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SPIN TRAPPING STUDY ON THE GENERATION MECHANISM OF ACTIVE OXYGEN RADICALS IN THE ENZYMATIC REDUCTION OF QUINOID ANTITUMOR AGENTS

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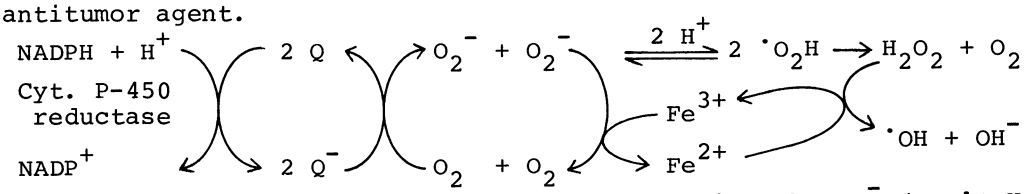
Generation of O_2^- from enzymatic reduction of purified aclacinomycin A with cytochrome P-450 reductase and NADPH in the presence of O_2 was demonstrated by spin trapping method in phosphate buffer. Addition of Fe^{3+} ion into the above system led to the detection of both O_2^- and $^{\cdot}OH$ spin adducts.

It has been proposed that the active oxygen radicals play an important role in the cleavage of DNA strands by antitumor agents.[1] Spin trapping method gives the proof of the generation of superoxide anion and hydroxyl radicals (O_2^- and $^{\cdot}OH$, respectively).[2] Applications of the method for quinoid antitumor agents, which mediate the generation of H_2O_2 and O_2^- during their reductions in the presence of O_2, have been reported by several investigators.[3,4] In their studies, however, there seems to be several problems in assigning the spin adducts from the reason pointed out by Finkelstein et al.[5] Furthermore, Komiyama et al. proposed a new mechanism for $^{\cdot}OH$ generation, in which $^{\cdot}OH$ could be produced from the system containing no Fe ion.[4] Our recent works[6,7] demonstrated that $^{\cdot}OH$ was generated from the reduction of adriamycin, a quinoid antitumor agent, by cytochrome P-450 reductase and NADPH in the presence of Fe^{3+}-ADP and O_2, while no $^{\cdot}OH$ generation occurred in the absence of Fe ion. The adriamycin-mediated $^{\cdot}OH$ generating system caused a significant λ-DNA cleavage.[7] Electron transfer mechanism in the system was proposed as follows, where Q is a quinoid antitumor agent.



In this paper, the above scheme is confirmed by detecting the O_2^- (or $^{\cdot}O_2H$) and $^{\cdot}OH$ radicals in the absence of and presence of Fe ion, respectively.

Purified aclacinomycin A (ACM, Aclarubicin hydrochloride) was kindly gifted from Sanraku-Ocean Co., Ltd. α-Phenyl-N-t-butylnitrone (PBN, Eastman Kodak Co.)

and 5,5-dimethyl-1-pyrroline N-oxide (DMPO, Aldrich Chem. Co.) were used as spin traps. DMPO was purified by distillation under vacuum. Sodium phosphate buffer (pH 7.5) was passed through a chelex-100 column to reduce the trace level of Fe^{3+} ion. Cytochrome P-450 reductase was prepared from rat liver microsome.[8] Superoxide dismutase (SOD) and bovine liver catalase were purchased from Sigma Chem. Co. and Calbiochem, respectively. Commercial catalase was dialyzed against 3 l of 10 mM (1 M = 1 mol dm^{-3}) Tris-HCl buffer (pH 7.4) before use. The Fe^{3+}-ADP complex was prepared so as to inhibit the precipitation of Fe^{3+} ion as hydroxide.[9] All sample solutions were prepared in test tube under aerobic condition at room temperature and the reaction was initiated by the addition of NADPH. Although ACM forms a complex with Fe ion in Tris-HCl buffer, no formation of the complex was observed in phosphate buffer like adriamycin.

