Potassium Monopersulfate and a Water-Soluble Manganese Porphyrin Complex, [Mn(TMPyP)](OAc)$_5$, as an Efficient Reagent for the Oxidative Cleavage of DNA†

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ABSTRACT: Reported studies indicate that the association of potassium monopersulfate with [Mn(TMPyP)](OAc)$_5$, a water-soluble manganese porphyrin complex, leads to an efficient reagent for the oxidative cleavage of DNA. Single-strand breaks (SSBs) are observed on double-stranded DNA at manganese porphyrin concentrations as low as 0.5 nM with a short incubation time of 1 min. The number of SSBs linearly varies with the concentration of the manganese complex, and potassium monopersulfate is at least 3 orders of magnitude more efficient as oxygen source than hydrogen peroxide. Cleavage efficiency is optimal in the pH range 7.5–9.0 for a NaCl concentration between 80 and 150 mM or for a $MgCl_2$ concentration of 10 mM. At very low manganese porphyrin concentration and by increasing the incubation time a catalytic cleavage activity of the complex is evidenced: up to 5 SSBs per manganese porphyrin are observed. The high cleavage activity of the monopersulfate–manganese porphyrin system makes it a good candidate for DNA-footprinting experiments.

Metalloporphyrin complexes have been largely used in the chemical modeling of monooxygenases during the past decade (McMurry & Groves, 1986; Meunier, 1986; Tabushi, 1988). The successful association of various oxygen atom donors (iodosylbenzene, sodium hypochlorite, hydrogen peroxide, alkyl hydroperoxides, or potassium monopersulfate) to synthetic metalloporphyrins led to a better understanding of the chemistry of metal–oxo species and their implications in monooxygenases, peroxidases, and catalase. In addition, it has been possible to use two of these oxygen surrogates, iodosylbenzene and potassium hydrogen persulfate, to evidence the possible role of high-valent iron–oxo entities in the bleomycin–iron mediated DNA breaks (Hecht, 1985; Stubbe & Kozarich, 1987; Pratviel et al., 1986, 1989).

Besides the use of metalloporphyrin complexes as biomimetic catalysts in the oxygenation of hydrocarbons, these molecules have been largely employed in biological studies. Natural or synthetic metalloporphyrins have been investigated as tumor-localizing labels for the detection of cancer cells. These studies include the use of radiolabeled metalloporphyrins to delineate tumors (Foster et al., 1985) and of paramagnetic metalloporphyrins as tumor-specific contrast agents for magnetic resonance imaging (Megnin et al., 1987).

Interactions of porphyrins and metalloporphyrins with nucleic acids have also been extensively studied during the past 10 years. The DNA affinity of these flat tetrapyrrolic macrocycles is high: binding constants are in the range 10^5–10^6 M^{-1}. The size and charge of peripheric groups attached at the meso position of tetraarylporphyrins and the presence or the absence of axial ligands on the inserted metals determine the interaction site on the polynucleotide (Banville et al., 1986; Carvlin & Fiel, 1983; Pasternack et al., 1983a). *meso*-Tetrakis(4-*N*-methylpyridiniumyl)porphyrin ($TMPyPH_2$)[1] is an intercalating agent as are its metalated derivatives, like Cu(II), Ni(II) or Pd(II), whereas Mn(III) and Fe(III) complexes of $TMPyPH_2$ bind to the outside of DNA with a significant preference for A-T sequences compared to G-C sequences (Pasternack et al., 1983b; Ward et al., 1986a).

DNA damages can be created by porphyrin class derivatives (i) by photoactivation of hematoporphyrin-type molecules (Moan, 1986; Kessel, 1984) or *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin and its zinc analogue (Praseuth et al., 1986) or (ii) by oxidative activation of iron- or manganese–porphyrin complexes, this latter category of damages being less documented than the first one. The oxidative activation of metalloporphyrins can be performed by molecular oxygen in the presence of a reducing agent (Fiel et al., 1982; Aft & Mueller, 1983) or iodosylbenzene (Ward et al., 1986b). Metalloporphyrins linked to intercalating agents (Lown et al., 1986; Hashimoto et al., 1986) or oligonucleotides (Le Doan et al., 1987) are also able to cleave DNA.

In this paper, we report the efficient cleavage of DNA by a cationic manganese porphyrin, [*meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrinato]manganese(III) pentaacetate, [[Mn(TMPyP)](OAc)$_5$], in association to potassium monopersulfate, $KHSO_5$ [for a preliminary paper, see Fouquet et al. (1987)]. Single-strand breaks (SSBs) are created on duplex DNA at manganese porphyrin concentration ranging from 0.5 to 250 nM in the presence of 10 μM $KHSO_5$ within 1 min. Such an efficient DNA cleaver might have some future in DNA-footprinting experiments. All experimental conditions are detailed in the present paper.

Experimental Procedures

Materials. Electrophoresis grade agarose was purchased from Bio-Rad Laboratories; phage ϕX174 supercoiled DNA

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[1] Abbreviations: $TPyPH_2$, *meso*-tetrapyridylporphyrin; TPyP, dianion of $TPyPH_2$; $TMPyPH_2$, *meso*-tetrakis(4-*N*-methylpyridiniumyl)-porphyrin; TMPyP, dianion of $TMPyPH_2$; Mn-TMPyP, shortening of [Mn(TMPyP)](OAc)$_5$; $KHSO_5$, potassium hydrogen persulfate or Oxone; SSB, single-strand break; *S*, mean number of SSBs per DNA molecule; DSB, double-strand break; bp, base pair; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DMF, dimethylformamide; DMSO, dimethyl sulfoxide.

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(500 μg/mL; storage buffer, 10 m Tris-HCl, pH 7.4, 5 mM NaCl, 0.1 mM Na_2EDTA) and the restriction endonuclease *Pst*I were obtained from Bethesda Research Laboratories. Tris, Hepes, Pipes, arsenic acid, cacodylic acid, and disodium and monopotassium phosphates for the preparation of the different buffers were from Sigma. Potassium monopersulfate is the triple salt $2KHSO_5$, $KHSO_4$, K_2SO_4, known under the trademark Oxone and was from Alfa-Ventron. Amberlite IRN78 resin was from Prolabo, and ion-exchanger I and plates RP-18 $F_{254}S$ were from Merck. All other common chemicals were purchased from Prolabo or Merck; water used for all the solutions was distilled twice.

