Hydroxylation, Epoxidation, and DNA Cleavage Reactions Mediated by the Biomimetic Mn-TMPyP/O_2/Sulfite Oxidation System†

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Autoxidation of sulfite is catalyzed by the water-soluble complex (*meso*-tetrakis(4-*N*-methylpyridiniumyl)-porphyrinato)manganese(III) (Mn-TMPyP), leading to an intermediate species capable of hydroxylation of *p*-isopropylbenzoic acid, epoxidation of carbamazepine, and generation of single-strand breaks both in plasmid ΦX174 DNA and in a 167-base-pair fragment of pBR322. ^{18}O-Labeling studies confirm that the high-valent manganese−oxo intermediate undergoes oxo−hydroxo tautomerism in competition with oxidation of substrate or reduction by excess sulfite. In contrast to the classical reductive activation of dioxygen by metalloporphyrins (dithiothreitol or ascorbate in the presence of O_2), a mechanism is proposed in which $SO_3^{\bullet-}$, formed by oxidation with Mn^{III}, is trapped by O_2, leading to a species ($SO_5^{\bullet-}$) capable of generating a Mn^{V}═O complex. Importantly, these studies point to the use of an alternative, biocompatible oxidation system compared to the preformed oxidant $KHSO_5$.

Introduction

The development of procedures for the hydroxylation of alkanes or the epoxidation of olefins with molecular oxygen is an important industrial and synthetic goal.[1] Since the pioneering work of Tabushi,[2,3] biomimetic oxygen atom transfers based on metalloporphyrin catalysts have been developed using either oxygen atom donors or molecular oxygen and a reducing agent.[4,5] In biology, O_2 is the oxidant in combination with NAD(P)H as reducing agent, although several biomimetic examples of ascorbate or thiols as the reductant have also been reported.[6] In contrast, the use of sulfite, SO_3^{2-} (and HSO_3^- in neutral, aqueous solution), as the reductant for O_2 has hardly been investigated for applications in catalytic oxidation.[7] Mechanistic studies of sulfite autoxidation have demonstrated the important role that transition metals such as manganese may play in the one-electron processes associated with the reaction, but the majority of these systems lead to formation of sulfoxyl radicals as the key products of sulfite autoxidation.[8−14] In contrast, recent studies of methemoglobin and manganese porphyrins suggest that these catalysts may provide access to high-valent metal−oxo species.[7,15,16]

Here we report the possibility of promoting hydroxylation and epoxidation reactions involving manganese(V)−oxo porphyrin species, reminiscent of the reactive intermediate described for cytochrome P-450, via sulfite autoxidation catalyzed by Mn-TMPyP.[17−19] In addition, the potential application of this system in biological oxidation is also illustrated by results on oxidative DNA cleavage.[20] *Importantly, these studies point to the use of an alternative, biocompatible oxidation system compared to the preformed oxidant KHSO*$_5$. To determine whether the oxygenation reaction occurs via a metal−oxo intermediate or via free radicals (see refs 21 and 22 for reviews on this controversial debate), we used the *oxo−hydroxo tautomerism* mechanism[23,24] (previously named "redox tautomerism") as an easy method to

† Dedicated to Professor John A. Osborn on the occasion of his 60th birthday.

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(16) Chen, S.-M. *J. Electroanal. Chem. Interfacial Electrochem.* **1996**, *407*, 123−130.
(17) Mn-TMPyP stands for the pentaacetate of the diaquamanganese(III) derivative of *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin. See the following references for details on this efficient DNA cleaver.
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Scheme 1. Mechanisms To Account for the Labeling Observed in Hydroxylation and Epoxidation Reactions in Experiments Using $H_2{}^{18}O$ (● = ^{18}O)