Reduction of ACM (0.6 mM) with cytochrome P-450 reductase and NADPH in the presence of O_2 and PBN exhibited an ESR spectrum (Fig. 1a) of the spin adduct, which was readily assigned to PBN-O_2H with reference to the hyperfine splitting (hfs) constants, a^N = 1.48 and a^H_β = 0.28 mT.[2] The adduct was labile and disappeared within 4 min. Although the concentration of $^\cdot O_2H$ is assumed to be less than 1% of that of O_2^- at pH 7.5, with pK_a = 4.88 for $^\cdot O_2H$,[10] both of O_2^- and $^\cdot O_2H$ are probably the origin of PBN-O_2H, because the reactivity of O_2^- is less than $^\cdot O_2H$.[11] No signal due to the $^\cdot$OH adduct (a^N = 1.55 and a^H_β = 0.27 mT)[2] was observed, showing no splitting in the $\Delta m_I^N = \pm 1$ lines. Under the same condition, except the presence of Fe^{3+}(0.075 mM)-ADP, both O_2^- and $^\cdot$OH adducts were detected simultaneously (Fig. 1b). Since PBN-OH is more persistent than PBN-O_2H, the PBN-OH signal remained after the disappearance of the PBN-O_2H signal, but vanished about 10 min after the initiation of reaction. When the system was brought into a high concentration of Fe^{3+} ion, only a PBN-OH was observed.

Catalase and SOD were used to identify these spin adducts. Since catalase catalyzes the decomposition of H_2O_2 to H_2O and O_2, the participation of H_2O_2 in the reaction can be proved by the effect of catalase. SOD quenches O_2^- and then generates H_2O_2 very rapidly ($2O_2 + 2H^+ \longrightarrow H_2O_2 + O_2$). Addition of catalase (20 μg/ml) to the above Fe^{3+}(0.075 mM) containing system prior to the reaction caused a complete inhibition of the production of PBN-OH, but little for the PBN-O_2H production (Fig. 1c). On the other hand, a catalytic amount of SOD (0.5 μM), added to the system instead of catalase, completely inhibited the production of PBN-O_2H (Fig. 1d). This system, however, showed a set of quartet lines ($a^N \simeq a^H$ = 1.49 mT), which was the same as observed in the enzymatic reduction of PBN.[6] These results clearly indicate that $^\cdot$OH is generated by the interaction between Fe^{2+} ion (produced from the reduction of Fe^{3+} by enzyme and/or O_2^-) and H_2O_2 (Fenton's reaction).

Detection of O_2^- and $^\cdot$OH were also carried out by using DMPO as the spin trap. Enzymatic reduction of ACM in the presence of O_2 and DMPO afforded the spectrum of Fig. 2a, indicating the generation of both O_2^- and $^\cdot$OH spin adducts of DMPO. The hfs constants, a^N = 1.42, a^H = 1.14, and a^H = 0.13 mT for DMPO-O_2H and $a^N = a^H$ = 1.50 mT for DMPO-OH, essentially coincide with the reference values.[2] Under this condition, the signal due to DMPO-O_2H was labile and the

spectrum was replaced by the quartet lines of DMPO-OH (Fig. 2b). The transformation of DMPO-O_2H into DMPO-OH has been observed in reduction systems.[12] Therefore, the observation of DMPO-OH in Fig. 2a does not mean the $^{\cdot}OH$ generation in the main course of ACM reduction. A lack of the PBN-OH signal in Fig. 1a supports the above interpretation. An addition of Fe^{3+} (0.075 mM) to the above system prior to the reaction weakened the DMPO-O_2H signal, accompanying an enhancement of the DMPO-OH signal (Fig. 2c) and, at higher concentrations more than 0.2 mM of Fe^{3+}, the DMPO-O_2H signal vanished. Catalase (20 μg/ml) added to the above system with 0.075 mM Fe^{3+} seemed to inhibit the formation of $^{\cdot}OH$, but not the transformation of DMPO-O_2H into DMPO-OH (Fig. 2d). On the other hand, SOD completely put out the DMPO-O_2H signal (Fig. 2e). The spectrum of Fig. 2e could be obtained by the reaction of DMPO with Fe^{2+}-phosphate[13] and from the enzymatic reduction of Fe^{3+}-ADP in phosphate buffer. However, the DMPO-OH