Spectrometric Methods. NMR spectra were recorded on a Bruker (250 MHz) spectrometer operated in the Fourier transform mode (FT). UV–visible spectra were obtained with a Varian Cary 219 spectrophotometer.

Synthesis of the Cationic Porphyrins $TPyPH_2$, $TMPyPH_2$, and Mn-TMPyP. $TPyPH_2$ and $TMPyPH_2$ were synthesized according to a previously reported method (Pasternack et al., 1972). The manganese (Mn^{III}) cationic porphyrin was obtained as follows: 150 mg (242 μmol) of $TPyPH_2$ was refluxed in 20 mL of DMF with 10 equiv of $Mn(OAc)_2 \cdot 4H_2O$. A 10-fold excess of 2,4,6-collidine was added to capture released protons. The reaction was monitored by UV–visible spectroscopy and was stopped when the Soret band shift was complete (from 420 to 462 nm). After the solvent was removed under vacuum, the crude product was washed with cold water and chromatographed on a dry neutral alumina column (25 × 2.5 cm) with $MeOH/CH_2Cl_2$ (3/97). The metalloporphyrin was methylated in 15 mL of DMF with a large excess of methyl iodide (20 equiv) for 3 h at 45 °C. Iodide anions were exchanged by acetate ions over an Amberlite IRN78 resin (pretreated by 2 M acetic acid) in MeOH solution, and finally the metalloporphyrin was precipitated by addition of a mixture $MeOH/Et_2O$ (1/4) giving 225 mg of Mn-TMPyP, pentaacetate salt (yield 90%). The purity of this product was checked by thin-layer reverse-phase chromatography [plates RP-18 $F_{254}S$, elution with $HCOOH/MeOH/H_2O$ (1/4/5)]: $Rf = 0.81$; no traces of $TPyPH_2$ ($R_f = 0.30$) or its methylated form $TMPyPH_2$ ($Rf = 0.33$) were detected. Visible spectrum of Mn-TMPyP (H_2O) λ ($\epsilon_M \times 10^{-3}$, M^{-1} cm^{-1}): 462 (101), 560 (10.5), 588 (4.6) nm.

DNA Cleavage Experiments. (*a*) *Reaction Conditions for DNA Cleavage and Agarose Gel Electrophoresis.* φX174 DNA digestion conditions for standard experiments were as follows: commercial DNA was diluted to 50 μg/mL in phosphate buffer (10 mM, pH 7.5) or in double-distilled water in some experiments when mentioned. The reaction involved 5 μL of φX174 DNA (50 μg/mL, 74.8 μM in base pairs), 5 μL of 50 mM phosphate buffer, pH 7.5, 5 μL of metalloporphyrin in a solution of 50 mM phosphate buffer, pH 7.5, and 5 μL of $KHSO_5$ diluted in the same buffer. Preincubation of DNA and metalloporphyrin was performed for about 15 min, and the digestion time in the presence of $KHSO_5$ was 1 min at 20 °C.

Metalloporphyrin-mediated DNA cleavage was monitored by agarose gel electrophoresis. Reactions were quenched by 5 μL of a "stopping reagent". The stopping reagent consisted of 250 mM Hepes buffer, pH 7.4, containing 75% glycerol and 0.05% bromophenol blue. We have checked that 50 mM Hepes buffer, pH 7.4 (final concentration in the quenched reaction samples), inhibits completely the cleavage reaction. Similar inhibition could be obtained with Pipes buffer at the same molarity and pH. Reaction mixtures were then run in 0.8% agarose slab horizontal gel containing ethidium bromide, 1 μg/mL, at constant current (25 mA for 15 h), in 89 mM Tris–borate buffer and 2.5 mM EDTA, pH 8.3. Bands were located by UV light (254 nm), photographed, and quantitated by microdensitometry. The correction coefficient 1.47 ± 0.30 was used for decreased stainability of form I DNA vs form II and III (see below).

(*b*) *Quantification of DNA Cleavage.* Different amounts of φX174 DNA form I were subjected to agarose gel electrophoresis, and the peak area was measured by densitometry. A linear relationship is obtained between the peak area measured and the amount of DNA applied to the gel under 500 ng of material. Since supercoiled DNA has a lower ability to bind ethidium bromide, it has been necessary to multiply the values obtained for form I DNA by a correction factor. To determine the correction coefficient, we compared the fluorescence of ethidium bromide for the same quantity of form I and form III DNA. Form I DNA was cleaved directly to form III by the restriction enzyme *Pst*I (only one site of cleavage on phage φX174 for this enzyme). Fifteen units of *Pst*I were incubated with 250 ng of φX174 form I DNA in 50 mM Tris-HCl buffer, pH 8, 10 mM $MgCl_2$, and 50 mM NaCl (20-μL final volume) at 37 °C for 30 min. At the end of the incubation time the reaction mixtures were loaded into the wells of an agarose gel and electrophoresed. The fluorescence of form I and form III bands before and after cleavage was measured by densitometry of electrophoresis gels. The mean value (15 experiments) for this correction factor was 1.47, and the standard error was 0.3. The same factor was also applied to form II, assuming that the binding ability to the relaxed circular form is the same as that of the linear form.

(*c*) *Variation of the Concentration of $KHSO_5$.* A typical reaction mixture contained 5 μL of φX174 DNA (50 μg/mL), 5 μL of 1 μM Mn-TMPyP in 50 mM phosphate buffer, pH 7.5, 5 μL of various concentrations (4-fold the final desired concentration) of $KHSO_5$ in 50 mM phosphate buffer, pH 7.5, and 5 μL of 400 mM NaCl diluted in the same buffer (see general protocol for more details).

(*d*) *Variation of the Concentration of [Mn(TMPyP)]-$(OAc)_5$.* A typical reaction mixture contained 5 μL of φX174 DNA (50 μg/mL), 5 μL of various concentrations (4-fold the final desired concentration) of Mn-TMPyP in 50 mM phosphate buffer, pH 7.5, 5 μL of 40 μM $KHSO_5$ dissolved in 50 mM phosphate buffer, pH 7.5, and 5 μL of 400 mM NaCl diluted in the same buffer (see general protocol for further details).

