Mechanism of DNA Cleavage Induced by Fe²⁺ Autoxidation

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This work investigated the difference between Fe²⁺ autoxidation-induced and Fenton-type cleavage of pBR322 plasmid DNA. [•]OH generation reactions in the absence and presence of H₂O₂ under various conditions were also investigated. Although both the Fe²⁺ autoxidation and Fenton-type reactions showed DNA cleavage and [•]OH generation, there were significant differences in their efficiencies and reaction rates. The rate and efficiency of the cleavage reaction were higher in the absence of 1.0 mM of H₂O₂ than in its presence in 20 mM phosphate buffer. In contrast, the [•]OH generation reaction was more prominent in the presence of H₂O₂ and showed a pH-independent, fast initial reaction rate, but the rate was decreased in the absence of H₂O₂ at across the entire tested pH range. Studies using radical scavengers on DNA cleavage and [•]OH generation reactions in both the absence and presence of H₂O₂ confirmed that both reactions spontaneously involved the active oxygen species [•]OH, O₂^{•-}, ¹O₂ and H₂O₂, indicating that a similar process may participate in both reactions. Based on the above observations, a new mechanism for the Fe²⁺ autoxidation-induced DNA cleavage reaction is proposed.

Key Words : Iron autoxidation, Fenton reaction, Reactive oxygen species, Hydroxyl radical

Introduction

Reduced iron ions are toxic. They damage various biomolecules due to their ability to produce reactive oxygen species (ROS). Living organisms utilize proteins such as transferrin and ferritin to store labile iron ions and thereby avoid accumulation of excess iron ions.^{1,2} The concentration of Fe²⁺ ions in cells is increased under stress, thus inflicting oxidative damage on various biomolecules, including proteins, DNA, lipids and tissue cells.^{3,4} Of all ROS induced by Fe²⁺, the causative agent of DNA damage is believed to be the [•]OH.⁵⁻⁸

It has been proposed that free Fe³⁺, once bound adventitiously to DNA, can be reduced by superoxide radical, O₂^{•-} (Eq. 1).⁹ Subsequently, highly reactive [•]OH is generated upon interaction with endogenously produced hydrogen peroxide (H₂O₂) under Fenton conditions (Eq. 2).¹⁰⁻¹³ In addition, it is also proposed that excess hydrogen peroxide could scavenge [•]OH as in Eq. (3).⁸

The kinetics of Fe²⁺ oxidation is very slow but it can be readily oxidized to Fe³⁺ in response to complex formation with chemicals containing phosphate moiety, e.g., pyrophosphate, polyphosphate, DNA, and nucleotides in the di- or triphosphate form.¹⁴⁻¹⁸ Additionally, several studies have identified the production of superoxide and [•]OH as a result of the oxidation of Fe²⁺ in which molecular oxygen is involved instead of H₂O₂.^{18,19}

On the other hand, some reports have proposed the

generation of reactive [•]OH mainly by the direct interaction between metal ions and molecular oxygen, rather than the Fenton reaction. Such an interaction produces a highly reactive iron-oxygen complex, for instance ferryl- or perferryl- complexes (Eqs. 4. and 5), that equals or surpasses the oxidizing potential of the [•]OH.^{8,20-22}

Recently, Flemmig and Arnhold observed that Fe²⁺-induced pBR322 plasmid DNA cleavage was affected by the nature of buffer components; phosphate buffer produced higher cleavage efficiency than bicarbonate did. Some component in the buffer solution was proposed to bind to Fe²⁺ and influence its autoxidation ability.²³ Cleavage efficiency was attenuated by the addition of superoxide dismutase (SOD). Therefore, it was also suggested that a superoxide radical is somehow involved in the cleavage reaction.

