Singlet Oxygen Takes Part in 8-Hydroxydeoxyguanosine Formation in Deoxyribonucleic Acid Treated with the Horseradish Peroxidase–H_2O_2 System

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Treatment of calf thymus deoxyribonucleic acid (DNA) with the horseradish peroxidase–H_2O_2 system resulted in efficient formation of 8-hydroxydeoxyguanosine (8-OH-dG) residues. It was concluded that singlet oxygen was the reactive species involved, based on experiments using active oxygen scavengers and D_2O. For 8-OH-dG formation, a higher-ordered polynucleotide structure seems to be required: double stranded DNA was a better substrate for the reactive species than single stranded DNA, and monomeric deoxyguanosine underwent C8-hydroxylation to a lesser extent.

Keywords horseradish peroxidase–H_2O_2; DNA; 8-hydroxydeoxyguanosine; singlet oxygen; active oxygen scavenger

It is known that active oxygen species generated from a variety of environmental and physiological sources cause oxidative deoxyribonucleic acid (DNA) damage such as base hydroxylations and strand breaks, *etc.*[1] 8-Hydroxydeoxyguanosine (8-OH-dG) formation is one such type of DNA damage. Since the 8-OH-dG residue in DNA is known to induce misreading during DNA synthesis *in vitro*,[2] this damage is considered to be mutagenic and/or carcinogenic. We previously reported that 8-OH-dG was formed in DNA when treated with bleomycin–Fe(II)[3] or 4-(acyloxyamino)quinoline 1-oxide.[4,5] The present study revealed that the horseradish peroxidase–H_2O_2 system gives rise to an appreciable formation of 8-OH-dG residues in DNA and that singlet oxygen takes part in 8-OH-dG formation. Experimental work leading to this unexpected finding is described.

Materials and Methods

Chemicals and Enzymes Calf thymus DNA, nuclease P1 from *Penicillium citrinum* and superoxide dismutase were purchased from Sigma Chemical Co. *E. coli* alkaline phosphatase was from Boehringer Mannheim GmbH. Horseradish peroxidase and catalase were from Tokyo Kasei Kogyo Co., Ltd.

Treatment of DNA with Horseradish Peroxidase–H_2O_2 The general procedure is as follows. A reaction mixture (1 ml) containing 1 mM·(P) DNA (6.5 OD/ml), 36.6 mM NaCl, 15 mM tris(hydroxymethyl)aminomethane (Tris)–HCl buffer (pH 7.0), and various concentrations of horseradish peroxidase–H_2O_2 was incubated at 37 °C for 60 min. Then, the solution was dialyzed against 200 ml of cold H_2O for 6 h. During dialysis, H_2O was changed every 2 h. After DNA was recovered by lyophilization, 300 μl of nuclease P1 solution (20 μg in 300 μl of 20 mM CH_3COONa buffer (pH 4.8)) was added, and the mixture was incubated at 37 °C for 30 min. Then, 300 μl of the alkaline phosphatase solution (2.6 units in 300 μl of 100 mM Tris–HCl buffer (pH 7.5)) was added and the mixture was further incubated at 37 °C for 60 min. For deproteinization, 500 μl of $CHCl_3$ were added and the mixture was shaken vigorously for 10 min. After centrifugation (3000 rpm, 10 min), the upper layer was separated and used for analysis of 8-OH-dG.

Quantification of 8-OH-dG The amount of 8-OH-dG in the DNA digest was determined by use of an high performance liquid chromatography (HPLC) apparatus (Jasco, Tri Rotar-V) equipped with a UV detector (Uvidec-10-V) and an electrochemical detector (Tosoh, EC-8010). The 8-OH-dG content was quantified by an electrochemical detector with the electrode potential at 0.6 V, and the contents of other nucleosides were quantitated by UV absorbance at 254 nm. A LiChrospher 100 RP-18(e) column (Merck, 4 × 125 mm) was used and eluted with 10 mM NaH_2PO_4–8% MeOH at a flow rate of 0.7 ml/min.

Each experiment was repeated two to three times to check reproducibility, and data points represent the means of at least duplicate samples of an experiment.