(*e*) *Variation of the Concentration of the Phosphate Buffer.* A typical reaction mixture contained 5 μL of φX174 DNA (diluted in double-distilled water, 50 μg/mL), 5 μL of various concentrations (4-fold the final desired concentration) of phosphate buffer, pH 7.5, with or without 400 mM NaCl, 5 μL of 1 μM Mn-TMPyP dissolved in double-distilled water, and 5 μL of 40 μM $KHSO_5$ also dissolved in double-distilled water (see general protocol for more details).

(*f*) *Variation of the pH of the Reaction Buffer.* A typical reaction mixture contained 5 μL of the φX174 DNA (diluted in double-distilled water, 50 μg/mL), 5 μL of 160 mM (or 16 mM) phosphate buffer with 400 mM NaCl, 5 μL of 400 nM manganese porphyrin solution in double-distilled water, and 5 μL of 1.2 μM $KHSO_5$ in double-distilled water. The digestion of φX174 DNA was carried out as described in the general procedure, but the pH of the medium varied. Various buffer solutions were prepared separately before being added to the reaction. For pH values higher than 8.4, adjustment is done by addition of 0.1 M NaOH. This significantly altered

neither the final Na^+ concentration nor the phosphate concentration. For pH 5, 0.1 M HCl was added to acidic phosphate solution.

(*g*) *Importance of the Nature of the Buffer.* A typical reaction mixture consisted of 5 μL of φX174 DNA (diluted in double-distilled water, 50 μg/mL), 5 μL of 160 mM buffer solution (phosphate, cacodylate, Tris, Hepes, Pipes, or arseniate), pH 7.5, 5 μL of 1 μM manganese porphyrin, and 5 μL of 40 μM $KHSO_5$. Manganese porphyrin and $KHSO_5$ were dissolved in double-distilled water.

(*h*) *Variation of the Concentration of Salts.* The effect of ionic strength on the cleavage activity was investigated by increasing the concentration of NaCl or $MgCl_2$ in the medium. NaCl or $MgCl_2$ was diluted in phosphate buffer at a concentration corresponding to 4 times the final concentration in the reaction medium, and 5 μL of these solutions was added to the test tubes. The reactions are carried out exactly as described in the general procedure. The final concentrations of manganese porphyrin and $KHSO_5$ were 250 nM and 10 μM, respectively. The experiment was done in 5 or 40 mM phosphate buffer, pH 7.5 (final concentration). A phosphate concentration of 5 mM was chosen to minimize the influence of the additional Na^+ ions coming from the phosphate buffer itself.

(*i*) *Influence of the Preincubation Time.* Five microliters of φX174 DNA (50 μg/mL), 5 μL of 50 mM phosphate buffer, pH 7.5, with 400 mM NaCl, and 5 μL of porphyrin were mixed; 5 μL of $KHSO_5$ (vide infra for concentration) was added to initiate the cleavage reaction either immediately or after various preincubation times. Both $KHSO_5$ and porphyrin solutions were in 50 mM phosphate buffer, pH 7.5. Preincubation of reactants, in the dark at ambient temperature, varied from 0 to 30 min. In all cases the reaction time was 1 min as usual. Final concentrations of manganese porphyrin were 250, 100, and 20 nM. The corresponding concentration of $KHSO_5$ was 10 μM except for the assay at 100 nM porphyrin, where the added $KHSO_5$ was 300 nM (final concentration).

(*j*) *Variation of the Incubation Time.* Reaction conditions were as indicated in the general protocol with final concentrations of 5 or 50 nM for Mn-TMPyP and of 10 μM for $KHSO_5$ in the presence of 100 mM NaCl and with a preincubation time of 30 min.

(*k*) *Effect of Temperature.* Reaction conditions were as indicated in the general protocol with final concentrations of 250 nM for Mn-TMPyP and 10 μM for $KHSO_5$ and in the presence of 100 mM NaCl. Three preincubation times (1, 10, and 30 min) and three temperatures (2, 20, and 37 °C) were tested.

(*l*) *Comparative Efficiency of $KHSO_5$ and H_2O_2.* Reaction conditions were as indicated in the general protocol, with final concentrations of 250 nM for Mn-TMPyP, 0.3, 1, 5, and 10 μM for $KHSO_5$, or 0.01, 0.05, 0.5, 2, 10, and 50 mM for H_2O_2 in the presence of 100 mM NaCl. Preincubation time was 30 min. Supplementary experiments were carried out in the presence of 5, 25, 100, and 500 μM imidazole when the concentration of H_2O_2 was 2 and 50 mM.

Results

Effect of $KHSO_5$ Concentration. First of all, the data illustrated by Figure 1 indicate that the manganese–porphyrin/monopersulfate system cleaves the double-stranded supercoiled φX174 DNA by means of single-strand breaks (SSBs). When form I gradually disappears, the concomitant appearance of form II is observed. It is only when the number of SSBs becomes sufficient that the band corresponding to form III can be seen on electrophoresis gels. So the double-strand breaks (DSBs) responsible for these linear forms of DNA have to be considered as resulting from coincidence of two random SSBs.

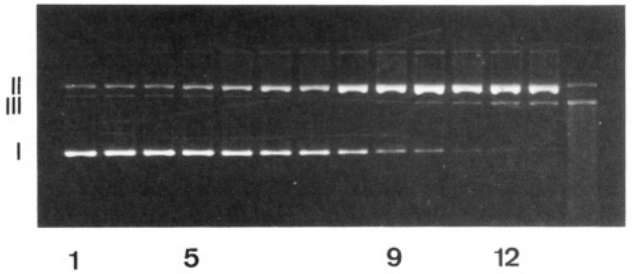

FIGURE 1: Cleavage of φX174 form I DNA by Mn-TMPyP at increasing concentration of $KHSO_5$. DNA was electrophoresed on agarose gel and treated as described under Experimental Procedures. DNA concentration is 18.7 μM (bp); Mn-TMPyP concentration is 250 nM (1 Mn-TMPyP for 75 bp). Lane assignment: (1) DNA control; (2) Mn-TMPyP control; (3) 5 μM $KHSO_5$ control; cleavage reaction in the presence of various concentrations of $KHSO_5$, (4) 0.01 μM; (5) 0.05 μM; (6) 0.08 μM; (7) 0.1 μM; (8) 0.2 μM; (9) 0.3 μM; (10) 0.45 μM; (11) 0.6 μM; (12) 0.8 μM; (13) 1 μM; (14) 5 μM. The various forms of plasmid DNA (form I, II, or III) are indicated in the figure.