These alternative mechanisms were proposed based on the fact that the Fenton reaction is an insufficient source for [•]OH due to the low concentration of physiological H₂O₂, which is approximately 10⁻¹⁰ M in red blood cells²⁴ and 10⁻⁸ M in the inner mitochondrial membrane²⁵ and liver cells.²⁶ Additional evidence supporting this process is observed in the rate constants for the oxidation of Fe²⁺ by O₂^{•-} and of the Fenton reaction, which are estimated to be 10⁶-10⁷ M⁻¹s⁻¹ and 10³-10⁵ M⁻¹s⁻¹, respectively. These physiological H₂O₂ concentrations are insufficient for the initiation of the Fenton reaction^{27,28} although the rate constants for the generation of [•]OH by Fe²⁺-O₂ and Cu⁺-O₂ complexes are comparable to that of the Fe²⁺-driven Fenton reaction.²¹

Although H₂O₂ is most likely the major promoter of DNA

damage, the exact nature of Fe^{2+} -induced DNA cleavage is still a matter of debate. In order to understand the characteristics of metal ion-induced DNA cleavage, we investigated the effects of pH, buffer components, and radical scavengers on the Fe^{2+} -mediated cleavage of pBR322 plasmid and $\cdot\text{OH}$ generation reactions in the absence and presence of H_2O_2 . Based on our results, we propose a new mechanism for the Fe^{2+} autoxidation-induced DNA cleavage reaction.

Materials and Methods

Materials. The pBR322 plasmid DNA stock solution (1.0 mg/mL) containing 10 mM Tris-HCl and 1.0 mM EDTA at pH 8.0 was purchased from New England Biolabs (Massachusetts, USA). Calf-thymus DNA (ctDNA) was purchased from Worthington Biochemicals (New Jersey, USA) and was dissolved in the required buffers containing 100 mM NaCl and 1.0 mM EDTA by exhaustive stirring at 4 °C. Dissolved DNA was dialyzed several times at 4 °C against the required buffers. The ctDNA concentration was determined spectrophotometrically using the absorption coefficient, $\epsilon_{260\text{nm}} = 6700 \text{ M}^{-1} \text{ cm}^{-1}$. FeCl_2 , glycerol, H_2O_2 , SOD from *E. coli*, bovine liver catalase, and terephthalic acid (TPA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of FeCl_2 in distilled water were prepared immediately prior to use. The H_2O_2 concentration was determined using a spectrophotometer at $\epsilon_{230\text{nm}} = 74 \text{ M}^{-1} \text{ cm}^{-1}$.²⁹ Buffer solutions with different pHs were prepared by mixing the same concentrated acidic and basic forms until the required pH was obtained.

Cleavage of pBR322 Plasmid DNA with Iron Ion. The pBR322 plasmid DNA was diluted to a final concentration of 100 ng/ μL with the required buffer solutions. The reaction mixture (12 μL) consisted of 200 ng of pBR322 plasmid DNA and FeCl_2 mixed in 20.0 mM NaH_2PO_4 buffer at pH 7.0, unless otherwise indicated. Since preparation order is crucial, the metal ion was always added last. After incubation at the required temperature, a 4 mL solution comprised of 50% glycerol (v/v), 40 mM EDTA, 0.025% bromophenol blue and 0.025% Xylene cyanol FF was added to stop the reaction. The reaction mixtures were then subjected to 1% agarose gel electrophoresis. The samples were placed in a horizontal slab gel apparatus in TAE buffer (40 mM Tris, 20 mM sodium acetate and 2 mM EDTA) set at 20 V for 12 hours. The gel was stained with ethidium bromide for 15 minutes followed by destaining in TAE buffer for 2-3 hours. Finally, the gels were photographed using a UV transilluminator.

Quantification of DNA Cleavage Efficiency. The integrated densities of the bands indicating pBR322 plasmid DNA in each lane were quantified by Total Lab TL100 image analysis software (Nonlinear Dynamic Ltd., USA). Since the lower binding constants of ethidium bromide to the supercoiled form, open circular and linear DNA were all comparable, the intensities of the supercoiled DNA were corrected by a factor of 1.4.³⁰

Fluorescence Detection of $\cdot\text{OH}$ Generation. Hydroxylation of non-fluorescent TPA yields 2-hydroxy-terephthalate which emits strong fluorescence around 430 nm when excited at 323 nm. TPA was dissolved in the required phosphate buffer to make a 10 mM stock solution and was diluted to a final concentration of 400 μM . All spectral measurements were performed after completion of the reaction at 37 °C. Time-dependent measurements were performed at room temperature and were started by the addition of FeCl_2 . A Jasco FP-777 fluorimeter was used for all measurements with 5/5 nm of excitation and emission slit widths. Emission spectra were obtained with the excitation at 323 nm and time-dependent intensity was measured at 426 nm.