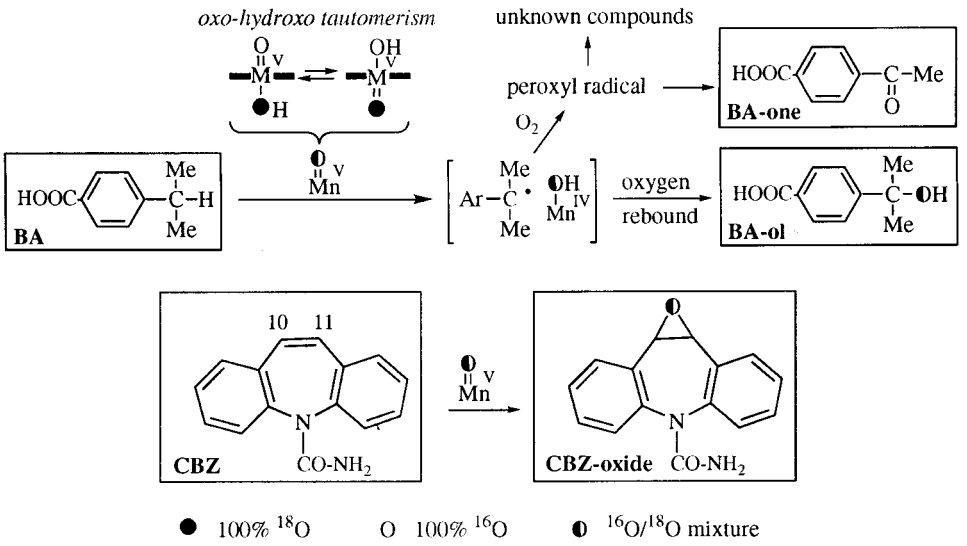


Table 1. Products Obtained in the Oxidation of 4-Isopropylbenzoic Acid (BA) and Carbamazepine (CBZ) Catalyzed by Mn-TMPyP in the Presence of $KHSO_5$ or Na_2SO_3/O_2

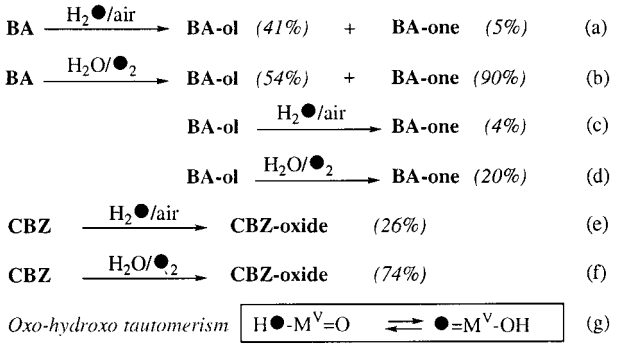
substrate[a]	mode of activation	concn, mM	conversion, %	product yield,[b] %		selectivity in identified products
				BA-ol	BA-one	BA-ol+BA-one
BA	$KHSO_5$	1	48	20	15	73
		10	93	10.5	41	55.5
	Na_2SO_3	1	34.5	8	14	63
		10	60	8	32	67
		2[c]	90	5	45.5	56
		10[d]	<2	n.d.[e]	n.d.	<2
				CBZ-oxide		CBZ-oxide
CBZ	$KHSO_5$	5	99	80		79
	Na_2SO_3	5	35	25		71
		1[f]	50	33		66

[a] Unless otherwise noted, substrate and Mn-TMPyP concentrations are 500 μM and 10 μM, respectively. [b] Yields were calculated from the initial amount of substrate after 10 min of reaction at room temperature. [c] [Mn-TMPyP] = 2 μM; [**BA**] = 100 μM. [d] Control experiments without O_2, sulfite, or catalyst. [e] Not detected. [f] [Mn-TMPyP] = 4 μM; [**CBZ**] = 100 μM.

obtain evidence for an oxygen atom transfer controlled by the metal center. This mechanistic approach was recently exemplified with various metalloporphyrins, with several oxygen atom donors, and in different types of oxidation reactions.[25−29]

Results and Discussion

Oxidation of Isopropylbenzoic Acid, BA. Catalytic hydroxylation of isopropylbenzoic acid, **BA**, was performed in aerated aqueous solutions with Mn-TMPyP as catalyst and sodium sulfite as reducing agent. During oxidation of **BA** (Table 1, Scheme 1), substrate conversion reached up to 90% depending on the experimental conditions, and production of both the hydroxylated compound **BA-ol** and the ketone derivative **BA-one** (resulting from an additional C−C bond cleavage) was observed. These same products have been obtained in the oxidation of **BA** with Mn-TMPyP/$KHSO_5$.[29] No reaction occurred in deaerated solution, showing that the catalytic oxidation was oxygen dependent. As shown in eqs a and b for