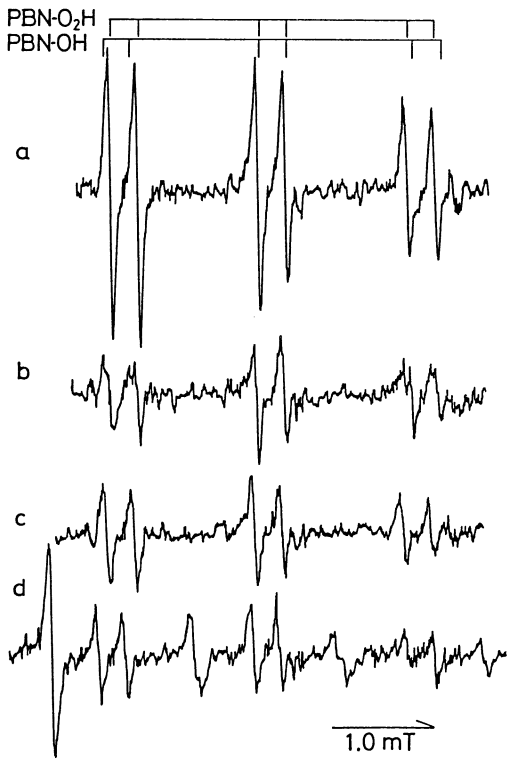


Fig. 1. Detection of O_2^- and $^{\cdot}OH$ by PBN (88 mM) during the enzymatic reduction (0.2 unit/ml cytochrome P-450 reductase and 0.2 mM NADPH) of ACM in the absence of and presence of Fe ion in 0.1 M phosphate buffer (pH 7.5). a: 0.6 mM ACM, b: 0.6 mM ACM + 0.075 mM Fe^{3+} + 1.25 mM ADP, c: b + 20 μg/ml catalase, and d: b + 0.5 μM SOD.

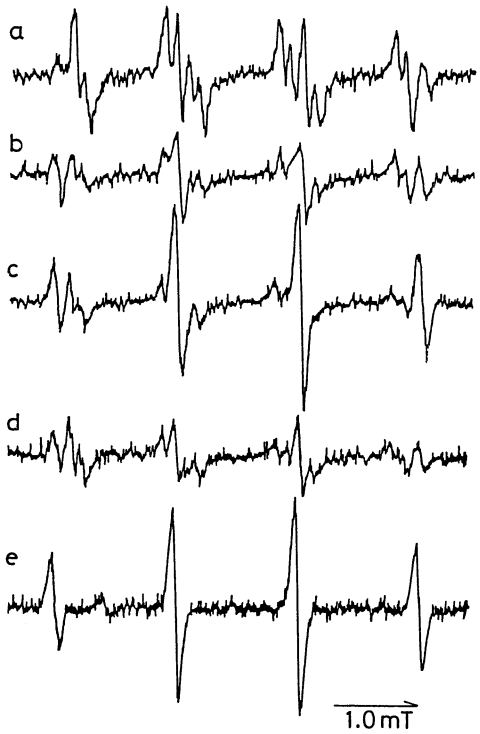


Fig. 2. Trapping of O_2^- and $^{\cdot}OH$ using DMPO (100 mM) during the reduction of ACM by cytochrome P-450 reductase (0.2 unit/ml) and NADPH (0.2 mM) in the absence of and presence of Fe ion in 0.1 M phosphate buffer (pH 7.5). a: 0.6 mM ACM; detected at 2 min (at the position of the first line) after the initiation of the reaction, b: the same components as those of a; observed at 7 min after the initiation of the reaction, c: 0.6 mM ACM + 0.075 mM Fe^{3+} + 1.25 mM ADP, d: c + 20 μg/ml catalase, and e: c + 0.5 μM SOD.

signal produced by the sequence other than ·OH trapping could not be quenched by the addition of catalase.

The present results clearly prove that the Fe ion is essential to the ·OH generation during the course of enzymatic reduction of quinoid antitumor agents. This means that metal ions play an important role in the cleavage of λ-DNA by quinoid antitumor agents. The production rate of ·OH in the reaction between quinone anion radical and H_2O_2 is probably slow like the Haber-Weiss reaction ($H_2O_2 + O_2^- \longrightarrow {}^{\cdot}OH + OH^- + O_2$).[14] The similar results have been obtained for the systems of adriamycin, mitomycin (Kyowa Hakko Co., Ltd.), daunomycin (Meiji Seika Co., Ltd.), and carbazilquinone (Sankyo Co., Ltd.), though their ESR intensities due to the spin adducts differ according to the agents. Details of the study will be reported elsewhere.

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