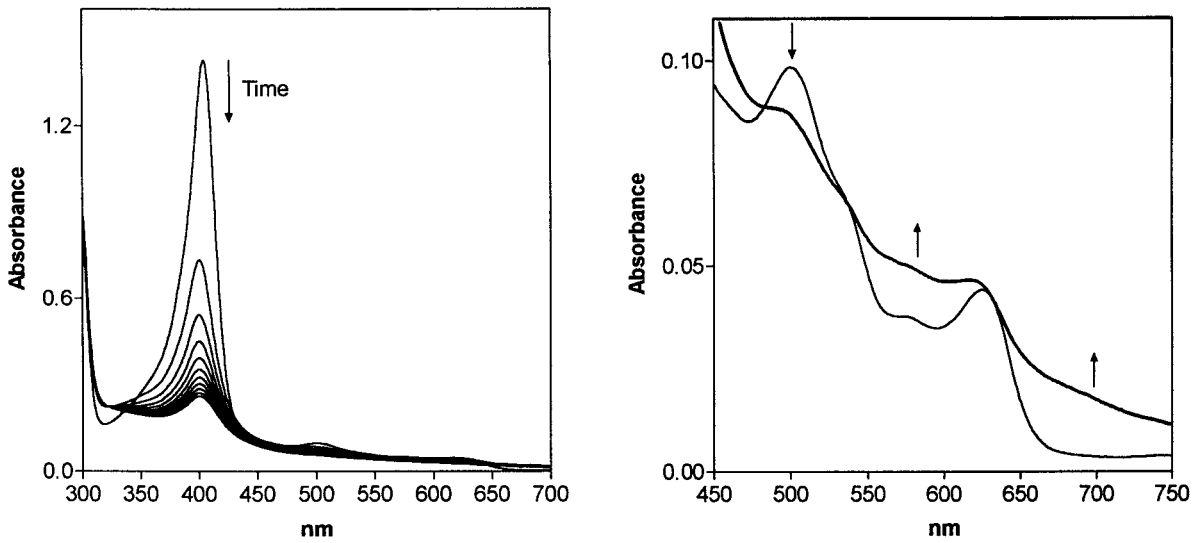

Figure 4. (A) Electronic spectral changes of the PS2.M−hemin complex (~7 μM) upon mixing with H_2O_2 (1 mM) at pH = 6.2 and 20 °C. Spectra were recorded before and after mixing, with a 1 min time interval. Arrow indicates the change in Soret intensity. (B) Rest state complex (thin line) and the initial increase in the absorbance envelope over $A_{450-750nm}$ (thick line). Arrows indicate the initial direction of spectral change after 1 min reaction time. After the accumulation of the initial spectrum, bleaching of the complex was observed and $A_{450-750nm}$ decreased across the entire envelope (reaction conditions as above for A).

reactions of PS2.M−hemin complex with H_2O_2 was only significantly affected when H_2Q was present in the mixture prior to the addition of peroxide, Figure 6. Overall, the substantive difference between uncomplexed hemin and the PS2.M−hemin complex suggested that in the absence of a reducing agent the reaction of the PS2.M−hemin complex with H_2O_2 resulted in irreversible modification of the nucleosides of PS2.M. That the reducing substrate inhibited the time-dependent hypochromicity of the Soret band under conditions where H_2Q was present prior to peroxide addition likely indicated an interaction of the peroxide with the heme iron through an inner sphere electron-transfer process.

EPR Spin Trapping Studies. In an attempt to further characterize the radical species detected by EPR upon reaction of H_2O_2 and the PS2.M−hemin complex, separate reactions were carried out in the presence of the spin trapping reagents DMPO and MNP. The spin trapping of radicals readily allows their detection at room temperature,[18] and the associated hyperfine coupling constants obtained from these stable radical adducts can be employed as diagnostic tools to assign identity to the primary radical species. When hydrogen peroxide was added to a PS2.M−hemin complex in the presence of DMPO (PS2.M−hemin complex:H_2O_2:DMPO ~1:1:10 mol/mol/mol), a weak EPR spectrum was obtained (data not shown). The DMPO adduct showed hyperfine coupling to a single nitrogen (a^N) and hydrogen (a^H_β) atoms with coupling constants a^N = 7.0 and a^H_β = 3.6 G, respectively: no other EPR signal was detected. These hyperfine couplings were similar to those reported[19−21] for the hydroxamic acid nitroxide DMPOX (5,5-dimethyl-pyrrolidone-2-oxyl), an oxidation product of DMPO. This product could be derived either from the decomposition of previously formed radical adduct (e.g., peroxyl or hydroxyl radical adducts DMPO−OOH or DMPO−OH, respectively) or by direct oxidation of DMPO via the peroxidase activity of the PS2.M−hemin complex.[19−21] However, no other EPR signals could be distinguished even when the concentration of DMPO was increased from 1 to 2 hundred-fold excess over the PS2.M−hemin complex. Importantly, authentic DMPO−OH was readily detected in separate reactions of iron(II) and H_2O_2 (at Fe(II):H_2O_2:DMPO ~1:10:10 mol/mol/mol) indicating that the lack of a DMPO−OH EPR signal in the reactions performed using PS2.M−hemin and peroxide was not due to an inability to detect the DMPO−OH adduct (data not shown). In addition, no carbon-centered methyl radicals (potentially generated from the degradation of trace DMSO by the putative hydroxyl radical) were detected in the presence of DMPO under any conditions.[22]

That DMPO was unable to trap the free radical observed on addition of H_2O_2 to the PS2.M−hemin complex does not necessarily rule out the formation of a radical species. In addition to the possibility that a radical adduct may be degraded to DMPOX,[19−21] other factors can affect the detection of radical adducts (e.g., stability of the spin adducts, the radical lifetime, and the rate of the trapping reaction). Moreover, it has been proposed that instability of DMPO adducts probably result from fast intramolecular electron-transfer reactions between adducts and some functionalities from the enzyme system and/or products of the reaction between heme and H_2O_2.[23] Therefore, we attempted to characterize the reaction of PS2.M−hemin complex with H_2O_2 by using the nitroso spin trap MNP. Nitroso spin-trapping agents have an advantage over nitrones (e.g., DMPO) in that adducts attach directly to the nitroxide nitrogen atom. This type of adduct can give rise to additional hyperfine splitting constants and may yield information on the type of primary free radical that is generated in the reaction mixture (e.g., primary, secondary, or tertiary carbon).