Results

Fe^{2+} -induced pBR322 Plasmid DNA Cleavage in the Presence and Absence of H_2O_2 . Fe^{2+} ion-mediated pBR322 plasmid DNA cleavage in the absence and presence of H_2O_2 are compared in Figure 1. Untreated plasmid DNA exhibited two bands; one corresponding to a circular supercoiled form with high electrophoretic mobility and the other to an open circular form with reduced mobility. The amount of supercoiled DNA bands was calculated to be 93.5% and 6.5% of the nicked circular form. The Fe^{2+} -induced supercoiled DNA cleavage clearly exhibited much higher efficiency in the absence of H_2O_2 than in its presence (Figure 1(a)). In the absence of H_2O_2 , supercoiled DNA almost completely converted to the nicked circular form within 30 minutes in the Fe^{2+} concentration as low as 50 μM . The band analysis showed that 88.2% of the nicked circular and 2.7% of the linear form were generated while 9.1% of the supercoiled form remained. Above 50 μM of Fe^{2+} , the supercoiled form

Figure 1. Effect of H_2O_2 on Fe^{2+} -induced pBR322 plasmid DNA cleavage. Plasmid DNA (200 ng) was treated in 20 mM phosphate buffer, pH 7.0 with or without 1.0 mM of H_2O_2 . (a) FeCl_2 concentration was increased as indicated in the absence and presence of 1.0 mM H_2O_2 . The sample was incubated for 30 minutes at 37 °C. (b) Time-dependent cleavage of pBR322 plasmid DNA at a fixed concentration of 0.1 mM of FeCl_2 in the presence and absence of H_2O_2 . The sample was incubated in iced water.

Figure 2. (a) Effect of N_2 bubbling on the Fe^{2+} -induced pBR322 plasmid DNA cleavage in the presence of 0.1 mM $FeCl_2$ in 20 mM phosphate buffer, at pH 7.0 and 37°C. The sample was gently bubbled by pure N_2 gas for 30 minutes. (b) The DNA bands of supercoiled (square) and nicked (circle) forms were quantified and plotted against reaction time with (blue line) and without (red line) N_2 bubbling.

was completely disappeared. The generation of the linear form increased gradually with increasing Fe^{2+} concentration, and finally band smearing appeared at 1.0 mM Fe^{2+} , indicating that the linearized form was converted to shorter DNA fragments. On the other hand, the presence of 1.0 mM of H_2O_2 alleviated the cleavage efficiency compared to that in the absence of H_2O_2 . The time-dependent pBR322 plasmid DNA cleavage in the absence and presence of 1.0 mM H_2O_2 are compared in Figure 1(b). Both reactions were carried out in ice since the reactions were too fast at higher temperatures. Comparing the two reactions, Fe^{2+} exhibited faster cleavage reaction in the absence of H_2O_2 than in its presence. The supercoiled form completely disappeared within 5 minutes in the absence of H_2O_2 while it was remained up to 1 hour in the presence of H_2O_2 . Whether the mechanism behind the cleavage in the presence and absence of H_2O_2 is the same or different, it is clear that Fe^{2+} -induced DNA cleavage does not require the presence of exogenous H_2O_2 .

Contribution of O_2 in the Fe^{2+} -mediated Plasmid DNA Cleavage. For the Fenton reaction, H_2O_2 is the source of reactive $\cdot OH$ as shown in Eq. (2). However, it is possible that the oxygen molecules dissolved in aqueous solution are the molecular source for the synthesis of ROS in the absence of H_2O_2 (Figure 1). Hence, we tested the role of dissolved oxygen by comparing the extent of cleavage with or without nitrogen bubbling. As shown in Figure 2(a), supercoiled pBR322 plasmid DNA began being converted to an open circular form by 0.1 mM Fe^{2+} ions within 10 minutes. After 120 minutes of mixing, about 65% of the supercoiled form was cleaved. The residual nicking seems to be due to the trace of oxygen molecules. However, the removal of dissolved oxygen molecules by nitrogen bubbling significantly reduced the DNA cleavage efficiency; 35% of plasmid DNA was cleaved after 120 minutes, a percentage almost twice than that of the non- N_2 bubbled reaction. This result suggests that dissolved oxygen molecules participate in Fe^{2+} -induced DNA cleavage in the absence of H_2O_2 . Considering the time-dependent curve of the cleavage product in Figure

2(b), Fe^{2+} induced faster and more efficient cleavage reaction with the help of dissolved oxygen molecules: the cleavage reaction was completed within 10 minutes. In contrast, in the presence of N_2 bubbling, the yields of cleavage products were gradually increased over time until they plateaued after 1 hour. This observation suggested that dissolved oxygen is an efficient reactant for Fe^{2+} ion-mediated DNA cleavage reaction.