Table I: Cleavage of φX174 DNA Plasmid (Form I) in the Presence of Various Concentrations of $KHSO_5$[a]

	form %			*S*			
concn (μM)	I	II	III	a	b	c	d
0	86	14	0				
0.01	78	22	0	0.10		0.16	
0.05	75	25	0	0.14		0.22	
0.08	60	40	0	0.36		0.66	
0.1	62	38	0	0.33		0.59	
0.2	43	57	0	0.69		1.45	
0.3	27	70	3	1.15	3.5	2.9	4.5
0.45	21	73	6	1.4	4.7	3.7	6.3
0.6	10	79.5	10.5	2.1	6.3	6.4	8.5
0.8	5	81.5	13.5	2.7	7.1	8.7	9.6
1	3.5	81.5	15	3.2	7.6	10.3	10.2

[a] Form I φX174 DNA (18.7 μM bp) was incubated with 250 nM Mn-TMPyP and 100 mM NaCl in the presence of indicated concentrations of $KHSO_5$ at 20 °C during 1 min. *S* = mean number of single-strand scissions per DNA molecule; *S* calculated from eq 3 (a), from eq 4, $h = 29$ (b), from eq 7 (c), or from eq 4, $h = 16$ (d).

When the phage DNA is exposed to increased doses of $KHSO_5$ in the presence of Mn-TMPyP at 250 nM, the concomitant decay of supercoiled DNA (form I) is measured (Figure 1; Table I). In the experimental range of $KHSO_5$ concentrations, these variations can be described by the relation (Figure 2a)

$$\ln I_0/I = 3.3[\text{persulfate}] \qquad (1)$$

where I_0 is the initial molar fraction of form I and I is the fraction of remaining form I DNA molecules after a 1-min incubation with the cleaving reagent; [persulfate] is expressed in micromolar terms.

If the cleavage reaction is first order in $KHSO_5$ and in the absence of form III release, for a small variation d*S* of *S* (*S* = mean number of single-strand breaks per DNA molecule), the concomitant variation dI of the amount of form I can be described as

$$dI = -dS\frac{k_1 I}{k_1 I + k_2 II}$$

k_1 and k_2 being the proportionality coefficients for the accessibility of cleavage sites of form I and form II, respectively.

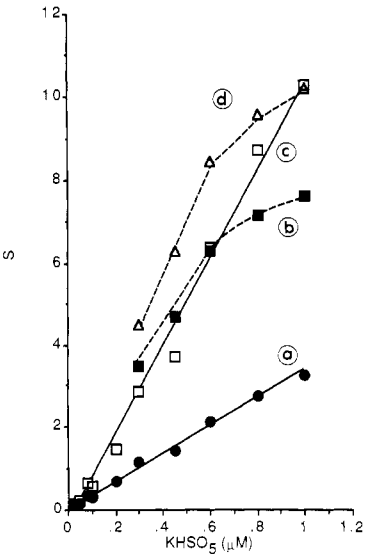

FIGURE 2: Single-strand breaks as a function of potassium monopersulfate concentration in cleavage of ϕX174 DNA by Mn-TMPyP/$KHSO_5$. ϕX174 DNA (18.7 μM bp) is incubated 1 min at ambient temperature with 250 nM Mn-TMPyP and the indicated concentrations of $KHSO_5$ in the presence of 100 mM NaCl. a (●), b (■), c (□), d (△): see Table I footnote.

II is the fraction of phage molecules in relaxed circular form (form II). With $I + II = 1$, this expression can be written

$$dS = -\frac{k_1 - k_2}{k_1} dI - \frac{k_2}{k_1}\frac{dI}{I}$$

The integration gives

$$S = \frac{k_2}{k_1} \ln \frac{I_0}{I} + (I_0 - I)\frac{k_1 - k_2}{k_1} \quad (2)$$

Then, when the accessibility for cleavage is the same on both form I and form II, then $k_1 = k_2$ and the equation simplifies to

$$S = \ln I_0/I \quad (3)$$

This last expression represents the equation used by most of the authors to estimate the mean number of single-strand scissions per DNA molecule (S) (Hertzberg & Dervan, 1984). But one has to keep in mind that this simplified equation is only valid for cleaving systems whose cleavage efficiency is not modulated by DNA topology. In addition, when the cleavage reaction proceeds to the formation of linear DNA molecules (form III), the number of single-strand breakages S could be calculated (Hertzberg & Dervan, 1984; Freifelder & Trumbo, 1969) from

$$I + II = \{1 - [S(2h + 1)/2L]\}^{S/2} \quad (4)$$

where h is the distance between nicks on opposite strands needed to produce a linear molecular (16 bp according to Freifelder and Trumbo (1969) for low ionic strength conditions] and L is the total number of DNA base pairs in ϕX174 (5386 bp). For the highest values of $KHSO_5$ concentrations, the variations of S are still linear, but the value of the slope is consequently higher (Figure 2, curve d):

$$S = 14.2[\text{persulfate}] \quad (5)$$

If we take into account the value of $h = 29$ more recently proposed by van Touw et al. (1985), the corresponding equation is then rescaled as (Figure 2, curve b)

$$S = 10.7[\text{persulfate}] \quad (6)$$

The difference between eq 1 and eq 5 or 6 can be explained if we consider that the metalloporphyrin-based system has more affinity for form II or form III than for form I or that the detorsion of the supercoiled DNA reveals cleavage sites previously hidden in the more constrained form I. This phenomenon is described by higher values for k_2 than for k_1. Consequently, the ratio k_2/k_1 is dependent of the estimated value of h, the distance between two SSBs on opposite strands. Taking into account the value of 16 [that used by Hertzberg and Dervan (1984) in a study on a DNA cleaver producing mainly SSBs] or of 29 ± 6 [that used by Van Touw et al. (1985) in a study on γ-irradiation of DNA, which produces a large amount of direct DSBs besides the SSBs], the k_2/k_1 ratio will be 5.5 in the first case and 4.0 in the second case. Without going deeper into a discussion that is far beyond the present study, we decided to choose the value of 4 for the ratio k_2/k_1 in eq 2. With this value, a rather well fitted relationship is observed between S and the concentration of persulfate all through the concentration range when we use either eq 4 or 2. In these conditions eq 2 can be simplified to

$$S = 4 \ln I_0/I + 3(I - I_0) \quad (7)$$

The corresponding curve (c) is indicated in Figure 2. When the results are expressed by using eq 4 ($h = 29$), the curve b fits correctly the data (see Figure 2).

