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Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents

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ABSTRACT

The C-8 position of deoxyguanosine (dGuo) was hydroxylated by ascorbic acid in the presence of oxygen (O_2) in 0.1 M phosphate buffer (pH 6.8) at 37°C. Addition of hydrogen peroxide (H_2O_2) remarkably enhanced this hydroxylation. The Udenfriend system [ascorbic acid, Fe^{II}, ethylenediaminetetraacetic acid (EDTA) and O_2] was also effective for hydroxylation of dGuo in high yield. Guanine residues in DNA were also hydroxylated by ascorbic acid. Other reducing agents, such as hydroxylamine, hydrazine, dihydroxymaleic acid, sodium bisulfite and acetol, were also effective for the hydroxylation reaction, as were metal-EDTA complexes (Fe^{II}-, Sn^{II}-, Ti^{III}-, Cu^{I}-EDTA). An OH radical seemed to be involved in this hydroxylation reaction in most of the above hydroxylating systems, but another reaction mechanism may also be involved, particularly when dGuo is hydroxylated by ascorbic acid alone or ascorbic acid plus H_2O_2. The possible biological significance of the hydroxylation of guanine residues in DNA in relation to mutagenesis and carcinogenesis is discussed.

INTRODUCTION

We have found that 8-hydroxy-isopropylideneguanosine was formed when isopropylideneguanosine was incubated with heated glucose.[1] To elucidate the mechanism of the hydroxylation of the guanosine derivative at the C-8 position, we investigated the abilities of various reagents to hydroxylate deoxyguanosine (dGuo, 1). A mixture of ascorbic acid, Fe^{II}, ethylenediaminetetraacetic acid (EDTA) and O_2 (Udenfriend system)[2] was found to be effective for this reaction. This system was chosen since it has been reported that it is effective for hydroxylation of many aromatic compounds at electronegative positions.[3] Ascorbic acid is also known to be involved in the hydroxylations of aromatic compounds *in vivo*[4,5] and in the hydroxylation of the proline residue in collagen biosynthesis.[6] Ascorbic acid has biological activities such as antiviral[7] and mutagenic[8] activities, which are generally thought to result from DNA cleavage by free radicals produced by ascorbic acid.[9] These activities might involve hydroxylation of guanine residue of DNA at position C-8. Therefore, as described in this paper, we

studied the hydroxylation of deoxyguanosine by ascorbic acid in more detail. We also examined the hydroxylations by other reducing agents, including metals. The mechanism of this reaction and its biological significance are discussed.

EXPERIMENTAL SECTION

Synthesis of 8-hydroxydeoxyguanosine by the Udenfriend system

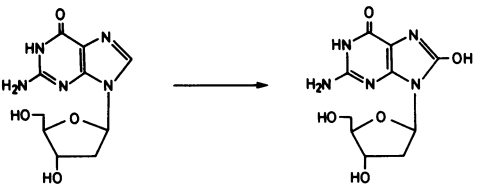
Standard samples of 8-hydroxyguanosine (8-OH-Guo) and 8-hydroxydeoxyguanosine (8-OH-dGuo) were prepared on a large scale from guanosine (Guo) and deoxyguanosine (dGuo), respectively, with the Udenfriend system for use in quantitative determinations by high performance liquid chromatography (HPLC). The 8-OH-Guo synthesized with the Udenfriend system showed the same spectral characters and chromatographic behavior as an authentic sample of 8-OH-Guo synthesized from 8-bromoguanosine by the method by Ikehara et al.[10] This is the first report of the synthesis of 8-OH-dGuo.

For the synthesis, deoxyguanosine (1 g) was dissolved in 780 ml of 0.13 M sodium phosphate buffer (pH 6.8) in a 1 l Erlenmyer flask and 140 ml of 0.1 M ascorbic acid, 65 ml of 0.1 M EDTA and 13 ml of 0.1 M $FeSO_4$ were added successively. Oxygen gas was then bubbled through the solution, controlling the temperature of the solution at 37°C in a water bath. After 3-hr reaction in the dark, the solution was adjusted to pH 3.7 with 1 N HCl and charcoal powder (10 g) was added with stirring. The charcoal was recovered by filtration and packed in a column (2 x 13 cm). The column was washed with H_2O (500 ml) and then material was eluted with 500 ml of aqueous acetone (1:1, v/v). The eluate was evaporated to dryness and the residue was fractionated by preparative HPLC [column: TSK-GEL LS-410 ODS SIL (2.15 x 30 cm) from Toyo Soda Ld., solvent: 15% aqueous MeOH]. The small peak eluted just after the main peak of dGuo was collected and evaporated to dryness. The residue was crystallized from water. Yield: 95 mg. MS (pentatrimethylsilyl derivative): m/z, 643 (M^+), 628 (M^+-CH_3), 383 (b+H), 368 (b+H-CH_3). ^{1}H-NMR (DMSO-d_6): δ (ppm) 10.87 (1H, s, 1-NH or 8-OH), 10.82 (1H, s, 1-NH or 8-OH), 6.52 (2H, s, NH_2), 6.15 (1H, t, J=7.5 Hz, 1'-H), 5.23 (1H, d, J=4.3 Hz, 3'-OH), 4.92 (1H, dd, J=4.7, 4.8 Hz, 5'-OH), 4.45 (1H, m, 3'-H), 3.86 (1H, m, 4'-H), 3.61 (2H, ABX, 5'-H), 3.09 (1H, m, 2'-H), 2.04 (1H, m, 2'-H). UV (H_2O): λ_{max} (ε), 245 (12,300), 293 (10,300).