Inhibition of Cleavage by Scavengers. Various scavengers were tested for the identification of ROS that participate in Fe^{2+} -mediated DNA cleavage in the presence and absence of H_2O_2 (Figures 3(a) and 3(b), respectively). The scavengers used in this work were glycerol, sodium azide, catalase, and SOD, which are known to suppress the actions of the $\cdot OH$, H_2O_2 , singlet oxygen, and superoxide radical, respectively. In this experiment, the efficiencies with which these scavengers inhibit radicals are not discussed due to the variation in scavenging capacities and loading concentrations. The addition of all scavengers exhibited similar inhibitory effects on the cleavage reactions, regardless of the H_2O_2 existence, indicating that all ROS namely, the $\cdot OH$, superoxide radical, singlet oxygen and H_2O_2 , may participate equally in Fe^{2+} -mediated cleavage both in the absence and presence of H_2O_2 . Under Fenton conditions, it is known that both hydroxyl and superoxide radical participate in the DNA

Figure 3. Effect of scavengers on pBR322 plasmid DNA cleavage in the (a) absence and (b) presence of 1.0 mM H_2O_2 . Plasmid DNA (200 ng) was treated by 0.2 mM of $FeCl_2$ in 20 mM phosphate buffer, pH 7.0. All samples were incubated in ice for 30 minutes.

Figure 4. (a) Effect of pH on Fe^{2+} -induced pBR322 plasmid DNA cleavage in the absence of H_2O_2 . All experiments were performed in 20 mM phosphate buffers with different pHs prepared as described in the experimental part. FeCl_2 concentration was increased as indicated. All samples were incubated at 37 °C for 30 minutes. (b) Time-dependent pBR322 plasmid DNA cleavage at various pHs in the absence of H_2O_2 . The samples were incubated at 37 °C and the reaction stopped at the indicated time. $[\text{FeCl}_2] = 1.0$ mM.

cleavage mechanism, although superoxide radical does not attack DNA directly.^{6,7}

pH-dependence of pBR322 Cleavage. It has been reported that Fe^{2+} , although stable at acidic pH, is rapidly autoxidized to Fe^{3+} at a neutral and alkaline pH.^{15,31,32} Therefore, the solution pH was expected to affect the cleavage. Figure 4(a) shows the pH-dependent pBR322 plasmid DNA cleavage produced by Fe^{2+} ion. All reaction mixtures were incubated at 37 °C for 24 hours with various Fe^{2+} ion concentrations to maximize the differences. Without Fe^{2+} , solution pH did not induce significant nicking or cleavage of the plasmid DNA. At a glance, it is notable that the cleavage efficiency was much higher under acidic conditions, although both the open circular and linearized products were generated at all three pHs. The cleavage efficiencies depended largely on the Fe^{2+} concentration at all pHs. Supercoiled DNA was completely cleaved after 24 hours of incubation under acidic conditions, even at the lowest Fe^{2+} concentration (0.1 mM). At Fe^{2+} concentrations above 0.3 mM, no band was observed as all plasmid DNA was seemingly cleaved to smaller pieces. The cleavage efficiency was decreased with increasing pH. Increasing the Fe^{2+} concentration at pH 7.0 resulted in a gradual decrease of the supercoiled form up to Fe^{2+} concentration of 0.5 mM. However, there was no sign of DNA fragmentation, indicating that the majority of plasmid DNA remained in either the open circular or linearized form. At a higher pH, the supercoiled form remained up to 1.0 mM Fe^{2+} , due to a significant decrease in cleavage ability. Here, the majority of plasmid DNA remained in the open circular form, while only a small amount of supercoiled and linearized forms was observed.