Eq. a-g. ● = ^{18}O; O = ^{16}O (between brackets: per cent of ^{18}O incorporated).

experiments performed either in $H_2{}^{18}O$ or under $^{18}O_2$, the incorporation of ^{18}O in **BA-ol** was close to 50% (in eqs a−f, the molar percent of ^{18}O introduced in oxidation products is indicated in italics).[30] This result may be explained by an oxo−hydroxo tautomerism involving the metal−oxo and the trans axial hydroxo ligand (eq g and Scheme 1) and strongly supports a metal-controlled O atom transfer.[23,25−29] Under the same experimental conditions, neither dithiothreitol nor ascorbate was capable of converting substrate significantly (<3%),[31] allowing us to discard a simple role of reductant for sulfite as in a P-450 catalytic cycle (Scheme 2B). In fact, the formation of a high-valent metal−oxo complex likely results from transfer of one

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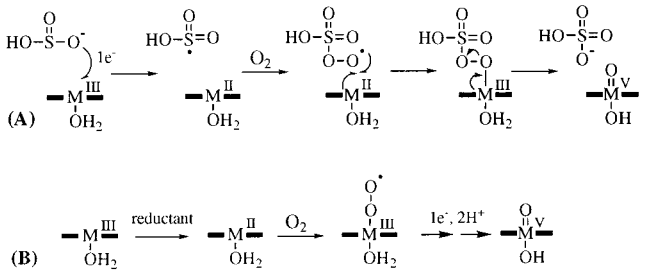
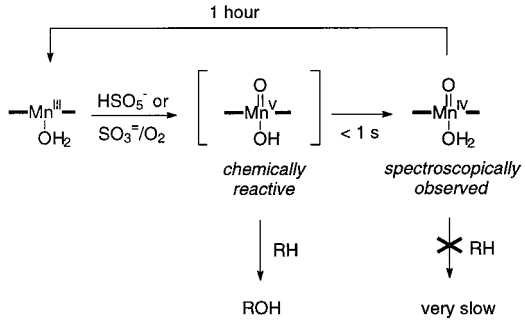
(30) The level of carbonyl oxygen exchange with water determined for an authentic sample of **BA-one** under the conditions of the catalytic reaction was close to 6.5%.
(31) When dithiothreitol or ascorbate was used instead of sulfite at 10 mM (**BA** oxidation) or 5 mM (**CBZ** epoxidation), in one or five consecutive additions, substrate conversion was always below 3%.

Scheme 2. (A) High-Valent Metal−Oxo Complex Formation from Oxygen Activation in the Presence of Sulfite and the Water-Soluble Metalloporphyrin Mn-TMPyP[a] and (B) Alternative Reductive Dioxygen Activation Route[a]



[a] At pH 5, bisulfite is the main species.

Scheme 3



electron from sulfite to the manganese porphyrin and trapping of the sulfite radical by dioxygen to give a monopersulfate radical which finally reacts with the manganese(II) porphyrin to afford a manganese(V)−oxo porphyrin complex and sulfate (Scheme 2A), analogous to the proposed formation of iron(V)−oxo methemoglobin with sulfite.[7] In addition, the extent of **BA-one** produced in the catalytic oxidation of **BA**, with no significant incorporation of ^{18}O in experiments performed in $H_2{}^{18}O$ (eq a) but with 90% ^{18}O incorporation in the presence of $^{18}O_2$ (eq b), indicated that an oxygen-dependent route competed in a large part with the oxygen rebound route; the intermediate tertiary carbon radical (Scheme 3) could escape from the solvent cage and react with dioxygen to give a peroxide radical with further evolution to **BA-one** and to other unknown products (Table 1). During oxidation of **BA**, direct conversion to **BA-one** from **BA-ol** may be considered as a minor route since we observed only a limited incorporation of ^{18}O label during experiments performed on a reference sample of **BA-ol** with $H_2{}^{18}O$ (eq c) or under $^{18}O_2$ (eq d). In any case, the product distributions observed at 90% conversion of **BA** with O_2/Na_2SO_3 and at 93% conversion with $KHSO_5$ are essentially the same (Table 1), consistent with the formation of a common manganese−oxo intermediate.