Thus, when peroxide was added to the PS2.M−hemin complex in the presence of MNP (PS2.M−hemin complex:

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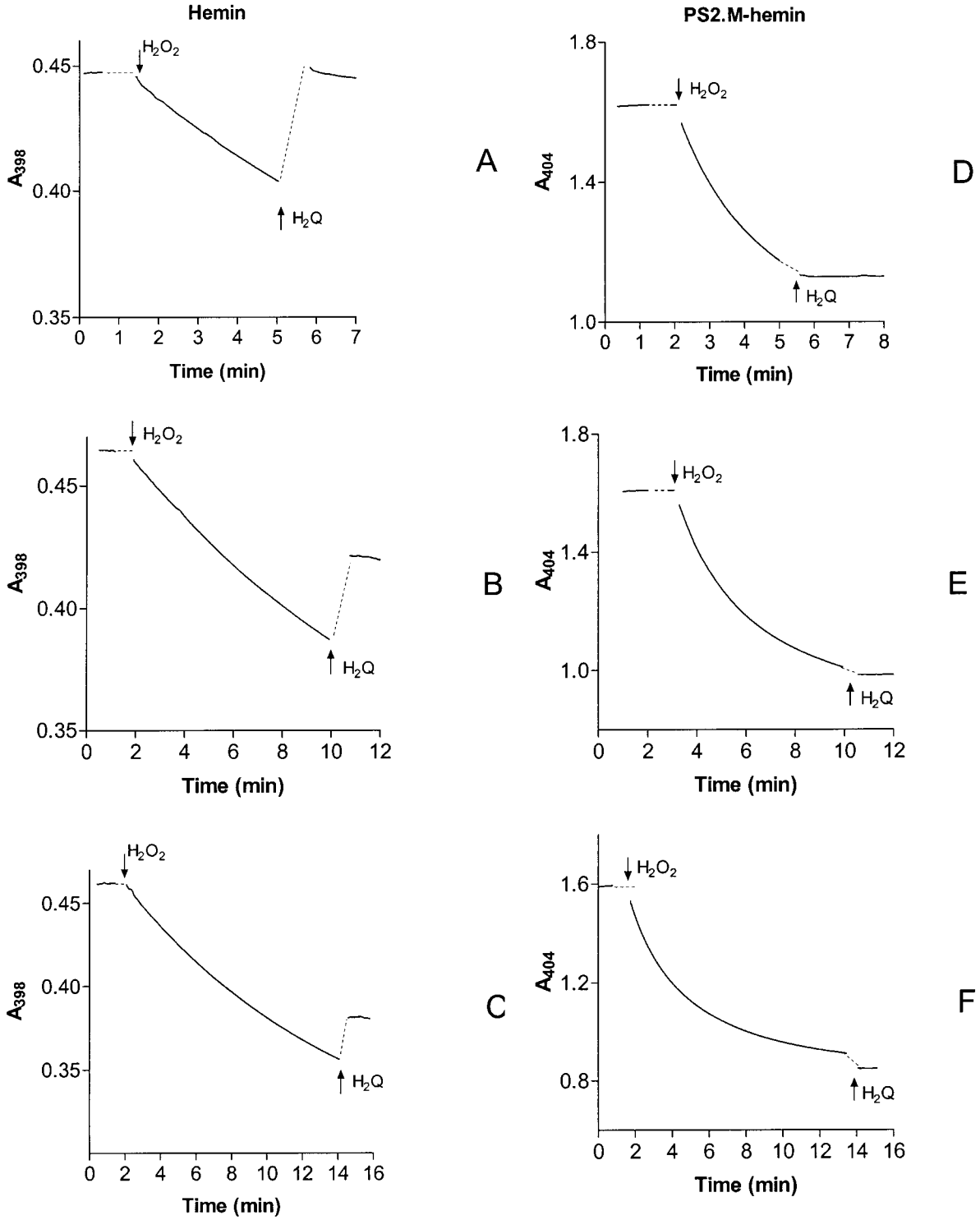

Figure 5. Effect of H_2Q (hydroquinone) addition on the Soret peaks of hemin after reaction with H_2O_2 for 3.5 (A), 8 (B), and 12 min (C), respectively, and of the PS2.M−hemin complex after reaction with H_2O_2 for 3.5 (D), 8 (E), and 12 min (F), respectively. Broken lines represent periods of cell manipulation.