RESULTS

Hydroxylation of dGuo by the Udenfriend system

Many aromatic compounds have been hydroxylated at electronegative



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positions by the Udenfriend system.[3] When a solution of dGuo (1) in 0.1 M phosphate buffer (pH 6.8) was shaken with this system at 37°C under air, 8-hydroxydeoxyguanosine (8-OH-dGuo, 2) was formed and detected by HPLC as shown in Fig. 1 (The compound 2 may tautomerize to 8-keto form in aqueous solution but at present we tentatively call the compound as 8-hydroxydeoxyguanosine). The time course of formation of 8-OH-dGuo is shown in Fig. 2. It was found that 8-OH-dGuo was also formed with either ascorbic acid or Fe^{II}-EDTA alone, although in lower yield (Fig. 2). However, when these two reagents were combined at the same concentrations to form the Udenfriend system, three times more 8-OH-dGuo was formed than the additive yield with

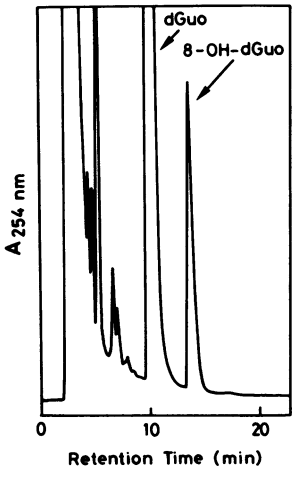

Fig. 1. Detection of 8-OH-dGuo by HPLC in a reaction mixture of dGuo with the Udenfriend system.

Reaction mixture (1 ml) containing 2.5 mM dGuo, 14 mM ascorbic acid, 6.5 mM EDTA, 1.3 mM $FeSO_4$ and 0.1 M sodium phosphate (pH 6.8) was shaken at 37°C under air in the dark. After an appropriate time, 20 µl of the reaction mixture was injected into an HPLC column [instrument: Shimadzu LC-3A, column: Merck Hybar column, LiChroscorb RP-18, 5 µ, solvent: 10% MeOH, 10 mM ammonium acetate (pH 5.3), flow rate: 0.8 ml/min].

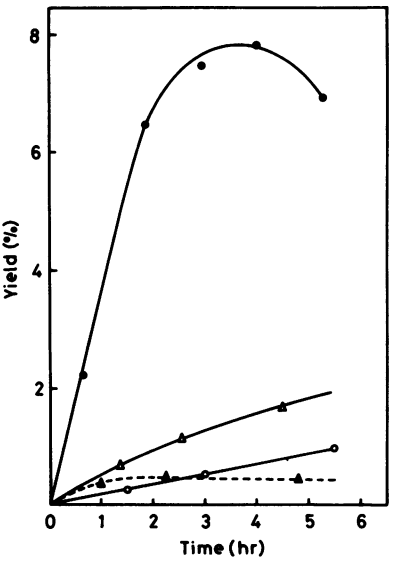

Fig. 2. Formation of 8-OH-dGuo by the Udenfriend system and other reagents.
The yield was calculated from the peak height of 8-OH-dGuo on HPLC. The reaction mixture contained 2.5 mM dGuo, 0.1 M sodium phosphate (pH 6.8) and ●—● 14 mM ascorbic acid + 1.3 mM $FeSO_4$ + 6.5 mM EDTA, Δ—Δ 14 mM ascorbic acid, o—o 14 mM ascorbic acid + 1.3 mM $FeSO_4$, or ▲—▲ 1.3 mM $FeSO_4$ + 6.5 mM EDTA.

each reagent separately (Fig. 2). This result suggests that a special ternary complex was formed between ascorbic acid and Fe^{II}-EDTA that stimulated formation of an active oxygen which might be responsible for the hydroxylation. Addition of Fe^{II} alone inhibited the hydroxylation (Fig. 2).