Epoxidation of CBZ. From **CBZ** oxidation data (Scheme 1 and Table 1), the present system Mn-TMPyP/O_2/Na_2SO_3 was shown to epoxidize this substrate like the Mn-TMPyP/$KHSO_5$ system,[23] but the conversion was lower. The analysis of the **CBZ-oxide** (26% of ^{18}O incorporation in experiments performed in $H_2{}^{18}O$, eq e) demonstrated that a high-valent manganese−oxo species was formed as the epoxidizing agent. The lower product yield compared to that observed with $KHSO_5$ under these conditions[23] can be explained by a competitive reduction of Mn^V=O to Mn^{IV}=O by excess sulfite ion.[27,32−37] The lower ^{18}O incorporation, which corresponds to an incomplete oxo−hydroxo tautomerism, was further found in the use of $^{18}O_2$ and $H_2{}^{16}O$, where the complementary amount of label incorporation

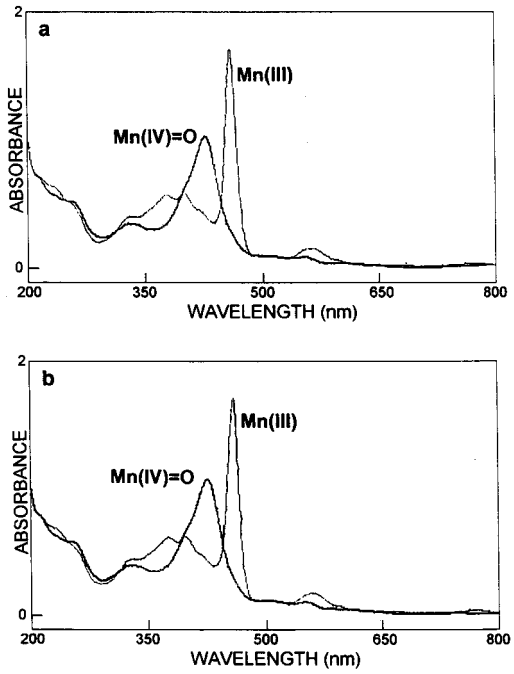


Figure 1. Electronic spectra of 15 μM Mn-TMPyP in aqueous solution (pH 7, 10 mM phosphate) immediately (<1 min) after addition of 100 μM Na_2SO_3 (final concentration) (a) or 30 μM $KHSO_5$ (b). See Scheme 3 and text for discussion.

was observed (74% vs 26%) (eq f vs eq e). Thus, only a fraction of the Mn^V=O species has undergone oxo−hydroxo tautomerism before either reduction by sulfite or epoxidation of the olefin. A parallel mechanism has been described in which NO_2^- oxidation competes with oxo−hydroxo tautomerism and olefin epoxidation using Mn^V=O.[27]

The ultimate formation of manganese(IV)−oxo species during autoxidation of sulfite is also supported by spectroscopic experiments in the absence of substrate. Addition of 100 μM Na_2SO_3 to a 15 μM solution (pH 7, 10 mM NaP_i) of Mn-TMPyP (λ_{max} = 462 nm) led to immediate conversion to the manganese(IV)−oxo complex (λ_{max} = 425 nm), which decayed over a period of minutes to Mn^{III} (Figure 1a). Identical behavior was observed upon addition of 30 μM $KHSO_5$ in place of Na_2SO_3 (Figure 1b). The Mn^V=O species was presumably formed initially, but its lifetime is too short ($t_{1/2}$ < 1 s)[27] for observation by conventional spectroscopy (Scheme 3). The Mn^{IV}=O species is not a good epoxidizing agent; addition of **CBZ** (0.5 mM) to the solution containing predominantly Mn^{IV}=O did not accelerate the rate of decomposition giving the 462 nm band (Mn^{III}-TMPyP).