Results

Formation of 8-OH-dG in DNA Treated with Peroxidase–H_2O_2 Calf thymus DNA was treated with horseradish peroxidase (0.2 mg/ml) and H_2O_2 (1.5 mg/ml) in Tris–HCl buffer (pH 7.0) at 37 °C for the indicated times, then subjected to quantitative analysis of 8-OH-dG thereby produced, as described in Materials and Methods. As shown in Fig. 1, the amount of 8-OH-dG increased with an increase in incubation time, and the reaction was almost complete after a 1 h incubation. Next, the dependence of

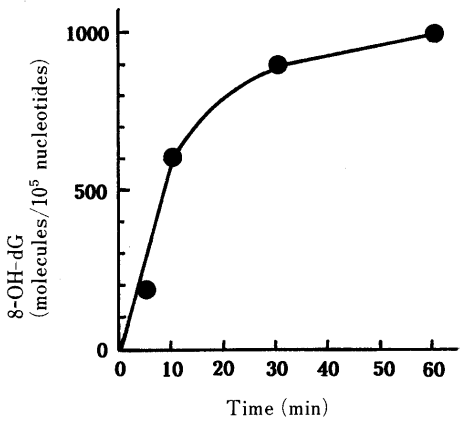

Fig. 1. Dependence on the Reaction Time

DNA was incubated with peroxidase (0.2 mg/ml)–H_2O_2 (1.5 mg/ml) in Tris–HCl buffer (pH 7.0) at 37 °C for the indicated time. See Materials and Methods.

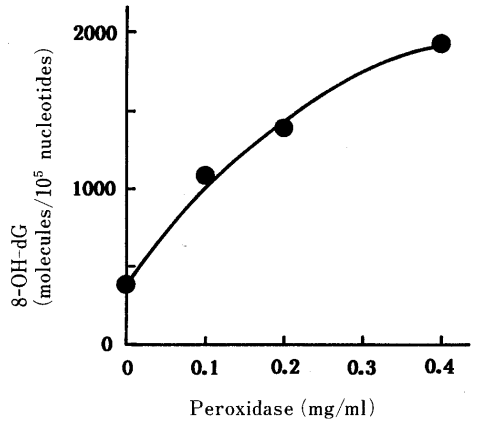

Fig. 2. Dependence on the Concentration of Peroxidase

H_2O_2 (3 mg/ml) and 1 h incubation were employed. See the legend of Fig. 1.

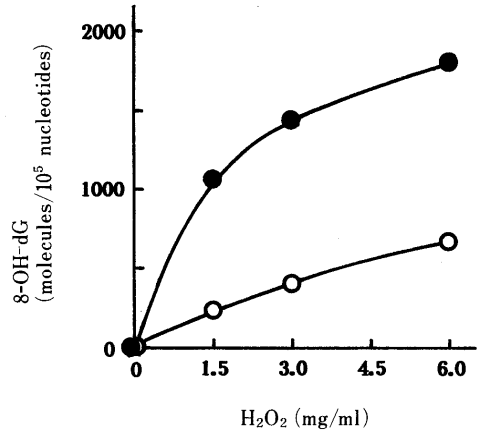

Fig. 3. Dependence on the Concentration of H_2O_2

DNA was incubated with H_2O_2 in the presence (—●—) or absence (—○—) of peroxidase (0.2 mg/ml). See the legend of Fig. 1.

TABLE I. Dependence on a Higher-Order Structure of DNA

Treatment[a]	8-OH-dG[b]	
Peroxidase (mg/ml)/H_2O_2 (mg/ml)	ds-DNA	ss-DNA[c]
0.1/0.75	435	n.t.
0.2/1.5	1100	860
0.3/2.25	1835	n.t.
0.8/6.0	2370	1220

a) See Materials and Methods. *b*) Molecules/10^5 nucleotides. *c*) ss-DNA was prepared by heating ds-DNA at 100 °C for 1 h and then cooled immediately in ice-cold water. n.t.: not tested.

8-OH-dG formation on the concentration of peroxidase was examined by treating DNA with 3 mg/ml of H_2O_2 for 1 h. As shown in Fig. 2, 8-OH-dG formation increased with an increase in the peroxidase concentration. When peroxidase (0.2 mg/ml) which had been denaturated at 100 °C for 1 h was used, the amount of 8-OH-dG was reduced to 39% of that of the undenatured control. Thirdly, the concentration of peroxidase was fixed at 0.2 mg/ml and the dependence on the H_2O_2 concentration was examined. As shown in Fig. 3, the amount of 8-OH-dG increased with an increase in the concentration of H_2O_2 employed. Without H_2O_2, peroxidase alone was unable to generate 8-OH-dG. On the other hand, H_2O_2 alone resulted in 8-OH-dG formation in a dose-dependent manner, but to a much lesser extent, as shown in Fig. 3. In subsequent experiments, the concentration ratio (w/w) of H_2O_2 *vs.* peroxidase was fixed at 7.5. It is to be noted that *tert*-butyl hydroperoxide (1.5 mg/ml) could not be substituted for H_2O_2 in 8-OH-dG formation, even in the presence of peroxidase.

Dependence of 8-OH-dG Formation on the Structure of DNA In order to examine the dependence of 8-OH-dG formation on a higher-order structure of DNA, double-stranded (ds-) and single-stranded (ss-) DNA was treated with peroxidase–H_2O_2 (Table I). At a higher concentration (H_2O_2 6.0 mg/ml, peroxidase 0.8 mg/ml), ds-DNA produced twice the amount of 8-OH-dG as ss-DNA. At a lower concentration (H_2O_2 1.5 mg/ml, peroxidase 0.2 mg/ml), ds-DNA also produced more 8-OH-dG than ss-DNA, but the difference was not as great as observed with the higher concentration.