H_2O_2:MNP ~1:1:20 mol/mol/mol), a weak triplet EPR signal was reproducibly detected, Figure 7A. The maximal concentration of the initial spin adduct was determined to be ~150 μM as judged by peak area comparison of the corresponding signal from a standard solution of TEMPO obtained under identical conditions. The EPR signal was broadened relative to that for the simple nitroxide TEMPO at similar concentrations (data not shown), indicative of the radical exhibiting restricted rotational motion on the EPR time scale. Such broadening is also suggestive of MNP trapping a radical on a relatively large molecule such as the PS2.M oligonucleotide. The formation of this immobilized nitroxide was completely dependent on the presence of the PS2.M oligonucleotide, H_2O_2, and MNP, Figures 7C−E. The addition of MNP to a mixture of H_2O_2 with hemin alone resulted in an EPR spectrum showing only background di-*tert*-butylnitroxide (DTBN, vide infra), which was significantly different from that obtained in the presence of the PS2.M, Figure 7F. Importantly, substituting the PS2.M−hemin complex with the complex of hemin with the control oligonucleotide (see Materials and Methods) only gave the sharp lines corresponding

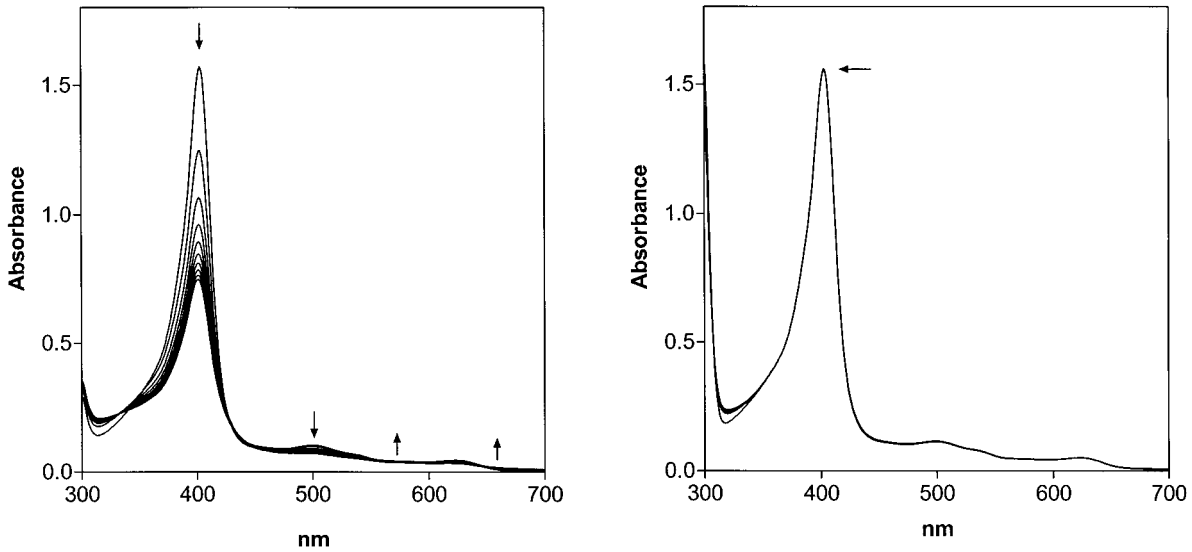

Figure 6. Effect of the presence of H_2Q (1 mM hydroquinone) on the reaction of the PS2.M−hemin complex and H_2O_2. UV−visible absorption spectra were accumulated as a function of time (every 1 min) following the addition of peroxide. Arrows indicate the initial scan and the direction of the absorbance change with time.

to the DTBN EPR signal upon incubation with H_2O_2 (data not shown). Together, these data are consistent with the MNP trapping a radical associated with the PS2.M oligonucleotide.

Simulation of the EPR signal obtained from the MNP adduct of the PS2.M−hemin complex/H_2O_2 mixture indicated the presence of a radical with hyperfine couplings to single nitrogen and hydrogen atoms, Figure 7B. The coupling constants of the MNP adduct were determined to be $a^N = 14.6 \pm 0.1$ G and $a^H = 1.8 \pm 6$ G. Simulations of the nitrogen hyperfine coupling showed no significant variation due to the peak broadening, whereas the smaller hydrogen coupling could be varied significantly over a range of up to 6 G without a loss in overall correlation of the simulation (R consistently remained ~0.85). As a result of this lack of sensitivity in the hydrogen coupling, only the nitrogen hyperfine value was used to determine the identity of the MNP adduct. MNP adducts of oxygen-centered alkoxyl- or peroxyl-radicals yield a^N values ≥ 27 G.[24,25] In contrast, a^N values of 14−16 G are typical of MNP adducts derived from secondary or tertiary carbon-centered radicals.[26,27] Therefore, the MNP adduct obtained from reaction of the PS2.M−hemin complex with H_2O_2 was assigned as a spin-trapped carbon-centered radical.

In an attempt to increase the concentration of the MNP adduct, the reaction mixtures of the PS2.M−hemin complex with H_2O_2 and MNP were incubated at 20 °C for various time intervals before analysis by EPR, Figure 8. After 30 s of incubation a broad three-line spectrum was detected, which was identical to that measured previously, Figure 8A. However, at longer incubation times a second sharp triplet EPR signal was detected, which increased in intensity in a time-dependent fashion, Figures 8B−E. Simulation of the EPR signal corresponding to the sharp triplet indicated a free radical with coupling to only a single nitrogen ($a^N = 17.0 \pm 0.2$ G, mean ± SD, $n = 3$) (data not shown). Other studies have previously reported that in the presence of excess MNP decomposition of the initial MNP adduct can lead to the formation of DTBN as a secondary radical product.[27,28] The reported coupling constant for DTBN is $a^N = 16.9$ G,[28] which is almost identical to the value observed here. Therefore, for reaction times <2 min, it is likely that we measured the EPR spectrum of an MNP radical adduct formed on the PS2.M oligonucleotide, whereas the DTBN spectrum was observed after longer incubation periods. Interestingly, immobilized MNP adduct(s) have been also observed when horseradish peroxidase (HRP) was incubated together with H_2O_2 and MNP, strongly suggesting that an MNP radical adduct could be formed on the peroxidase protein itself.[28]

Chemical Probing Studies. The detection of a broadened EPR signal likely localized on the complex itself indicates that oxidative damage might be localized to the PS2.M oligonucleotide. The PS2.M oligonucleotide folds in the presence of potassium ions to form a guanine-quadruplex (G-quadruplex) structure,[29,30] which is the proposed active folding required for hemin binding and for the enhanced peroxidase activity of the PS2.M−hemin complex. A proposed structural model (Y. Li and D. Sen, unpublished data) for the folded PS2.M oligonucleotide is shown in Figure 9. In this figure, the individual bases are shown and numbered from the 5′-position (see Materials and Methods section), with guanine nucleosides (G) highlighted as filled circles. The filled sphere represents a single potassium ion coordinated between the G-quartet layers II and III. The hatched square shows the plane of the bound hemin group intercalated between layers I and II.