All the calculations developed in the preceding paragraphs take into account the accessibility differences of manganese porphyrin for more or less constrained forms of DNA (supercoiled versus relaxed forms). However, an alternative hypothesis cannot be definitively rejected and would lead to a smaller number of SSBs for the same experimental data (the percentages of DNA forms are the real bases for discussions on S values). If the manganese porphyrin derivative is able to strongly recognize particular base-pair sequences as preferential sites of cleavage, then the probability of having a DSB resulting from two close independent SSBs on opposite strands would largely increase. Then eq 3 is correct, and eq 7 overestimates the S values. Therefore, an improvement of the statistical model corresponding to eq 4 should be necessary, but it requires a complete knowledge of the interactions of manganese porphyrin derivatives with supercoiled and relaxed forms of DNA that is beyond the scope of the present study and will be developed in future studies. Having these restrictions in mind, we decided to choose the first hypothesis, i.e., calculations with eq 4 and 7, as a practical method to quantify the S value. But we are aware that further studies have to be undertaken to ascertain or modify this pragmatic approach.

Variation of the Concentration of Mn-TMPyP. Data related to the influence of the manganese porphyrin concentration on the cleavage of DNA are reported in Table II. For each complex concentration, the corresponding number of SSB per DNA molecule (S) calculated from eq 7 and from eq 4 with $h = 29$ is indicated in Table II. The first line corresponds to the control experiment where DNA is incubated with $KHSO_5$ only. No breaks are observed when the metalloporphyrin is incubated alone for 1 min at the various concentrations described here. Figure 3 illustrates the linear relationship between the manganese porphyrin concentration and the number of SSB per DNA molecule. It should be pointed out that DNA breaks are observed for Mn-TMPyP concentrations as low as 0.5 nM. Within 1 min of incubation time and Mn-TMPyP concentrations above 60–80 nM, form I of ϕX174 DNA is fully converted to form II and form III. The number of DNA breaks per molecule of manganese porphyrin ($=S[\text{DNA}][\text{Mn-TMPyP}]^{-1}$) is close to 0.5 [we will

Table II: Cleavage of ϕX174 DNA in the Presence of Various Concentrations of Mn-TMPyP[a]

	form %			S	
concn (nM)	I	II	III	a	b
0 (control)	72	28	0		
0.5	66.5	33.5	0	0.15	
1	60	40	0	0.35	
3	51	49	0	0.75	
5	47	53	0	0.95	
10	38.5	60	1.5	1.5	
15	46	53.5	0.5	1.0	
20	31	67	2	2.15	
30	33.5	64.5	2	1.9	
50	12	81	7	5.3	5.0
60	4.5	86	9.5		6.0
80	0	60	24.5		13.5
100	1.5	54.5	27.5		14
120	0	42	34.5		17
150	0	39.5	35.5		18
250	0	<3	<3		>35

[a] Form I ϕX174 DNA (18.7 μM bp) was incubated with the indicated concentrations of Mn-TMPyP at 20 °C during 1 min in the presence of 10 μM $KHSO_5$ and 100 mM NaCl; S = mean number of single-strand scissions per DNA molecule; S calculated from eq 7 (a) or from eq 4 with h = 29 (b).

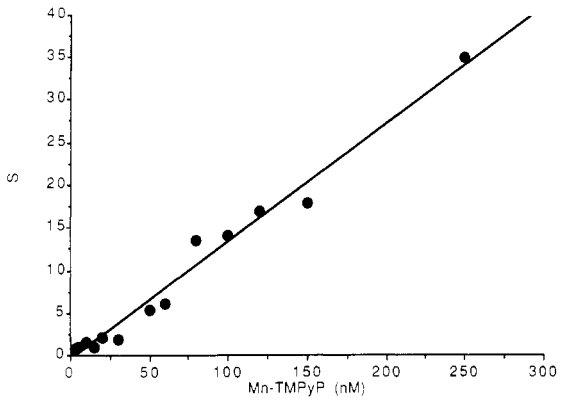

FIGURE 3: Influence of Mn-TMPyP concentration on the cleavage of ϕX174 DNA in the presence of potassium monopersulfate. ϕX174 DNA (18.7 μM bp) is incubated 1 min at ambient temperature with the indicated concentrations of Mn-TMPyP and 10 μM $KHSO_5$ in the presence of 100 mM NaCl.

see below that higher catalytic activities are observed at low manganese porphyrin concentration for incubation times higher than 1 min (see Variation of the Incubation Time)].

Dependence of the Reaction on Solution Conditions. When the concentration of phosphate buffer varies from 5 to 40 mM, a significant increase (about 5-fold) of the cleaving activity of the artificial nuclease is measured. The same general effect is also observed in the presence of 100 mM sodium chloride. At higher phosphate buffer concentration (100 mM) an inhibitory effect takes place (consequently, 40 mM has been selected as a better value for the phosphate buffer concentration in most of the experiments described herein). Apart from the increase of the ionic strength itself, a competition between phosphate and water as proximal ligand for the chelated metal might be proposed as a possible explanation for the inhibitory effect. Such phenomenon has been previously studied to explain the low nuclease activity of bleomycin in vitro (Dabrowiak et al., 1979; Burger et al., 1979). In the present case, we checked this hypothesis by careful visible spectroscopy analyses. Visible absorption spectra of a solution of the manganese porphyrin (3 μM) in water or in 40 or 100 mM phosphate buffer, pH 7.5, were measured at ambient temperature. No differences are noticeable on the various spectra, except a weak hyperchromic effect on the Soret band and a slight bathochromic effect on the minor bands α and β when the phosphate concentration increases. The measured ϵ_M values ($\times 10^{-3}$, M^{-1} cm^{-1}) at 462.5 nm are 100.8 ± 0.4, 104.2 ± 0.8, or 106.6 ± 0.5 for Mn-TMPyP solutions in water, 40 mM, or 100 mM phosphate buffer, respectively. This represents an increase of 3% for the ϵ_M value in 40 mM phosphate compared to that in distilled water and an increase of 6% in 100 mM phosphate. The ϵ_M value ($\times 10^{-3}$, M^{-1} cm^{-1}) at 560 nm (band α) of the manganese porphyrin in water is 10.5. In the presence of phosphate (40 or 100 mM) the ϵ_M remains the same, but a weak bathochromic effect is observed: the maximum shifts from 560 to 561.5 nm. From this study, the inhibitory effect of phosphate buffer concentration above 50 mM cannot be clearly attributed to the coordination of a phosphate anion at the axial position of the metalloporphyrin; it might be also correlated to a modification of the accessibility to DNA cleavage sites by small changes in DNA conformations depending on the buffer concentration.