DNA Cleavage. The present system was then successfully applied to the oxidative cleavage of supercoiled ΦX174 DNA. The nuclease activity of Mn-TMPyP in the presence of $KHSO_5$ or Na_2SO_3 (see Figure 2) was tested as described previously for $KHSO_5$.[18] A similar nuclease activity (direct strand breaks) was obtained for concentrations of 50 nM Mn-TMPyP/10 μM $KHSO_5$ (lane 4) or 250 nM Mn-TMPyP/10 μM sulfite (lane 6), indicating that the latter system is an efficient chemical DNA

(32) Such a reduction was previously observed with the nitrite ion.[27,33]
(33) Groves, J. T.; Marla, S. S. *J. Am. Chem. Soc.* **1995**, *117*, 9578−9579.
(34) The known redox potential of the sulfite ion ($SO_3^{\bullet-}/SO_3^{2-}$, 0.39 V vs SCE)[35] is below the estimated value for water-soluble Mn^{IV} porphyrins (>1.15 V vs SCE).[27,36,37]
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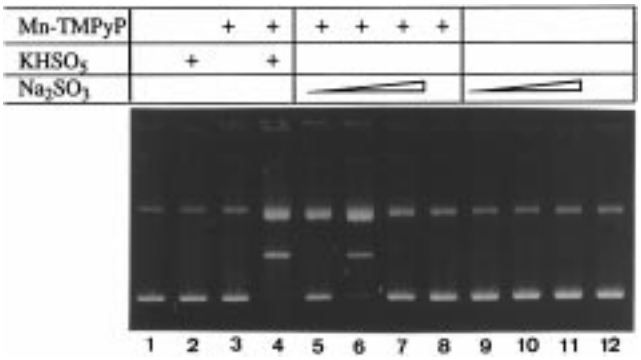

Figure 2. Cleavage of supercoiled ΦX174 DNA (19 μM bp) by Mn-TMPyP activated by $KHSO_5$ or Na_2SO_3 in 40 mM phosphate buffer, pH 7.5. The reaction times were 1 min ($KHSO_5$) or 10 min (Na_2SO_3) at room temperature. Lane 1: DNA only. Lane 2: 10 μM $KHSO_5$. Lane 3: 250 nM Mn-TMPyP (10 min). Lane 4: 50 nM Mn-TMPyP, 10 μM $KHSO_5$. Lanes 5−8: 250 nM Mn-TMPyP in the presence of 1 μM (5), 10 μM (6), 100 μM (7), and 1 mM (8) Na_2SO_3. Lanes 9−12: 1 μM (9), 10 μM (10), 100 μM (11), and 1 mM (12) Na_2SO_3.

cleaver able to operate under physiological conditions akin to those of metallobleomycin.[38,39] By comparison with previous results on activation of iron porphyrins in the presence of a variety of reducing agents where active intermediates are not clearly identified (hydrogen peroxide, peroxyl, hydroxyl, or superoxide radicals)[40,41] and on the basis of the above results, we can propose a high-valent oxo−manganese porphyrin as a likely intermediate in DNA cleavage promoted by the Mn-TMPyP/O_2/sulfite system (as previously shown for the Mn-TMPyP/$KHSO_5$ system[25]). In addition, we note that the higher concentrations of sulfite (lanes 7 and 8) inhibited the formation of DNA breaks, which is again consistent with sulfite reduction of the high-valent metal−oxo intermediate to inactive reduced metalloporphyrins. This suggests that ideal reaction conditions involve a low [HSO_3^-]:[O_2] ratio.

To perform a more detailed comparison of DNA oxidation from Mn-TMPyP-catalyzed reactions with HSO_5^- vs HSO_3^-/O_2, we carried out experiments with a 167-base-pair restriction fragment from pBR322. Use of polyacrylamide gel electrophoresis allowed comparison of the extents of reaction at specific nucleotides for the two oxidation systems, and the results are shown in Figure 3. As previously observed, Mn-TMPyP (500 nM) in the presence of $KHSO_5$ (750 nM) leads to direct strand breaks (Figure 3, lane 3) that are located on the 3′-side of each sequence consisting of three consecutive AT base pairs.[19,42,43] The direct strand breaks have been shown to be due to C5′-hydrogen atom abstraction from the deoxyribose group of the 3′-nucleoside adjacent the three-AT-base-pair site.[19,44] Treatment of the oxidized restriction fragment with hot piperidine revealed only a few additional alkali-labile sites in a guanine-rich segment near the 3′-end (Figure 3, lane 4). When the same experiments, with or without piperidine, were carried out with sulfite in place of monopersulfate, essentially identical results were obtained (Figure 3, lanes 8 and 9). The concentration of Na_2SO_3 required