To investigate the possibility that the carbon-centered radical localized on the PS2.M oligonucleotide may be involved in degradation of the oligonucleotide, we performed gel electrophoretic analysis of the PS2.M−hemin complex/H_2O_2 reaction mixture. Figure 10A shows a sequencing gel of the PS2.M oligonucleotide after reaction with H_2O_2, followed by piperidine treatment[30] (lanes 1−7) or without such treatment (lanes 8−13). The lack of significant cleavage in the absence of the piperidine treatment indicated that chemical modifications occurred mainly on the individual bases, probably as alkali-labile lesions, without breakage of the deoxyribose-phosphate backbone. However,

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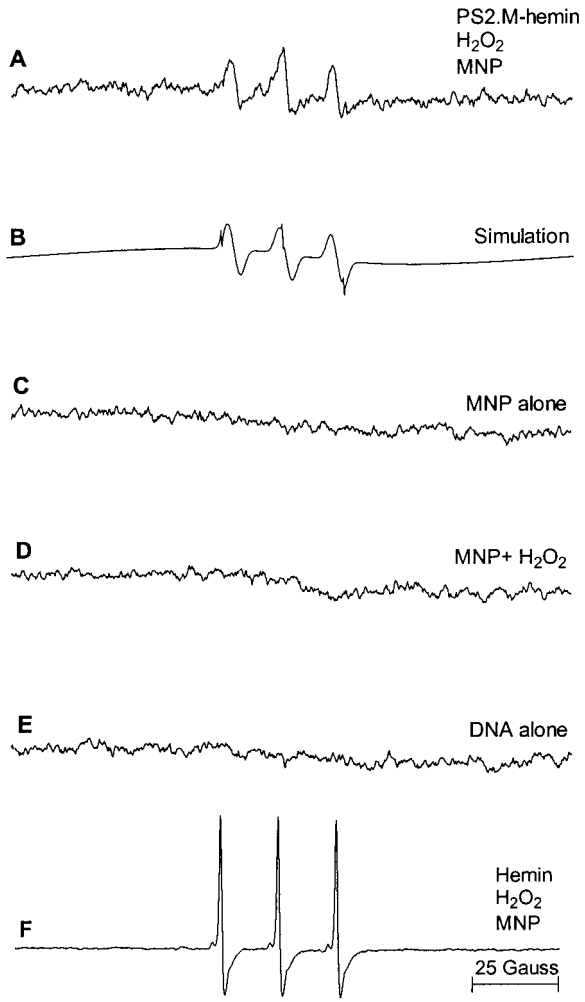

Figure 7. EPR spectra recorded following the addition of H_2O_2 to the PS2.M−hemin complex in the presence of MNP. (A**)** EPR spectrum of 1 mM PS2.M−hemin complex incubated with 1 mM H_2O_2 in the presence of 20 mM MNP. (B) Computer simulation of A gave a triplet with $a^N = 17.1$ G (line width 0.2 G) and a triplet with $a^N = 14.7$ G (line width 1.4 G). (C) As in A, but without the PS2.M−hemin complex and H_2O_2. (D) As in A, but without the PS2.M−hemin complex. (E) As in A, but without MNP and H_2O_2. (F) As in A, but without PS2.M oligonucleotide. The steady-state ratio of MNP adduct to PS2.M−hemin determined immediately after mixing was ∼0.15 mol/mol, indicating that only a fraction of the available complex is damaged upon addition of peroxide. Instrument parameters were as follows: microwave power 10 mW; modulation amplitude 1.0 G; scan range 160 G.

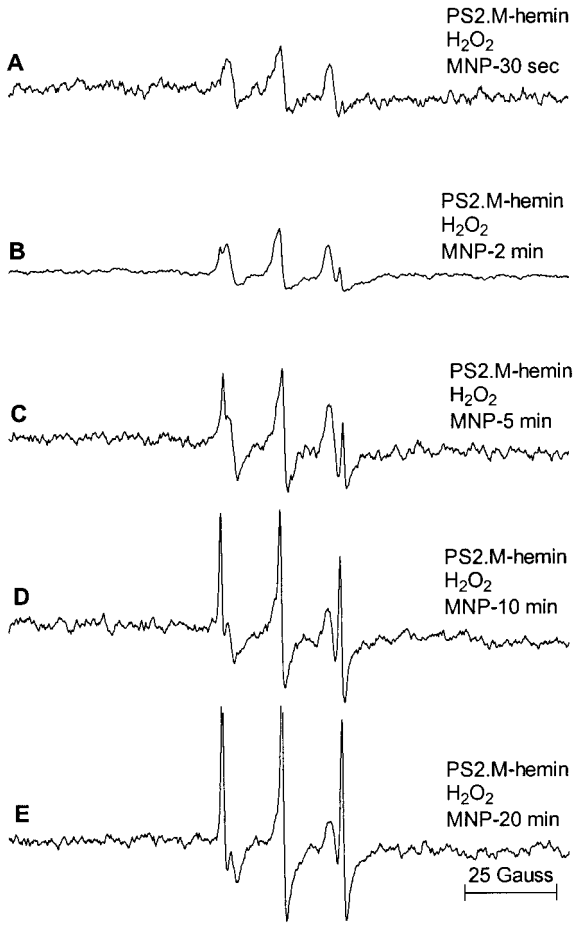

Figure 8. EPR spectra recorded from the reaction of H_2O_2 with the PS2.M−hemin complex in the presence of MNP after increasing incubation times. (A) 30 s; (B) 2 min; (C) 5 min; (D) 10 min; (E**)** 20 min. Reaction conditions and instrument parameters were as for Figure 7.