The average number of single-strand breaks mediated by 100 nM Mn-TMPyP and 300 nM $KHSO_5$ in 4 or 40 mM phosphate buffer in the presence of 100 mM NaCl has been determined for increasing pH values above 5. Except the potentiation effect of the buffer concentration itself, which has been discussed in the preceding paragraph, the following remarks can be made: (i) there is almost no nuclease activity for a pH value of ≤6.0; (ii) the cleavage efficiency of the Mn-TMPyP/$KHSO_5$ system increases from pH 6 to pH 7.5 and stabilizes above pH 8.0. For pH values above 9, the manganese porphyrin is able to cleave the phage DNA even in the absence of $KHSO_5$. At alkaline pH values, the amount of breaks by manganese porphyrin alone is almost the same as that obtained with the manganese porphyrin/$KHSO_5$ association. This phenomenon is tentatively attributed to the formation of Mn(IV)=O species generated from Mn(III)—OH at pH above 10 (Meunier, unpublished data). Such DNA breaks mediated by a metalloporphyrin in alkaline solutions where alkali-labile sites are revealed are currently under investigation in our laboratory. In conclusion, the optimal pH values corresponding to the best efficiency of the Mn-TMPyP/$KHSO_5$ system are between 7.5 and 9.0.

The DNA cleaving activity was tested and compared in five different buffer solutions: phosphate, cacodylate, Tris-HCl, Hepes, and arseniate. These buffers can thus be classified from the most to the least potent to support the nuclease activity of the porphyrin/$KHSO_5$ system as follows: arseniate and phosphate ≫ cacodylate > Tris. Hepes buffer totally inhibits the reaction. It was consequently chosen as stopping reagent for this reaction. The reason such an inhibition is observed is probably the competitive oxidation of buffer molecules by $KHSO_5$ itself (as is also the case, at a lower extent, for Tris-HCl buffer).

The cleavage activity varies as a function of the concentration of salts. The influence of the ionic strength on the nuclease activity of Mn-TMPyP/$KHSO_5$ has been studied with two different salts: NaCl (Figure 4) and $MgCl_2$. In both cases an optimal salt concentration is observed. The efficiency maximum is reached for a salt concentration between 80 and 150 mM for NaCl and near 10 mM for $MgCl_2$. In the case of NaCl, the phosphate concentration used was 40 or 5 mM; the same effect with the same maximum position is observed for both concentrations of phosphate. The maximal cleavage activity has been observed in the presence of 100 mM NaCl with 40 mM phosphate.

When ϕX174 DNA is incubated with 250 nM manganese porphyrin (1 Mn–porphyrin/76 bp) in the presence of 100 mM

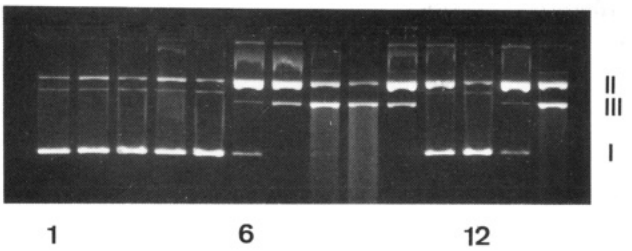

FIGURE 4: Effect of NaCl concentration on the cleavage efficiency of Mn-TMPyP/$KHSO_5$ system. ϕX174 DNA (18.7 μM bp) is incubated with 250 nM Mn-TMPyP and 10 μM $KHSO_5$ in 40 mM phosphate buffer, pH 7.5, at various concentrations of NaCl. Lane assignment: (1) DNA control; (2) Mn-TMPyP control, no NaCl added; (3) Mn-TMPyP control, 500 mM NaCl; (4) $KHSO_5$ control, no NaCl added; (5) $KHSO_5$ control, 500 mM NaCl; cleavage reaction at various concentrations of NaCl, (6) no NaCl added; (7) 25 mM; (8) 50 mM; (9) 100 mM; (10) 200 mM; (11) 500 mM; (12) 1000 mM; (13) 100 mM ($KHSO_5$ 1 μM); (14) 100 mM ($KHSO_5$ 5 μM).

NaCl, no increase of the cleavage activity as a function of preincubation time is observed. Consequently, a preincubation of 5 min has been considered sufficient to obtain the maximal activity. On the contrary, when DNA is incubated with 100 nM manganese porphyrin (1 Mn–porphyrin/190 bp) or with 20 nM manganese porphyrin (1 Mn–porphyrin/950 bp) in the presence of 100 mM NaCl, a progression of cleaving activity is noted over the first 30 min of the preincubation period (preincubation times tested were 0, 5, 10, 20, and 30 min). The longer the preincubation time is, the higher is the amount of breaks generated by the system: at 100 nM manganese porphyrin (300 nM $KHSO_5$), S increases from 1.2 to 8; at 20 nM manganese porphyrin (10 μM $KHSO_5$), S increases from 2.5 to 4.5.