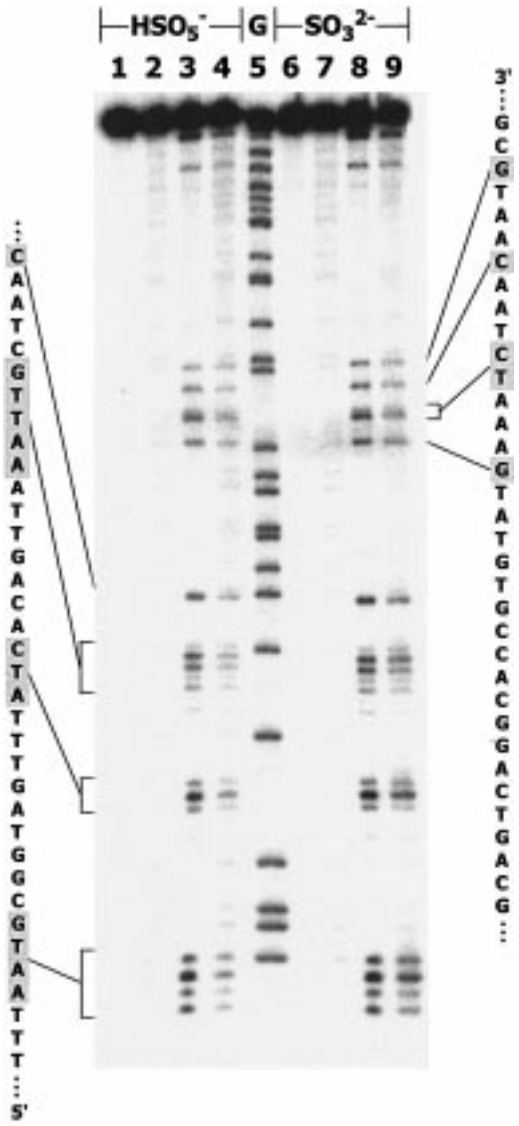

Figure 3. Polyacrylamide gel electrophoretic analysis of the cleavage of a 167-bp fragment from pBR322 by Mn-TMPyP and $KHSO_5$ or Na_2SO_3 in 10 mM phosphate/100 mM NaCl (pH 7.5). Lane 1: DNA + 750 nM $KHSO_5$. Lane 2: DNA + 750 nM $KHSO_5$, followed by piperidine treatment. Lane 3: DNA + 500 nM Mn-TMPyP and 750 nM $KHSO_5$. Lane 4: DNA + 500 nM Mn-TMPyP and 750 nM $KHSO_5$, followed by piperidine treatment. Lane 5: Maxam−Gilbert G sequencing lane.[48] Lane 6: DNA + 50 μM Na_2SO_3. Lane 7: DNA + 50 μM Na_2SO_3, followed by piperidine treatment. Lane 8: DNA + 500 nM Mn-TMPyP and 50 μM Na_2SO_3. Lane 9: DNA + 500 nM Mn-TMPyP and 50 μM Na_2SO_3, followed by piperidine treatment. Piperidine treatment consisted of reacting DNA samples with 60 μL of 0.2 M piperidine for 30 min at 90 °C.

to achieve the same level of DNA oxidation as with $KHSO_5$ was significantly higher, 50 μM compared to 750 nM, indicative of the lower efficiency of Mn^V═O formation via sulfite autoxidation. Nevertheless, the fact that the reactivity patterns are the same for the two oxidants strongly supports a common reactive intermediate believed to be the Mn^V═O species. The relatively low level of piperidine-labile guanine oxidation is further indication that free radical oxidants such as $SO_4^{\bullet -}$ are not involved, since the sulfate radical is well-known to react preferentially at guanine nucleobases.[20,45,46]

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Conclusion

On the basis of the results presented here, it is clear that biomimetic hydroxylation or epoxidation reactions can be mediated, in the presence of Mn-TMPyP as catalyst, by molecular oxygen and sulfite, two inexpensive and environmentally acceptable reagents. Use of isotopically labeled H_2O and O_2 allows a mechanistic interpretation consistent with oxo−hydroxo tautomerism of the key intermediate, a Mn^{V}═O species. This species is formed efficiently by oxygen atom transfer from $KHSO_5$, but also by Mn-catalyzed autoxidation of sulfite. Through examination of hydrocarbon oxidation (isopropylbenzoic acid and carbamazepine) and DNA oxidation (plasmid DNA and restriction fragments), as well as spectroscopic studies, support is found for the common intermediacy of a high-valent manganese−oxo species rather than diffusible free radicals typical of sulfite autoxidation.