when the samples were treated with 10% piperidine, specific sites of phosphodiester cleavage were observed. These bands corresponded to specific guanine nucleosides of the PS2.M sequence. The guanine-specific cleavage was dependent on the presence of hemin and the folded conformation of the oligonucleotide (i.e., in the presence of potassium required for the G-quadruplex folded structure). Only a weak background cleavage was observed in the absence of hemin or with the denatured "PS2.M−hemin complex" (i.e., in the absence of potassium ions). Additionally, after H_2O_2 treatment the PS2.M−hemin complex treated with piperidine was predominantly cleaved at *specific* guanine sites, Figure 10A, lanes 4−6. This cleavage pattern was highly specific at hemin/oligonucleoside ratios $r_0 \leq 0.8$ but became less distinct and increasingly similar to a general background cleavage at higher r_0. These results strongly suggested that the bound hemin interacted with specific guanine bases of PS2.M. Strand scission events occurred mostly at the G_{10}, G_{12}, and G_1/G_3 residues in the PS2.M sequence. Of special interest was the fact that G_{10} was a hot-spot for cleavage, Figure 10A, lane 6. Also, G_5 showed some preferential reactivity, but without any substantial difference for different hemin/DNA molar ratios, Figure 10A, lanes 4−6. The uniformity of G_5 reactivity, even in the presence of excess hemin, may indicate a different mechanism for the degradation of G_5, as compared to G_{10}, G_{12}, and G_1/G_3.

To ascertain that the observed guanine-specific cleavage was not simply due to the exposure of certain guanines to H_2O_2 in the solvent, we probed the G-quadruplex structure of the PS2.M oligonucleotide with and without bound hemin, using methylation protection assays, Figure 10B. The N7 positions of guanine bases in single-stranded (unfolded) as well as double-helical DNA are susceptible to methylation by dimethyl sulfate (DMS). However, guanines participating in G-quartet formation are protected from methylation.[31,32] Such an analysis takes advantage of the labilization of the base−sugar glycosidic bond upon alkylation of the guanine N7, which allows specific base-catalyzed DNA strand scission at the alkylated guanine. Thus, a DMS "footprinting" experiment probes the accessibility of specific guanine N7 positions. A comparison of lanes 2−3 with 4−5 (at [DMS] = 0.2%, v/v) or lanes 7−8 with lanes 9−10 (at [DMS] = 0.3%) showed that even in the absence of bound hemin all the guanines of the PS2.M sequence were protected

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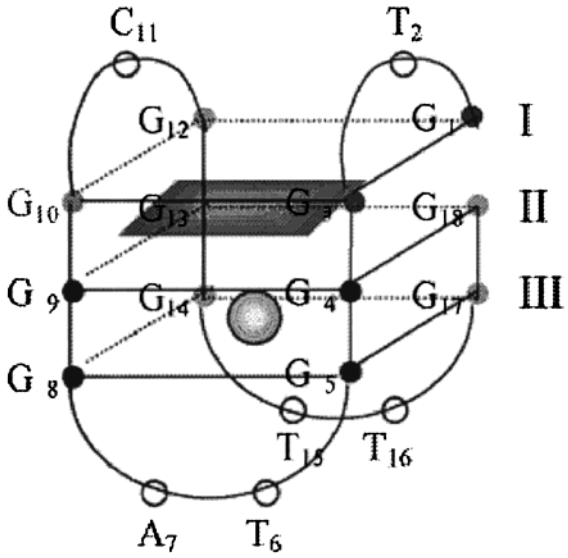

Figure 9. Proposed guanine−quadruplex model for the folded and catalytically active structure of the PS2.M−hemin complex (deoxyribozyme). The large solid sphere represents a potassium ion coordinated between the G-quartet layers II and III, and the hatched square represents the plane of the bound hemin, intercalated between layers I and II. The position of the hemin is shown schematically, since no detailed information is available about the extent of its intercalation between the G-quarets. Numbering of nucleic bases is in order from the 5′-position of the PS2.M oligonucleotide (see Materials and Methods). The guanines (G, indicated by small filled circles) that are likely to be in contact with the bound hemin are G1, G3, and G10.

from cleavage and therefore likely involved in forming G-quartets. There were some guanines, however, such as G_{10}, G_{12}, G_4, and G_5, which even in the context of a properly folded PS2.M showed a slight reactivity, Figure 10B, lanes 9−10. This might indicate a degree of flexibility in the folded PS2.M structure at these positions and the possibility that some guanines deviate from the standard G-quartet geometry (such as buckling out from the G-quartet plane−"wobbling" guanines). It is not an uncommon observation that one or more guanines in a G-quartet "wobble" with respect to the plane shared by the other guanines.[33−36] In the absence of potassium ions, the reaction of PS2.M with DMS simply afforded the Maxam−Gilbert guanine sequence ladder,[31] Figure 10B, lanes 2−3 and 7−8. From these data it was also apparent that when hemin was bound, certain guanines in PS2.M appeared to gain some "additional" protection from methylation. Lanes 9−10 of Figure 10B show that G_{10} and G_{12} were more protected in the presence of hemin, while G_5 and G_4 did not show significant changes. This observation suggests that G_{10} and G_{12} may be involved in hemin binding and that subtle changes in the overall tertiary structure of PS2.M are induced following the binding of hemin. Overall, similar results were obtained independent of the buffer systems used in the preparation of oligonucleotide stock solutions, e.g., compare corresponding experiments performed in MT (10 mM MES, 0.1 mM EDTA, pH 7.8) buffer and TE buffer (each containing 0.2% DMS), Figure 10B, lanes 2−5 and 11−13, respectively.