Stimulation of the hydroxylation by molecular oxygen and hydrogen peroxide

The hydroxylation of aromatic compounds by Udenfriend system is reported to require molecular oxygen (O_2).[3] To confirm that O_2 was involved in the hydroxylation of dGuo, we examined the reaction in the presence and absence of O_2. As shown in Fig. 3, the formation of 8-OH-dGuo was considerably greater when O_2 gas was bubbled through the reaction solution, than that under air. On the other hand, when O_2 was completely removed by bubbling the reaction mixture with N_2 gas, no hydroxylation was observed. Addition of hydrogen peroxide (H_2O_2) to the reaction mixture also greatly stimulated the hydroxylation of dGuo, although H_2O_2 itself had no hydroxylating activity. From these findings, it is likely that active oxygen is involved in the hydroxylation of dGuo.

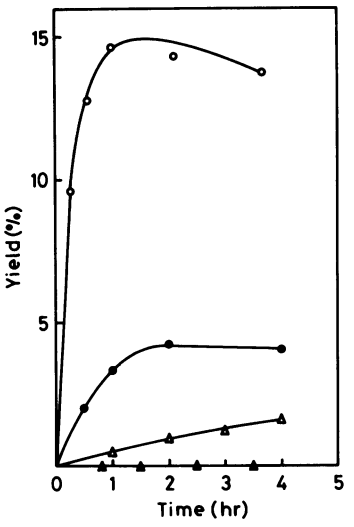

Fig. 3. Stimulation of the hydroxylation by O_2 and H_2O_2.
The reaction conditions and determination of the yield were as for Figs. 1 and 2. The reaction mixture contained 2.5 mM dGuo, 0.1 M sodium phosphate (pH 6.8) and o—o 14 mM ascorbic acid + 50 mM H_2O_2, ●—● 14 mM ascorbic acid + O_2 (bubbling), Δ—Δ 14 mM ascorbic acid, or ▲—▲ 14 mM ascorbic acid + N_2 (bubbling).

Formation of 8-OH-dGuo by other reducing agents and metals and its inhibition by EtOH

The formation of 8-OH-dGuo by other reducing agents was investigated. Various reducing agents, such as hydroxylamine, hydrazine, dihydroxymaleic acid, sodium bisulfite and acetol, were found to be effective for the hydroxylation. The yields of 8-OH-dGuo after treatments of dGuo with these agents for 1 hr are shown in Table 1. Hydroxylamine, hydrazine and dihydroxymaleic acid showed stronger hydroxylation activity than ascorbic acid. Hydroxylation of dGuo was also observed when an aqueous solution of dGuo was shaken with the Fe^{II}-, Sn^{II}-, Ti^{III}- or Cu^{I}-EDTA complex at 37°C. These metal-EDTA complexes were previously reported to be effective for hydroxylation of aromatic compounds.[11,12] The yields of 8-OH-dGuo with these metal-EDTA systems are shown in Table 2. Fe-EDTA gave the highest yields.

Next we studied the speices of oxygen radical involved in the formation of 8-OH-dGuo by comparing the yields of 8-OH-dGuo with and without 10% EtOH, added to the reaction mixture as an OH radical scavenger. As shown in

Table 1. Yields of 8-OH-dGuo with various reducing agents and their inhibitions by EtOH.

Reducing agent	Yield (%)	Yield (+EtOH) (%)	Inhibition by EtOH (%)
hydroxylamine	4.59	1.77	61.4
hydrazine	3.48	0.75	78.4
dihydroxymaleic acid	2.70	1.10	59.3
ascorbic acid	1.65	3.80	0
sodium bisulfite	0.58	0.02	96.6
acetol	0.23	0.09	61.0
Udenfriend system[a)]	5.98	0.29	95.2
ascorbic acid + H_2O_2[b)]	14.7	14.7	0

The reaction mixture (2 ml) containing 1.3 mM dGuo, 10 mM reducing agent and 0.1 M sodium phosphate buffer (pH 6.8) was shaken at 37°C under air. After reaction for one hour in the dark, 20 µl of the reaction mixture was injected into an HPLC column, and the yield of 8-OH-dGuo was determined. a) 10 mM ascorbic acid, 1 mM $FeSO_4$ and 5 mM EDTA. b) 10 mM ascorbic acid and 50 mM H_2O_2.

Tables 1 and 2, the formations of 8-OH-dGuo with most reducing agents and metal-EDTA complexes were inhibited by EtOH. For example, the hydroxylations with sodium bisulfite, the Udenfriend system, Fe^{II}-EDTA and Sn^{II}-EDTA were inhibited more than 95% by EtOH. These results suggested that the major active oxygen species responsible for the hydroxylation of dGuo is the OH radical. However, the hydroxylations with ascorbic acid, ascorbic acid plus H_2O_2 and the Cu^{I}-EDTA complex were not inhibited by EtOH. In fact, with ascorbic acid , the formation of 8-OH-dGuo was enhanced by EtOH, probably because the O_2 concentration was higher since O_2 is several times

Table 2. Yields of 8-OH-dGuo with metal-EDTA-complexes and their inhibitions by EtOH.