In biology, this work represents an original approach to replace the use of $KHSO_5$ as oxygen atom donor by a more "physiological" system that may be applicable to DNA oxidation within cells. Furthermore, the use of HSO_3^-/O_2 in place of HSO_5^- avoids the presence of a large excess of a strong peracid oxidant in the medium and may allow selective oxidations to occur in the presence of sensitive functional groups.

Experimental Section

Materials. Potassium monopersulfate (the triple salt $[KHSO_5]_2$-$[KHSO_4][K_2SO_4]$, Curox) was a gift from Peroxid Chemie GmbH. Mn-TMPyP$(AcO)_5$ was prepared according to ref 18. Sodium sulfite was purchased from Fluka. **BA**, **BA-one**, and **CBZ** were purchased from Aldrich. **BA-ol** was prepared according to ref 29. **CBZ-oxide** was a gift from Novartis. $H_2{}^{18}O$ (96.5 atom %) and $^{18}O_2$ (96.8% atom) were supplied by Leman (Saint Quentin en Yvelines, France). Supercoiled ΦX174 DNA was purchased from Gibco BRL, and electrophoresis grade agarose, from Sigma. 5′-End-labeled restriction fragments from pBR322 were obtained by digestion with *Eco*RI restriction endonuclease, followed by dephosphorylation with calf intestinal alkaline phosphatase (New England Biolabs) and treatment with γ-[^{32}P]-dATP and T4 polynucleotide kinase. A second digestion with *Rsa*I restriction endonuclease yielded a 167- and a 514-base-pair fragment. The 167-base-pair fragment was purified by 8% preparative nondenaturing gel electrophoresis and isolated by the crush and soak method. All enzymes were obtained from new England Biolabs. γ-[^{32}P]-dATP (3000 Ci/mmol) was purchased from Amersham, and radioactivity was quantified by scintillation counting.

Instrumentation. HPLC analyses were performed on a Waters Millipore chromatograph equipped with a U6K injector, a diode array detector (Kontron Instrument), and a 10 mm Nucleosil C18 column (Interchrom). The eluent was a mixture of methanol/water (1/1, v/v) containing 5 mM ammonium acetate and 17 mM acetic acid or methanol/water (6/4, v/v), and detection was performed at 240 or 215 nm (for analyses of **BA** or **CBZ** derivatives, respectively). GC/MS analyses were performed on a Hewlett-Packard 5890 instrument using electron impact ionization at 70 eV. The GC column used was a nonpolar capillary column (12 m × 0.2 mm HL-1, cross-linked methylsilicone gum), and the carrier gas was helium. The injector temperature was 250 °C, and analyses were performed at 80 °C for 2 min and then up to 200 °C (10 °C/min), which was maintained for 15 min. MS spectra of **CBZ-oxide** were obtained on a Nermag R10/10H instrument by using the electronic impact method at 70 eV. Spectroscopic studies were carried out with a Beckman DU 650.

Oxidations with $KHSO_5$. The reaction medium (1 mL of water/acetonitrile or methanol (9/1, v/v)) contained 66 mM phosphate buffer, pH 5, 500 mM substrate (introduced as a 25 mM solution in CH_3CN for **BA** and **BA-ol** or a 5 mM solution in MeOH for **CBZ**), and the desired concentration of $KHSO_5$. Mn-TMPyP (10 mM) was then introduced as a 1 mM solution in water in five consecutive additions of 2 nmol each. The first addition initiated the reaction. Reactions were performed at room temperature and monitored by HPLC. Yields were calculated after 10 min from the initial amount of substrate by comparison with calibration curves.