Importance of Polymeric Nucleotide Structure in 8-OH-dG Formation DNA (1 mM·(P)) was treated with peroxidase–H_2O_2 in the presence of 2′-deoxyguanosine (dG, 2 mM) or 2′-deoxyguanosine-5′-monophosphate (dGMP, 2 mM). In calf thymus DNA, one fifth of the DNA bases are guanine; therefore, the mixture contained about a 10-equimolar dG or dGMP concentration. After 1 h incubation, the DNA solution was dialyzed against H_2O and the amount of 8-OH-dG in DNA was quantified. (Table II). Even in the presence of such a large excess of the monomer, the amount of 8-OH-dG formed in DNA decreased slightly to 65—68% of that of the control. The amount of 8-OH-dG derived from monomeric dG was also analyzed after the reaction mixture was dialyzed against H_2O, and subsequently the outer solution was collected and concentrated to a small quantity (Table II). In the presence of DNA, the amount of 8-OH-dG derived from monomeric dG was 210 molecules per 10^5 dG. It is worth noting that this value almost accounts for the shortage of 8-OH-dG in DNA due to the presence of 10-equimolar dG; this shortage was (5100−3450=) 1650 molecules per 10^5 dG residues, while the 8-OH-dG formed from 10-equimolar monomeric dG amounted to 2100 molecules per 10^6 dG (calculated from 210 molecules/10^5 dG). In the absence of DNA, the 8-OH-dG derived from monomeric dG was far less than that derived in its presence.

TABLE II. Effect of Monomer on 8-OH-dG Formation

Treatment[a]	8-OH-dG	
	In DNA	IN monomeric dG
DNA	1020[b] (5100)[c]	—
DNA + dG (2 mM)	690[b] (3450)[c]	210[d]
DNA + dGMP (2 mM)	660[b] (3300)[c]	n.t.
dG (2 mM)	— —	20[d]

a) Peroxidase (0.2 mg/ml) and H_2O_2 (1.5 mg/ml) were employed. See Materials and Methods. *b*) Molecules/10^5 nucleotides. *c*) Molecules/10^5 dG residues. *d*) Molecules/10^5 dG. n.t.: not tested.

TABLE III. Effects of Buffer and pH[a]

pH	8-OH-dG[b]	
	Phosphate buffer	Tris–HCl buffer
5.0	575	—
7.0	355	1020
9.0	52	—

a) DNA was incubated with peroxidase (0.2 mg/ml)–H_2O_2 (1.5 mg/ml) in the indicated 15 mM buffer at 37 °C for 1 h. *b*) Molecules/10^5 nucleotides.

Dependence of 8-OH-dG Formation on the Buffer and pH of the Reaction Medium Formation of 8-OH-dG was examined in a phosphate buffer (15 mM, pH 7.0) which was substituted for the Tris–HCl buffer (15 mM, pH 7.0) (Table III). The amount of 8-OH-dG decreased to 34% of that in the Tris–HCl buffer. The effect of pH on 8-OH-dG formation was examined using a phosphate buffer. The amount of 8-OH-dG increased at pH 5 and decreased at pH 9. This pH dependence coincides with that reported for peroxidase activity of this enzyme.

Active Oxygen Species Involved in 8-OH-dG Formation In order to determine the reactive oxygen species involved

TABLE IV. Effect of Active Oxygen Scavenger[a]

Scavenger	8-OH-dG[b]	
	Tris–HCl buffer	Phosphate buffer
None	1050 (100)[c]	360 (100)[c]
SOD (100 μg/ml)	1370 (130)[c]	600 (167)[c]
D-Mannitol (10 mM)	1060 (101)[c]	380 (106)[c]
L-Histidine (10 mM)	60 (6)[c]	0 (0)[c]
Catalase (100 μg/ml)	0 (0)[c]	0 (0)[c]
Sodium azide (10 mM)	400 (38)[c]	
2,5-Dimethylfuran (10 mM)	1010 (96)[c]	

a) DNA was incubated with peroxidase (0.2 mg/ml)–H_2O_2 (1.5 mg/ml) in the presence of a scavenger in 15 mM buffer at 37 °C for 1 h. *b*) Molecules/10^5 nucleotides. *c*) Relative value (%).