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Discussion

Nature of the Axial Ligands for PS2.M-Bound Hemin. In this study, EPR and chemical probing studies have afforded insights into the nature of the active site of an oligonucleotide−hemin complex that exhibits enhanced peroxidase activity compared with hemin alone. Analysis of the EPR spectrum of the PS2.M−hemin complex revealed that the hemin cofactor had an axial high-spin ferric iron when bound to the DNA oligonucleotide. Moreover, the EPR spectra from solutions of the PS2.M−hemin complex were remarkably similar to that of ferric myoglobin, which has been reported to have a hexacoordinated heme iron with a histidine residue and a water molecule as the fifth and sixth ligands, respectively.[11−13] These results are consistent with our previous report that found that the PS2.M−hemin complex exhibited an electronic spectrum with near identical maxima to those of ferric globins.[2] These optical and EPR data together with our determination of a pK_a of ∼8.7 for the PS2.M−hemin complex strongly support the conclusion that one of the axial coordination positions of the heme iron is occupied by a water molecule.

As noted above, a minor rhombic distortion was detected in the high-spin EPR spectrum of the PS2.M−hemin complex at ∼4 K. Rhombic distortions to the axial symmetry of high-spin ferric complexes can be explained in terms of a degree of in-plane distortion exerted by the fifth and/or sixth axial ligand(s) on the heme iron.[37] In this regard, it is notable that a recombinant metmyoglobin variant that lacked the distal His residue (E7, His64 replaced by Gln; H64Q) exhibited an EPR spectrum with the same fundamental characteristics as that of the PS2.M−hemin complex.[12] On the basis of electronic and EPR spectra, it was concluded that the H64Q variant exists as a mixture of a five- and six-coordinate heme (the latter as the major species). The electronic spectrum of this Mb variant did, however, differ in some respects from that of the PS2.M−hemin complex. The former had a smaller *Soret* extinction coefficient and a weak shoulder near 390 nm, suggesting the presence of a mixture of five/six-coordinate heme species. In contrast, the UV−visible spectrum of the PS2.M−hemin complex (measured at 20 °C) appeared to be almost identical to that of the wild-type metmyoglobin, which is known to be solely six-coordinate and high-spin. Our chemical probing studies have suggested that specific guanine(s) within the PS2.M sequence may interact with the heme. The folded structure of the PS2.M oligonucleotide (see Figure 9 for proposed structure) in which such guanine(s) appear seems to be characterized by a degree of flexibility possibly accommodated by a "wobbling" of certain guanines within the guanine base quartets. It is also conceivable that an equilibrium may exist between two (or more) conformations of the PS2.M−hemin complex in which specific guanine(s) approach closer to the heme iron, thus influencing the axial symmetry of the high-spin ferric center.

Reaction of the PS2−Hemin Complex with H_2O_2. Addition of H_2O_2 to the PS2.M−hemin complex resulted in significant changes to its EPR spectrum compared with that of the resting state complex, cf., Figures 2 and 1B. Overall, the rather complex line shape of the EPR signal at $g = 1.995$ suggested that the radical might be derived from more than one component, with

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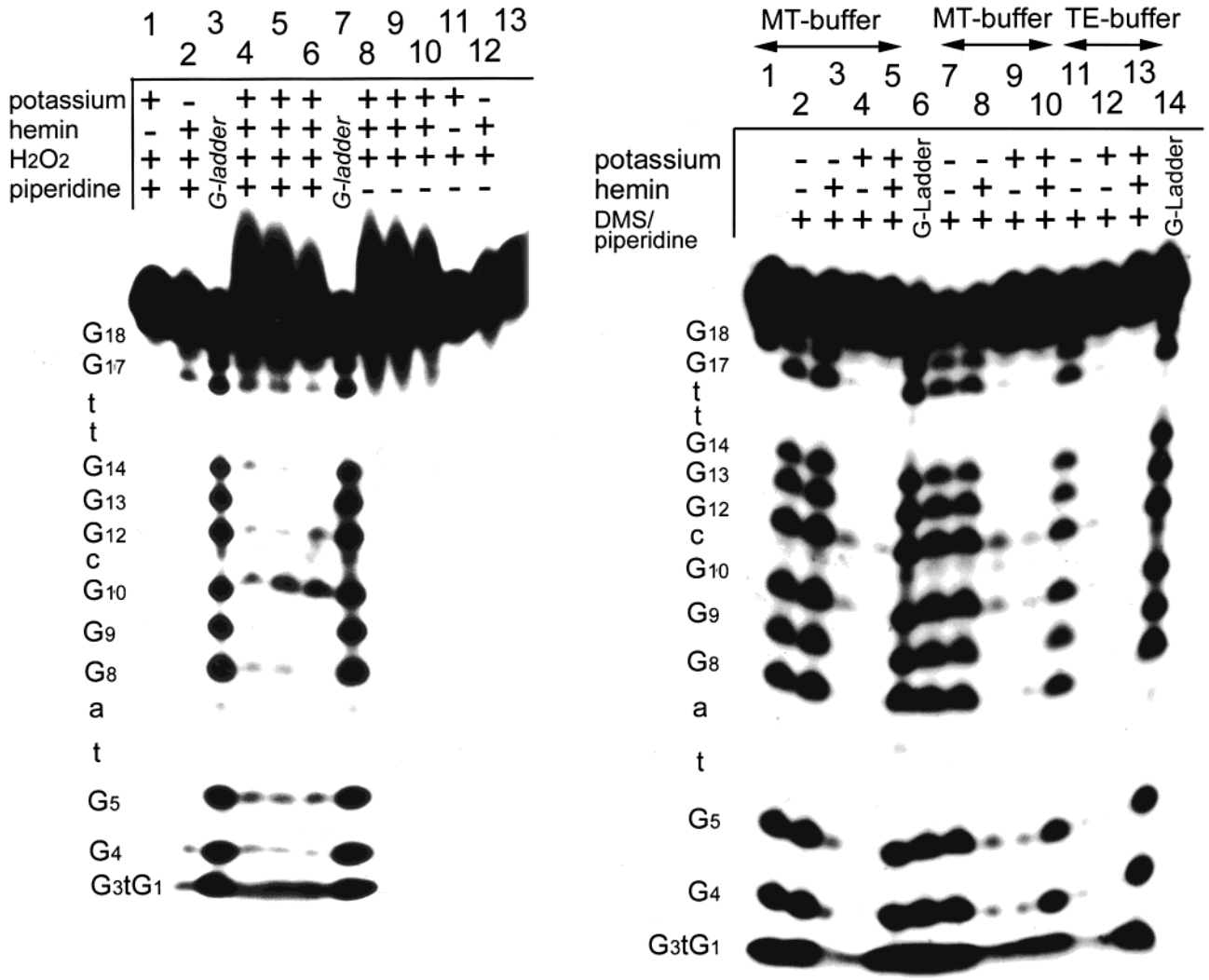