Oxidations with Sulfite. The conditions were the same as described for $KHSO_5$, except that the reaction was initiated by addition of sulfite instead of Mn-TMPyP. Thus, Na_2SO_3 was introduced in five consecutive additions, every 2 min. In all cases, control experiments without catalyst gave a conversion of <3%.

$H_2{}^{18}O$ experiments. Solutions of the required concentrations and volume of buffer (500 FM) plus Mn-TMPyP (10 mM) and Na_2SO_3 (1 mM for oxidation of **BA** and **BA-ol**, 5 mM for oxidation of **CBZ**) were prepared separately and lyophilized using a Speed-Vac. Appropriate volumes of $H_2{}^{18}O$ were added (in this case the total volume was 500 mL), and the reactions were carried out as described above. After 10 min, the mixtures were saturated with NaCl and extracted with three 1-mL portions of CH_2Cl_2. The products resulting from oxidation of **BA** and **BA-ol** were converted to methyl esters with diazomethane (prepared with an Aldrich MNNG diazomethane generation apparatus), concentrated, and then analyzed by GC/MS. The samples resulting from oxidation of **CBZ** were directly analyzed by MS after concentration. The percentage of ^{18}O incorporated in each product was determined by the relative abundances of the peaks at *m*/*z* 252 or 254 (M^+ of [^{16}O]-**CBZ-oxide** or [^{18}O]-**CBZ-oxide**), *m*/*z* 163 or 165 ($[M - CH_3]^+$ of [^{16}O]-**BA-ol** or [^{18}O]-**BA-ol** methyl esters), and *m*/*z* 179 or 181 ($[M - CH_3]^+$ of [^{16}O]-**BA-one** or [^{18}O]-**BA-one** methyl esters). The values obtained were corrected by considering the ^{18}O content of the labeled water.

$^{18}O_2$ Experiments. The conditions are the same as described for $H_2{}^{18}O$ experiments. The reaction mixtures were placed in a Schlenk tube, degassed by three consecutive freeze−thaw cycles under vacuum, and then repressurized with $^{18}O_2$ (for the reaction without oxygen, the mixture was repressurized with N_2). The required amounts of Na_2SO_3 solution (previously degassed and stored under nitrogen) were then added via a gastight syringe to initiate the reactions. After 10 min, the samples were treated and analyzed as for $H_2{}^{18}O$ experiments.

Cleavage of ΦX174 Form I DNA. Supercoiled ΦX174 DNA (19 μM bp, 125 ng) 40 mM in phosphate buffer, pH 7.5, was allowed to react with Mn-TMPyP, at room temperature, in the presence of $KHSO_5$ or Na_2SO_3 in a final volume of 10 μL. DNA was not preincubated with Mn-TMPyP. The addition of $KHSO_5$ or Na_2SO_3 started the reaction that lasted 1 min with $KHSO_5$ or 10 min with Na_2SO_3 and was stopped by the addition of 1 μL of 1 M Hepes buffer, pH 8. The concentrations of Mn-TMPyP, $KHSO_5$, and Na_2SO_3 are indicated in caption of Figure 2. A volume of 5 μL of loading buffer (containing bromophenol blue and 50% glycerol in 10 mM Tris/HCl buffer, pH 8) was added to the samples before they were deposited in the wells of an 0.8% agarose gel containing 1 μg/mL ethidium bromide. The electrophoresis migration was at 25 mA overnight in 89 mM Tris/borate buffer, pH 8.3, with 2.5 mM EDTA. Fluorescent bands of DNA were then photographed under UV light.

Cleavage of Restriction Fragments. DNA experiments were conducted as previously described.[20] The 167-bp restriction fragment from pBR322 (9 nCi) and 80 μM calf thymus DNA in 10 mM phosphate/100 mM NaCl buffer (pH 7.5) were treated with 500 nM Mn-TMPyP and incubated at room temperature for 10 min, followed by the addition of 750 nM $KHSO_5$ or 50 μM Na_2SO_3 in a final volume of 50 μL. The reactions were stopped after 10 min by the addition of 2 μL of 50 mM Hepes/250 mM EDTA (pH 7). The samples were extracted with 60 μL phenol/chloroform to remove Mn-TMPyP (which interferes with piperidine treatment[47]) and dialyzed.

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