Time-dependent cleavage reactions at three pH conditions with 1.0 mM of Fe^{2+} were also performed in Figure 4(b). At all pHs, the supercoiled form was cleaved to open circular and linear forms. The cleavage rate increased with decreas-

ing pH. At pH 5.4, the supercoiled form completely disappeared at 10 minutes and band smearing appeared at 30 minutes. After 1 hour of reaction, all bands disappeared and even the band smearing became faint, indicating that small DNA fragments were cleaved to pieces of nucleotides. Finally after 2 hours of reaction, neither bands corresponding to DNA itself nor that representing smeared DNA were observed. At pH 7.0, the supercoiled form was completely cleaved to open circular and small linear forms at 30 minute of reaction. After this time, no significant change was observed: the majority remained as the open circular form and minority as the linear form. At the highest pH, some of the supercoiled form remained even after 4 hours, after which no further significant change occurred.

Fluorescence Detection of $\cdot\text{OH}$ Generation. The $\cdot\text{OH}$ has been proposed as the active radical species in the Fe^{2+} -mediated DNA cleavage reaction. Therefore, the generation of $\cdot\text{OH}$ by Fe^{2+} at various concentrations was tested using the fluorescence method. The fluorescence measurement uses the $\cdot\text{OH}$ -induced conversion of non-fluorescent TPA to highly fluorescent 2-hydroxy-terephthalate (TPA-OH).³² Fe^{2+} concentration-dependent $\cdot\text{OH}$ generations in the presence and absence of H_2O_2 in H_2O and in 5 mM phosphate buffer were compared in Figure 5a. In both cases, the $\cdot\text{OH}$ generation in 5 mM phosphate buffer solution appeared to be fairly Fe^{2+} concentration-dependent while it was almost negligible in H_2O . In phosphate buffer, the $\cdot\text{OH}$ generation was far more efficient in the presence of H_2O_2 than in its absence. The fluorescence intensity of TPA-OH in the presence of H_2O_2 was about 9-fold higher than its absence at 10 μM of Fe^{2+} and about 5-fold higher at 200 μM of Fe^{2+} . The H_2O_2 concentration and the pH may also affect the $\cdot\text{OH}$ generation. Thus, the TPA-OH-originated fluorescence intensity was compared at various H_2O_2 concentrations at three different pHs (Figure 5(b)). The increase in the H_2O_2

Figure 5. (a) FeCl₂ concentration-dependent fluorescence intensity due to the formation of TPA-OH in 20 mM of phosphate buffer in the absence (circle, red curve) and presence (square, blue curve) of 1.0 mM of H₂O₂. All samples were prepared separately and incubated for at least 6 hours to ensure reaction completion. (b) H₂O₂ concentration-dependent TPA-OH generation at pH 5.4 (red), 7.0 (black) and 9.0 (blue). [FeCl₂] = 100 μM, [H₂O₂] = 0, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 and 10 mM. (c) Time-dependent fluorescence change of TPA-OH in 5 mM of phosphate buffer at various pHs. [FeCl₂] = 100 μM. Insert: time-dependent fluorescence change of TPA-OH in the presence of 1 mM of H₂O₂ under the same condition. (d) Inhibition of [•]OH generation by various scavengers in the absence of H₂O₂ at pH 7.0. Reaction mixtures were incubated at room temperature for at least 6 hours to ensure reaction completion. 5 units of SOD and catalase and 2 mM of glycerol and sodium azide were used.

concentration generally induced more [•]OH. The optimal H₂O₂ concentrations appeared to be dependent on the pH of the medium, in this particular case, phosphate buffer. At pH 5.4, the increasing fluorescence plateaued at 2 mM H₂O₂ and no further change was observed with increasing H₂O₂ concentration. In contrast, at pH 7.0, the maximum appeared at 1.0 mM of H₂O₂, followed by a slight decrease with further increasing H₂O₂ concentration. At pH 9.0, the [•]OH generation appeared to be less efficient. The fluorescence intensity increased gradually up to 10 mM of H₂O₂ addition. According to the previous report, the optimal H₂O₂ concentration for [•]OH generation was determined to be ~2.5 mM, but excess H₂O₂ scavenged [•]OH (Eq. 3), based on the observation of in vivo cellular survival and cellular DNA damage production experiments.⁸ This phenomenon is similar with the result obtained at pH 7 in our experiment. Considering the results, the lower pH was preferred in the

Fe²⁺-mediated [•]OH generation. This may be related to the low pH-preferable plasmid DNA cleavage (Figure 4a).