Metal-EDTA-complexes	Yield (%)	Yield (+EtOH) (%)	Inhibition by EtOH (%)
Fe^{II}-EDTA	2.9	0.04	98.6
Sn^{II}-EDTA	0.51	0.0	100
Ti^{III}-EDTA	0.33	0.14	57.6
Cu^{I}-EDTA	0.26	0.35	0

The reaction conditions and method for measuring the yield were as for Table 1, except that 5 mM Metal-EDTA was used instead of 10 mM reducing agent.

Table 3. Hydroxylation of guanine residues in nucleic acids by ascorbic acid.

Nucleic acid	8-OH-dGuo/10^4 dGuo
native calf thymus DNA	9.4
denatured calf thymus DNA[a)]	15.6
poly(dG-dC)·poly(dG-dC)	4.3
poly(dG-dC)·poly(dG-dC) + 4 M NaCl	5.5
poly G[b)]	22.2
dGuo (control)	149.0

A solution of 5 A_{260} units of each nucleic acid in 350 µl of 0.1 M sodium phosphate buffer (pH 6.8) containing 14 mM ascorbic acid was shaken at 37°C for 3 hr in the dark. The modified DNA was dialized against distilled water and recovered by EtOH precipitation. It was digested with nuclease P_1 and then E. coli alkaline phosphatase and analysed by HPLC. The quantity of 8-OH-dGuo or 8-OH-Guo was determined from its peak height. a) hyperchromicity 26%, b) for separation of 8-OH-Guo from Guo, 5% aqueous MeOH containing 10 mM ammonium acetate (pH 5.3) was used for elution.

more soluble in EtOH than in water. These hydroxylations, must occurs by some mechanism no involving an OH radical.

Hydroxylation of guanine residues in nucleic acid by ascorbic acid

Heat-denatured and native calf thymus DNA, poly(dG-dC)·poly(dG-dC) and poly G were treated with ascorbic acid in 0.1 M phosphate buffer (pH 6.8) at 37°C under air for 3 hr. Then the nucleic acids were isolated, digested successively with nuclease P_1 and alkaline phosphatase and analyzed by HPLC. The results in Table 3 show that guanine residues in heat-denatured DNA were more efficiently hydroxylated than those in native DNA. The hydroxylations of poly(dG-dC)·poly(dG-dC) in low salt (0.1 M sodium phosphate) and high salt (4 M NaCl) conditions were not significantly different. Modification of guanine bases was highest in poly G, probably because the guanine residues in poly G are sterically most open to hydroxylating reagents.

DISCUSSION

In this work we found that the C-8 position of dGuo was hydroxylated by ascorbic acid and other reducing agents under aerobic conditions. This type of modification is unique in that the active oxygen formed by reducing agents and O_2 reacts directly with one component of DNA. The guanine residue in DNA was also hydroxylated by ascorbic acid. On HPLC analysis, 8-OH-dGuo was the only detectable modified deoxynucleoside in DNA treated with ascorbic acid. Furthermore, base analysis of DNA before and after

ascorbic acid treatment showed no noticeable degradation of base residues. It is well established that the OH radical produced by ionizing radiation is responsible for DNA damage, such as strand breaks[13] or modification of pyrimidine bases.[14] Strand breaks were also observed on treatment of DNA with ascorbic acid in the presence of O_2.[15] Other reducing agents may also cause the same type of DNA damage in addition to hydroxylation of dGuo, because they produce the oxygen radical in the presence of O_2.

It is tempting to speculate that in addition to strand scissions and pyrimidine base modifications, hydroxylation of guanine residues in DNA is also involved in mutagenesis and carcinogenesis. This speculation is supported by the fact that many of the reducing agents and metals used for hydroxylation of dGuo in this study are mutagenic or carcinogenic.[8,16,17] Moreover, many mutagens and carcinogens are known to modify guanine residues in DNA at the C-8 position.[18] Furthermore, many mutagens and carcinogens are thought to act partly through the generation of oxygen radicals.[19] Ascorbic acid in the presence of oxygen was found to induce reverse mutations in S. typhimurium and chromosome aberrations in human fibroblast cells and to trigger DNA repair synthesis.[8] Ascorbic acid was also reported to have cocarcinogenic[20] and tumor promoting[21] activities. It is reported that 8-OH-Guo favors the syn-conformation.[22] Possibly structural and conformational alteration of the DNA molecule induced by 8-hydroxylation of an guanine residue may result in increased mispairing during DNA replication or DNA repair. Studies are now in progress to elucidate the biological significance of hydroxylation of guanine residues in DNA.

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