When the DNA cleavage experiment is stopped after various incubation times (from 15 s to 8 min), we observed that the number of single-strand breaks is roughly proportional to the time of reaction. A weak cleavage activity of $KHSO_5$ alone has been observed, especially for reaction times above 1 min. The turnover number of manganese porphyrin is better for low concentrations of this cleaving agent: at 5 nM it can increase up to 5 SSBs by molecule of manganese porphyrin. From these data it is clear that Mn-TMPyP behaves as a real catalyst molecule at low concentration, when the ratio manganese porphyrin/base pair is minimum (here, 1 Mn-TMPyP for 3700 bp). Since these 5 SSBs are obtained within 10 min, the catalytic activity of Mn-TMPyP is around 0.5 SSB/min.

When the temperature of both preincubation and incubation steps was varied from 4 to 37 °C, no significative difference in the cleavage efficiency could be evidenced. Increasing the time of preincubation from 1 to 30 min in this range of temperature also did not demonstrate a significative difference.

Comparative Efficiency of $KHSO_5$ and H_2O_2. As shown in Figure 5, $KHSO_5$ appears as an oxygen donor much more efficient than H_2O_2. Similar cleavage profiles on electrophoresis gels are obtained with concentrations of $KHSO_5$ about 10^3–10^4-fold weaker than that of H_2O_2. This is clearly illustrated in Figure 5. It has also to be noted that addition of imidazole (from 5 to 500 μM, i.e., 20–2000 imidazole molecules/Mn–porphyrin) does not change the low efficiency of H_2O_2 (two concentrations of H_2O_2 have been tested, 2 and 50 mM; data not shown).

Discussion

From data reported in Figure 1, it is clear that the DNA cleaving system Mn-TMPyP/$KHSO_5$ is able to cleave double-stranded supercoiled ϕX174 DNA by means of single-strand breaks. This can be evidenced by following the disappearance of form I as function of the oxidant concentration (Table I) and the concomitant formation of form II. Up to a form I conversion of 70%, [form I] + [form II] is equal to the initial concentration of form I. This indicates that the main event is the cleavage of supercoiled DNA by single-strand breaks and the linear form III is resulting only from two SSBs in a close vicinity (see Results for the mode of calculations of S, the mean number of single-strand breaks per DNA molecule). The same observation, i.e., DNA cleavage by SSBs, is also made when the cleavage of ϕX174 DNA is studied as a function of the manganese porphyrin derivative (see Table II).

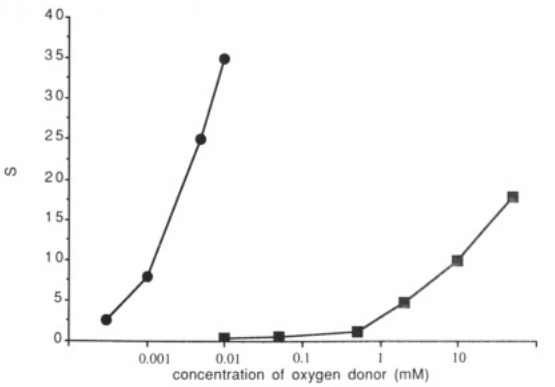

FIGURE 5: Comparative efficiency of $KHSO_5$ (●) and H_2O_2 (■) as oxygen atom donor on DNA cleavage mediated by Mn-TMPyP. ϕX174 DNA (18.7 μM) was incubated with 250 nM Mn-TMPyP and 0.3, 1, 5, and 10 μM $KHSO_5$ or 0.01, 0.05, 0.5, 2, 10, and 50 mM H_2O_2 in the presence of 100 mM NaCl. S was calculated from eq 7 or 4 as for Table II and Figure 3.

The second main fact is the very high efficiency of this artificial endonuclease system. Cleavage of supercoiled DNA is observed at manganese porphyrin concentrations as low as 0.5 nM associated to potassium monopersulfate at micromolar concentrations for a short incubation time (1 min).

The nuclease activity is highly dependent on the nature of the oxidant. Comparative studies (Figure 5) indicate that hydrogen peroxide, the biological single oxygen atom donor, is not as efficient as potassium monopersulfate to activate the metalloporphyrin bound to DNA; in our conditions, addition of imidazole did not improve the efficiency of H_2O_2 as has been observed in catalytic epoxidation reactions (Battioni et al., 1988). The same cleavage patterns on gel electrophoresis are obtained for hydrogen peroxide concentrations that are at least 10^3 times higher than that of potassium monopersulfate. In the absence of distal and proximal assistance for the heterolytic cleavage of the peroxidic O–O bond, the association of hydrogen peroxide and synthetic metalloporphyrins generates hydroxyl radicals as main active species rather than metal–oxo entities. Indeed, hydroxyl radicals are known as DNA cleavers. But it should be noted that DNA breaks by $HO^\bullet$ are usually observed at high concentrations of generating systems based on hydrogen peroxide: $Cu^I(OP)_2/H_2O_2$ is operated at 4 μM and 7 mM, respectively (Pope & Sigman, 1984) and Fe-EDTA/H_2O_2 at 10 μM and 1 mM, respectively (Tullius et al., 1987). Since it is a well-established ability of $KHSO_5$ to behave as an efficient oxygen donor in oxygenation reactions catalyzed by metalloporphyrins (Robert & Meunier, 1988) or metallobleomycins (Girardet & Meunier, 1987), it is tempting to attribute the observed DNA breaks by Mn-TMPyP/$KHSO_5$ to high-valent manganese–oxo species rather than to diffusable oxygen radicals derived from the oxidant. Such hypothesis is also supported by the recent finding that Mn-TMPyP/$KHSO_5$ is able to hydroxylate adenosine 5′-

monophosphate at the C_8 position of the base (Bernadou et al., 1988).