Time-dependent TPA-OH generation at different pHs both in the presence and absence of H₂O₂ in 5 mM of phosphate buffers was also measured in Figure 5(c). In the absence of H₂O₂, low pH produced more [•]OH, which is well consistent with the pH dependent plasmid DNA cleavage. But the reaction rates were turned out to be in reverse: higher pH showed faster reaction. In the presence of H₂O₂, the [•]OH generation efficiencies showed same pH dependent with those in its absence. The [•]OH generation is composed with two distinctive reactions independent of the buffer pH; very fast ones terminated in few seconds and very slow ones last for several hours and this trend is much clear at lower pHs. Although the initial rates are too fast to compare with each other, the slower ones seem to be similar with the overall reaction rates in the absence of H₂O₂: lower pH exhibits

Figure 6. (a) Time-dependent fluorescence change of TPA-OH in 5 mM of phosphate (red curve), bicarbonate (black), and cacodylate (green) buffers and in H₂O with 100 μ M of ctDNA (blue). The reactions were started by the addition of 100 μ M of FeCl₂. When the fluorescence intensities were stabilized, 100 μ M of ctDNA was added and the changes in the fluorescence intensity were continuously recorded. In the case of H₂O, ctDNA was included prior to the reaction start. (b) Time-dependent fluorescence change of TPA-OH in 5 mM phosphate (red, curve a), bicarbonate (black), and cacodylate (green) buffers and H₂O (blue) in the presence of 100 μ M of ctDNA. For comparison, 5 mM of phosphate (dotted violet curve marked by b) in the absence of ctDNA was also measured. Reactions were started by the addition of 100 μ M of FeCl₂. (c) Time-dependent pBR322 plasmid DNA cleavage in different buffers. Buffer concentrations were 5 mM, at pH 7.0 and 37 $^{\circ}$ C.

slower reaction.

The presence of various scavengers may inhibit the generation of $\cdot$ OH, which would decrease the fluorescence intensity of TPA-OH. The fluorescence spectra of TPA-OH in the presence of various scavengers in the absence of H₂O₂ are depicted in Figure 5(d). All the radical scavengers decreased the fluorescence intensity, suggesting that the $\cdot$ OH generation was suppressed by the presence of scavengers, indicating that the superoxide radical, H₂O₂, as well as singlet oxygen may participate in the process of $\cdot$ OH generation. The same trend was observed in the presence of H₂O₂ (data not shown), indicating that the mechanism behind the $\cdot$ OH generation reaction is unaffected by the presence of H₂O₂.

Buffer Components and DNA Effect on $\cdot$ OH Generation and DNA Cleavage. The buffer components have been reported to be critical in the Fe²⁺-mediated DNA cleavage and OH radical generation,^{23,32} as well as in the oxidation of Fe²⁺.^{34,37} The increase in the phosphate or bicarbonate buffer concentration has been reported to enhance both the $\cdot$ OH generation and DNA cleavage efficiency. Consequently, the involvement of a new intermediate, 'crypto- $\cdot$ OH', which may originate from the $\cdot$ OH-Fe-buffer component complex, in the cleavage process was proposed. Here, we assayed the

similar buffer effect on the Fe²⁺-mediated $\cdot$ OH generation and pBR322 plasmid DNA cleavage in the absence of H₂O₂. In Figure 6(a) shows a time-dependent increase in the fluorescence intensity upon TPA-OH generation, which was induced by the addition of Fe²⁺ to three different buffer systems and in water. For the control experiment, when Fe²⁺ and TPA were mixed with H₂O, no significant fluorescence change was observed (data not shown). In the absence of any buffering compounds when H₂O₂ was present, the fluorescence intensity increased very rapidly: the increase peaked within two minutes, suggesting the rapid formation of TPA-OH. The presence of buffering compounds namely, phosphate or bicarbonate ions, reduced the rate of the TPA-OH generation. In the cacodylate buffer, the fluorescence intensity remained almost constant. Observed reductions in the reaction rates suggested the interaction of buffering compound with Fe²⁺. In a comparison of the phosphate and bicarbonate buffers, the former exhibited higher fluorescence intensity than the latter, which is consistent with a previous report.³² $\cdot$ OH generation was faster in bicarbonate buffer, reaching completion within several minutes, than in phosphate, in which it lasted for about 20 minutes. In these two buffer cases, the addition of 100 μ M of ctDNA induced