TABLE V. Effect of D_2O[a]

D_2O (%)	8-OH-dG[b]
0	910 (100)[c]
30	946 (104)[c]
70	1750 (192)[c]

a) DNA was incubated with peroxidase (0.2 mg/ml)–H_2O_2 (1.5 mg/ml) in Tris–HCl buffer containing D_2O at 37 °C for 1 h. *b*) Molecules/10^5 nucleotides. *c*) Relative value (%).

in 8-OH–dG formation, the reaction was carried out in the presence of various types of active oxygen scavengers, *i.e.*, superoxide dismutase (SOD), catalase, D-mannitol, L-histidine, 2,5-dimethylfuran and sodium azide. As shown in Table IV, remarkable inhibitions were observed with catalase, L-histidine and sodium azide. The inhibition by catalase may arise from instantaneous decomposition of H_2O_2, as shown in Fig. 3, where H_2O_2 was crucial for 8-OH–dG formation. L-Histidine is known to react with singlet oxygen, as well as the OH radical, at a high reaction rate. Sodium azide is a strong singlet oxygen quencher. These results, together with the finding that D-mannitol, an OH radical scavenger, was not effective, suggest that singlet oxygen may be involved in 8-OH–dG formation. 2,5-Dimethylfuran is reported to be a reagent for singlet oxygen capture, but the reaction rate is very low. Among the scavengers employed, only SOD remarkably enhanced 8-OH–dG formation.

8-OH–dG Formation in D_2O In D_2O, singlet oxygen is more stable than in H_2O.[6] Therefore, if singlet oxygen is involved in 8-OH–dG formation, the reaction in D_2O should promote its formation. As shown in Table V, the amount that 8-OH–dG increased in 70% D_2O, was nearly twice the amount as in H_2O.

Discussion

Treatment of DNA with a horseradish peroxidase–H_2O_2 system known to oxidize the substrate AH_2 to HA–AH or A, resulted in unexpected 8-OH–dG formation, and there is a strong possibility that the reaction species involved is singlet oxygen. Horseradish peroxidase may have a similar activity to myeloperoxidase, which catalizes the following reaction: $H_2O_2 + X^- \rightarrow HOX + OH^-$, where X is a halogen. The HOX thus produced may react with H_2O_2 to generate singlet oxygen. Alternatively, as Kanofsky reported in a review,[7] the Russell mechanism, which demonstrated that the bimolecular reaction of peroxy radicals generates singlet oxygen, may be involved. Floyd *et al.* recently demonstrated that 8-OH–dG was formed in DNA treated with methylene blue plus light,[8] which is a typical method for generating singlet oxygen. They concluded that singlet oxygen participated in 8-OH–dG formation. We reported previously that the treatment of DNA with bleomycin–Fe(II) resulted in 8-OH–dG formation, although the reaction species involved was not conclusively determined.[3] Using the same scavengers employed in the present study, we found that singlet oxygen was also the reactive species in the case of bleomycin–Fe(II). Details will be published elsewhere.

The fact that monomeric dG produces 8-OH–dG in the presence of DNA suggests that the reaction proceeds indirectly. Thus, the phosphodiester moiety of DNA may be an initial target for singlet oxygen to generate a reactive intermediate. Studies on this matter are in progress.

The reason for the difference in 8-OH–dG formation between a phosphate buffer and the Tris–HCl buffer is still unclear. The decrease in 8-OH–dG formation in a phosphate buffer is not due to the low concentration of Cl^-. Addition of 50 mM NaCl in a phosphate buffer so as to adjust the level of Cl^- concentration to that of the Tris–HCl buffer did not raise the level of 8-OH–dG formation, but rather, brought it down further to some extent. Several studies describe the difference in the free radical-trapping property of phosphate and Tris–HCl buffers.[9,10]

SOD enhanced 8-OH–dG formation (Table IV). The metal of SOD may work as a catalyst to promote the Fenton-type oxidation reaction. In fact, when DNA was allowed to react with H_2O_2 (1.5 mg/ml) in the presence of SOD (100 μg/ml), 8-OH–dG formation was remarkably enhanced. This was also the case when heat-denatured (100 °C, 15 min) SOD was employed (data not shown). Therefore, the increase in 8-OH–dG might have stemmed from a mechanism involving OH radical hydroxylation.

In conclusion, there seem to be two mechanisms for 8-OH–dG formation by active oxygen species. In one case, as previously documented,[11] the OH radical directly attacks position-8 of the dG moiety, which is the site most susceptible to free radical substitution.[12] In the other case, as proposed in the present study, singlet oxygen initiates hydroxylation at the same site. It is of interest that the OH radical produces 8-OH–dG with monomeric dG more readily than the dG moiety of DNA,[11] whereas singlet oxygen reacts preferentially with the dG moiety of DNA. The molecular mechanism involved in singlet oxygen-mediated formation of 8-OH–dG is now open for future study. It is worth adding that 8-OH–dG is also produced through an ionic mechanism which operates in DNA modification by 4-(acyloxyamino)quinoline 1-oxide and its analogs, as we previously proposed.[4,5]

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