Manganese tetrakis(methylpyridiniumyl)porphyrin is known to preferentially interact by electrostatic forces with double-stranded DNA in the minor groove at AT-enriched sites (Ward et al., 1986a,b). Consequently, its nuclease activity is expected to be in strong dependence on phosphate buffer concentration, as well on addition of salt (NaCl or $MgCl_2$). The optimal phosphate buffer concentration is in the range 40–50 mM, and the cleavage activity is highly enhanced by 100 mM NaCl (Figure 4). The nuclease activity also reaches a maximum for a $MgCl_2$ concentration of 10 mM. *S* values up to 30 are obtained at 40 mM in phosphate buffer and 100 mM in NaCl. These reaction conditions have been considered the optimal standard conditions and might be taken as a good starting point for people interested in applying Mn-TMPyP/$KHSO_5$ in protein–DNA-footprinting experiments. In these standard conditions with 250 nM manganese porphyrin, a preincubation time of 5 min is apparently sufficient to obtain the maximum cleavage activity. However, at low manganese porphyrin concentration, at a Mn-TMPyP/bp ratio between 200 and 1000, the activity increases when the preincubation time increases up to 30 min. In these conditions, at low "cleaver/cleavage sites" ratio, it might be important to have a longer preincubation time to allow the selective equilibration of Mn-TMPyP with accessible and more fragile cleavage sites. Such phenomenon might be more important at very low manganese porphyrin concentrations. At nanomolar concentrations, the porphyrin complex behaves like an enzyme that, first, binds DNA and, second, moves along the double strand to choose the appropriate sequence before reacting. This hypothesis is also supported by studies on the influence of the incubation times at low Mn-TMPyP concentrations: up to 5 SSBs per Mn-TMPyP molecule are obtained at 5 nM in Mn complex within 10 min, indicating that the manganese porphyrin really acts as a catalyst. For concentrations of this reagent above 200 nM in manganese porphyrin and 1 μM in $KHSO_5$, it is noteworthy that a short incubation time of 1 min is sufficient to obtain a complete disappearance of form I DNA; as matter of comparison, 5 mM iodosylbenzene is required in DNA cleavage by Mn-TMPyP/PhIO at a manganese porphyrin concentration close to 200 nM, but with a 20-min incubation time (Ward et al., 1986b).

Mn-TMPyP has been compared to other metal derivatives of the same porphyrin ligand or to other manganese complexes with, for example, *meso*-tetrakis(4-carboxyphenyl)porphyrin (data not shown). The manganese derivative of $TMPyPH_2$ appears as a rather unique complex with respect to the activation of metalloporphyrins by $KHSO_5$. At 250 nM, Fe, Co, and Zn complexes of $TMPyPH_2$ are inactive compared to the manganese derivative. Such a difference is expected if the mechanism of the DNA breaks involves metal–oxo entity, since studies on catalytic alkane hydroxylations have revealed that manganese porphyrins are always more efficient as oxygen-transfer catalyst than iron (cobalt and zinc complexes are inactive in oxygenation reactions). The highest activity of manganese porphyrins can be attributed to the fact that, in association to a neutral proximal ligand, they are isoelectronic to iron porphyrins with a thiolato ligand, which is the design of cytochrome P-450 monooxygenases. Experiments with the anionic manganese porphyrin complex failed, even in the presence of divalent ions (calcium, magnesium, and zinc salts were used to favor a ternary association, Mn-anionic porphyrin/M^{2+}/DNA).

When homooligodeoxynucleotides are used to obtain some information on the cleavage process at the molecular level, it must be noted that no base-propenal derivatives have been identified by analytical HPLC, only released free bases are detected. At least, these data suggest that if the hydrogen atom at C'_4 is the target for the high-valent manganese–oxo species as observed for metallobleomycin-mediated DNA breaks, then the oxygen-dependent route which is the reaction pathway responsible for the formation of base propenals in bleomycin cleavage is not involved in the present case. An alternative target is the tertiary C–H bonds at C'_1, which is also situated inside the minor groove in B DNA, but more deeply, being accessible for cleaving agents that can partially intercalate between base pairs, like $Cu(OP)_2$, for example (Goyne & Sigman, 1987). In addition, one other possible target, the tertiary C–H bonds at C'_3, might be discarded since it is located in the major groove. We are currently developing studies on the molecular aspect of these oxidative DNA breaks catalyzed by manganese porphyrin complexes.

Conclusion

The association of potassium monopersulfate to manganese tetrakis(methylpyridiniumyl)porphyrin leads to an efficient DNA cleaving system. Studies performed on supercoiled ϕX174 DNA have shown that single-strand breaks are responsible for the observed DNA cleavage which is obtained at manganese porphyrin concentrations as low as 0.5 nM and at $KHSO_5$ concentrations in the micromolar range within 1 min at room temperature. Because of the high activity of Mn-TMPyP/$KHSO_5$ on double-stranded DNA and the preliminary data obtained on RNA (with a low or high double-stranded portions), the presently described system can be considered a general nucleic acid cleaver and consequently might find its own place among the recent new cleavers based on coordination complexes and useful in DNA- or RNA-footprinting experiments.

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Solution Conformation of d-CTCGAGCTCGAG by Two-Dimensional NMR: Conformational Heterogeneity at *Xho*I Cleavage Site

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ABSTRACT: Nonexchangeable proton resonances in the 500-MHz NMR spectrum of d-CTCGAGCTCGAG have been assigned by using two-dimensional correlated spectroscopy (COSY) and nuclear Overhauser enhancement spectroscopy (NOESY). ^1H–^1H coupling constants (J) in the deoxyribose rings have been measured by analyzing intensity and multiplet patterns in the phase-sensitive ω_1-scaled COSY spectra. A modification of the J-resolved technique, called amplitude-modulated J-resolved spectroscopy, has been described and used to increase the accuracy of J measurements. Absorption mode ω_1-scaled NOESY spectra at mixing times in the range 50–200 ms have been analyzed to monitor spin diffusion. A 50-ms spectrum has been used to estimate several interproton distances. The coupling constant and distance data have been used to arrive at sequence-specific sugar geometries and glycosidic torsion angles. The backbone structure has been refined by model building using the FRODO program, employing the sugar geometries and glycosidic torsion angles discussed above. The molecule shows interesting sequence-dependent variations in the structure. The cleavage site of the restriction enzyme *Xho*I exhibits unique differences in the sugar geometry and backbone torsion angles.

With recent advances in methods for synthesis of DNA segments and the physical tools for structure determination, the understanding of DNA structure has undergone radical changes. It is no longer believed that DNA is a regular double-helical structure. New evidence suggests that DNA exhibits wide structural diversity depending upon the base sequence and experimental conditions. It is envisaged that the information content in DNA with respect to its function may be coded and modulated not only by the base sequence but also by the associated structural diversities.

Recent 2D NMR studies have focused on DNA segments recognized by proteins in a highly specific manner (Ravikumar

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