significant decreases in the fluorescence intensity followed by a slight increase. The decrease was the result of dilution by addition of DNA solution. In contrast, the fluorescence intensity was dramatically increased by the addition of ctDNA in the cacodylate buffer.

The time-dependent increases in the fluorescence intensity upon formation of TPA-OH in various buffers in the presence of ctDNA are depicted in Figure 6(b). The reactions were initiated by the addition of Fe^{2+} to various buffer solutions containing ctDNA and TPA. The increases in fluorescence intensity in the phosphate and bicarbonate became slower than that observed in the absence of DNA (Figure 6(a)). In the cacodylate case, the profile of the increase in the fluorescence intensity resembled that when ctDNA was either initially present (Figure 6(b), green curve) or added during the reaction process (Figure 6(a), green curve). This observation suggested that the number of Fe^{2+} ions participating in the hydroxyl generation reaction was unaffected by whether ctDNA was initially present or added during the process, implying that either all Fe^{2+} binding sites of ctDNA were pre-occupied by cacodylate or cacodylate does not prevent the Fe^{2+} binding to DNA due to its very weak interaction with Fe^{2+} . The buffer component-dependent plasmid DNA cleavage reaction was carried out and the results are depicted in Figure 6(c). All three buffers exhibited efficient cleavage of supercoiled DNA. The linearized component was also observed, even at 10 minutes of reaction time. Of the buffer components, phosphate buffer showed the highest cleavage efficiency: production of the linear component was the most effective. Bicarbonate and cacodylate showed similar cleavage efficiencies. In the case of cacodylate, the cleavage efficiency is very similar with the same experiment performed in water without any buffering system (data not shown).

Discussion

Comparison of Fe^{2+} -induced DNA Cleavage in the Presence and Absence of H_2O_2 . Some studies reported that molecular oxygen is involved in Fe^{2+} -induced DNA cleavage²³ and $\cdot\text{OH}$ generation³³ in the absence of H_2O_2 , indicating that the molecular oxygen may act as the molecular source of ROS. The fact that nitrogen bubbling significantly suppressed the DNA cleavage efficiency (Figure 2), which was also consistent with prior reports, implied the production of the ROS by the molecular oxygen. The efficiency and the reaction rate of the DNA cleavage do not necessarily reflect the H_2O_2 -originated generation of $\cdot\text{OH}$. The rate and efficiency of the DNA cleavage in the absence of H_2O_2 were both higher than those in its presence (Figure 1). On the other hand, $\cdot\text{OH}$ generation was more efficient in the presence of H_2O_2 (Figure 5). These observations suggested the presence of two pathways in regard to the existence of H_2O_2 . The first appeared dominant in the presence of H_2O_2 . Fe^{2+} may form a complex with H_2O_2 so that the cleavage reaction would follow the Fenton mechanism as shown in Eqs. (2) and (3). In contrast, the appeared dominant in the absence of

H_2O_2 ; Fe^{2+} complexed with buffer components, which, in turn, may have interacted with molecular oxygen and used it as a substrate for ROS production. It has been proposed that the biologically active oxygen radical is not the free hydroxyl radical but the "crypto- $\cdot\text{OH}$ ",^{8,32,34} which explains the low efficiencies in $\cdot\text{OH}$ generation when the reaction was performed in water without any buffer components.