Figure 10. (A) A DNA sequencing gel showing guanine residue damaged following reaction of the PS2.M−hemin complex with H_2O_2 (see Materials and Methods). Lane 1: a sample without hemin. Lane 2: sample without potassium, $r_0 = 0.8$. Lanes 3, 7: guanine ladder of PS2.M. Lanes 4−6: The PS2.M−hemin complex, with hemin to PS2.M at r_0, 5, 2, and 1, respectively. Lanes 8−10: same as lanes 4−6, but the samples were not treated with piperidine. Lanes 11, 12: same as lanes 1, 2, but without piperidine treatment. Lane 13: the intact deoxyoligonucleotide PS2.M. (B) Guanine-methylation protection patterns of the folded PS2.M oligomer and of the PS2.M−hemin complex. Lane 1: intact deoxynucleotide PS2.M. Lanes 2, 3: G-specific cleavage for PS2.M and PS2.M−hemin under unfolded (denaturing) conditions ($[K^+] = 0$; [dimethyl sulfate] = 0.2%). Lanes 4, 5: methylation protection patterns for folded PS2.M and for PS2.M-aptamer hemin, respectively ([dimethyl sulfate] = 0.2%). Lanes 6, 14: guanine ladder for PS2.M. Lanes 7−10: same as above but with [dimethyl sulfate] = 0.3%. Lanes 11−13: same as above but in TE buffer instead of MT buffer.

similar g values (∼2) but different line widths. It is however unlikely that the primary products of the reaction of PS2.M−hemin with H_2O_2 are a PS2.M-Fe^{IV} species (i.e., compound II, which contains an EPR-silent low-spin ferryl iron) and the potently oxidative OH• radical. This is supported by our inability to trap the OH• with DMPO to yield DMPO−OH.[18,38] Importantly, we cannot rule out that DMPO−OH may form initially and subsequently be converted to DMPOX, which we readily detect by EPR, or that trace DMSO may compete with the spin trap for OH•.[39] However, as DMPO failed to yield DMPO−OH in reactions of PS2.M−hemin and H_2O_2 (even under conditions of increased trapping efficiency) we conclude that OH• is not produced in this reaction. That methyl radicals derived from OH•-mediated fragmentation of DMSO were not detected in the reactions containing either DMPO or MNP is also consistent with the idea that OH• radicals are not generated in this reaction system. Alternately, the latter may also indicate that the residual DMSO in the buffer was negligible and unable to compete with the added spin trap. Additionally, the presence of a large excess of H_2O_2, required to initiate significant spectral changes, prohibits the detection of a putative compound II.

The singlet EPR signal exhibited by the PS2.M−hemin complex in the presence of H_2O_2 is similar to that attributed to compound I of HRP.[5,40,41] Addition of H_2O_2 to HRP is reported to yield an asymmetric EPR signal, ∼15 G wide, with a g value of 1.995,[6] and these data compare well with those obtained for the PS2.M−hemin complex/H_2O_2 mixture, Figure 2. Distinct from compound II, HRP compound I possesses an odd number of electrons and may, therefore, be detected by EPR. Similar

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to our observation of a $g \sim 2$ radical in the reaction of the PS2.M−hemin complex/H_2O_2 mixtutre, the EPR signal of HRP compound I is also best detected at temperatures <20 K. Coupling to the iron center of HRP compound I results in short relaxation times that broaden the EPR signal to baseline at room temperature.[42] Therefore, it is conceivable that in the reaction of the PS2.M−hemin complex and H_2O_2 the radical detected here by EPR at low temperature may be a compound I-like radical (i.e., an oxo-ferryl porphyrin π-cation radical).

Despite the above EPR evidence supporting the formation of a compound I-like species in the reaction of the PS2.M−hemin complex and H_2O_2, other lines of evidence obtained here also argue against such an assignment. First, the $g \sim 2$ radical detected by low-temperature EPR showed extremely short relaxation times and was not detectable at temperatures >77 K. Therefore, the initial increase in $A_{450-750nm}$ detected at 20 °C is unlikely related to the formation of this radical. Second, the general decay in absorbance across the optical envelope observed upon reaction of the PS2.M−hemin complex and H_2O_2 indicates significant degradation of the heme group. Such degradation may contribute to the transient increase in absorbance initially detected at $A_{450-750nm}$ upon addition of H_2O_2. Furthermore, the observation that the addition of a reducing agent immediately after peroxide failed to regenerate the Soret intensity also argues strongly against the formation of a putative compound I in reaction mixtures of the PS2.M−hemin complex and H_2O_2. Next, the similarity between the $g \sim 2$ radical detected upon reaction of the PS2.M−hemin complex and H_2O_2 and the EPR signal of HRP compound I is not necessarily restricted to this protein. For example,[43] the EPR signal detected upon irradiation of salmon testes DNA is attributed to a guanine radical cation that exhibits similar EPR characteristics to the radical species detected in mixtures of the PS2.M−hemin complex and H_2O_2. In addition, irradiation of an oxidation product of guanine (7,8-dihydro-8-oxo-2′-deoxyguanosine) also yields a radical cation with a near identical EPR signal to that $g \sim 2$ radical detected here.[43] Finally, our results from the series of spin-trapping experiments with MNP indicate that a radical(s) likely forms on the PS2.M nucleotide and not on the heme group. Thus, the EPR signal we detect at low temperatures in reactions of the PS2.M−hemin complex and H_2O_2 may just as likely be derived from guanine as a compound I-like species. Collectively, these data prohibit a definitive assignment of identity of the $g \sim 2$ radical detected in the reaction of the PS2.M−hemin complex and H_2O_2 as a porphyrin π-cation radical.

Oxidative Damage to PS2.M in the Reaction of the PS2.M−Hemin Complex and H_2O_2. Chemical probing studies provided evidence that the PS2.M oligonucleotide suffered oxidative damage following reaction with H_2O_2. The greatest damage occurred at specific guanines (G_{10}, G_{12}, and G_1/G_3) in the PS2.M sequence. The release of guanine (and, to a lesser extent, of other free bases), which generally attends polynucleotide degradation brought on by oxidizing agents, can occur via the generation of diffusible oxygen radicals.[44,45] Such species might be generated from the peroxidation reaction of the PS2.M−hemin complex (as well as of hemin itself) following activation by peroxide. However, it is generally the case that diffusible oxygen-centered radicals (such as $OH^{\bullet}$) cause nonspecific DNA cleavage, i.e., at all accessible nucleotides, generating a nonspecific degradation pattern.[46] By contrast, the PS2.M−hemin complex showed a highly *specific* pattern of oxidative damage that was dependent on the binding of hemin to the folded PS2.M. In support of this, metalated DNA-damaging agents [e.g., Ni(III)-containing DNA-reactive molecules of the bleomycin family] have been shown to modify only those guanine residues in a DNA quadruplex whose N7 positions were coordinated axially to the metal center.[47] In other words, if an oxidant were to be generated in the vicinity of the heme group, then it is likely to damage those nucleosides that are in close proximity to it (including the relevant guanine bases of a G-quadruplex structure).[48−50]

The enhanced reactivity to the oxidant observed at specific guanine nucleosides is consistent with the substantially lower reduction potential of guanine compared with other DNA nucleosides[51,52] as well as with guanine-specific base oxidations that have been reported following exposure of single- and double-stranded DNA to various oxidizing species.[46,53−55] It has recently been reported that GG doublets and GGG triplets act as site specific traps in the long-range oxidative modification of DNA by one-electron oxidants through the double helix.[56] It is, therefore, conceivable that in the PS2.M oligonucleotide the preferred strand scission at G_{10} might be due to proximity to or even a specific axial interaction of this guanine residue with an oxidant produced in the proximity of the complexed heme group. In contrast, the hemin-independent cleavage at G_5 is probably due to oxidation by diffusible radicals. Since activation appears to take place only on the axial sites of the bound hemin, it is further conceivable that G_{10} might coordinate (directly or indirectly) the hemin iron within the potassium-folded, active G-quadruplex structure of PS2.M. Consistent with this proposal, it has been shown that nitrogens within guanine bases can indeed occupy an axial coordination position in metallo-complexes, such as in the case of the aforementioned bleomycin−nickel(III) complex.[46]

Conclusion and Biological Significance. EPR investigation of the PS2.M−hemin complex has shown that the heme iron is a hexacoordinated high-spin species in which the axial ligands are a water molecule and possibly a "wobbling" guanine. EPR analysis of the reaction of the PS2.M−hemin complex with H_2O_2 revealed the formation of an organic radical that exhibited a simple singlet EPR signal. Electronic absorption spectroscopy together with EPR spin-trapping experiments indicated that this species was unstable and led to the formation of a carbon-centered radical adduct, most likely on the guanine base(s) of the PS2.M oligonuclotide. In support of this, chemical-probing studies have shown that PS2.M underwent preferential oxidative cleavage at specific guanines. Such cleavage suggested a specific

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interaction (conceivably axial coordination) of these guanine(s) with the heme iron. Therefore, on the basis of the spectroscopic and chemical probing studies, we propose that coordination of a "buckled" guanine(s) to the heme iron, in conjunction with other features of the PS2.M active site, may contribute to its superior peroxidative activity.

PS2.M−hemin (and its RNA counterpart, rPS2.M−hemin)[3] thus represent a nucleic acid−hemin complex with a demonstrated peroxidase activity, much as a naturally occurring enzyme, such as HRP, is a complex of a hemin moiety and a polypeptide. The "RNA World" hypothesis[57] for the origin of life postulates that since RNA (and RNA-like polymers, but not proteins) is capable of both genetic and catalytic functions, primordial "life" may have consisted of RNA molecules capable of self-replication and also of catalysis of the constituent reactions of metabolic pathways. From this perspective PS2.M−hemin may be viewed as a prototype of a nucleic acid enzyme that utilizes hemin to catalyze oxidative reactions.

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