From the effect of the scavengers on the DNA cleavage reaction, which are shown in Figures 3(a) and 3(b), the same ROS were detected irrespective of the presence of H_2O_2 . In addition, a similar inhibitory effect was observed in $\cdot\text{OH}$ generation (Figure 5(d)). All these observations led us to conclude that the $\cdot\text{OH}$, superoxide radical, H_2O_2 , and singlet oxygen were produced during the process of Fe^{2+} oxidation both in the presence and absence of H_2O_2 . These results are in agreement with the previous proposal in which the Fenton mechanism can be evoked from the direct interaction between Fe^{2+} and molecular oxygen through Eqs. (6) and (8).^{15,35}

The steps denoted by Eqs. (6) and (7) are highly pH-dependent. Saran *et al.* conducted pH-dependent $\cdot\text{OH}$ generation in phosphate buffers by monitoring the fluorescence change of TPA and found that the most appropriate pH value was 6.3, while the efficiency was decreased by adjusting the pH values.³² Meanwhile, Svoboda *et al.* measured the pH-dependent autoxidation efficiency of Fe^{2+} in tris-maleate buffer.¹⁵ They observed that the autoxidation efficiency was increased with increasing the pH in the pH range 6.8–8.1. In our experiments, however, both $\cdot\text{OH}$ generation and DNA cleavage efficiencies showed the same pH dependency in the presence and absence of H_2O_2 : both reactions were preferable at acidic pH. These results seem to support Eqs. (6) and (7). As aforementioned, the time-dependent $\cdot\text{OH}$ generation at different pHs (Figure 5(c)), the reaction rate, and the efficiency did not exhibit consistent trends. The reaction rate was maximized at the highest pH, while the $\cdot\text{OH}$ yield was highest at the lowest pH. On the other hand, both reaction rate and efficiency presented similar trend in the cleavage reaction. This discrepancy suggests that the $\cdot\text{OH}$ may not be the only oxygen species responsible for DNA cleavage.

pH Dependency of DNA Cleavage and $\cdot\text{OH}$ Generation.

Previous studies observed that the autoxidation of Fe^{2+} has a strong pH dependency in phosphate buffer, resulting in the production of $\text{O}_2^{\cdot-}$ through the reaction shown in Eqs. (6)–(7).^{15,36} According to the reports, the formation of Fe^{3+} via the autoxidation was markedly increased with increasing pH, over the pH range 6.8–8.1. They attributed the pH effect on the catalytic activity to the increased binding constant of Fe^{2+} with HPO_4^{2-} (3981 M^{-1}) compared to that with H_2PO_4^- (501 M^{-1}),³⁷ because the ratio between $[\text{HPO}_4^{2-}]/[\text{H}_2\text{PO}_4^-]$

Scheme 1. Proposed Fe^{2+} autoxidation-induced DNA cleavage mechanism. Numbers indicate the equation cited in the text.

increased from 1.0 at pH 7.2 to 2.5 at pH 7.6. In our paper, we performed the $\bullet\text{OH}$ generation and DNA cleavage reactions in phosphate buffer at three different pHs: 5.4, 7.0, and 9.0. However, the observed pH dependencies exhibited exactly the opposite trends to those observed in the iron autoxidation: both DNA cleavage and $\bullet\text{OH}$ generation were predominant at decreasing pH. This discrepancy suggests that the action of at least two stepwise processes: the first step, responsible for the generation of $\text{O}_2^{\bullet-}$ via autoxidation of iron, which is denoted as process 6_a or 6_b in the Scheme 1; and the second step for the production of H_2O_2 and $\bullet\text{OH}$, through process 7 and 2 or 8 known as Haber-Weiss reaction. Thus, our results suggested that the first process may be preferred at higher pH for producing $\text{O}_2^{\bullet-}$ by iron autoxidation with the help of HPO_4^{2-} binding, while the second process seems to be preferred at low pH due to the requirement of two equivalents of protons. This conclusion is supported by the slow rate of $\bullet\text{OH}$ production at lower pH (Figure 5(c)), whereas the efficiency was higher. At high pH, $\text{O}_2^{\bullet-}$ can be generated quickly but the continuing process to generate $\bullet\text{OH}$ was prevented by the low concentration of available protons in the aqueous solution.

Buffer Component and DNA Effect. It is proposed that the complexation of Fe^{2+} ions and chelating agents, which include not only DNA moieties but also buffer components, enhanced the Fe^{2+} oxidation or $\bullet\text{OH}$ generation in the absence of H_2O_2 .^{15,23,32,35,38} Of these chelating agents, DNA has been reported to be the most efficacious for Fe^{2+} oxidation. In this paper, phosphate, bicarbonate, and cacodylate buffer components were tested for their capability in $\bullet\text{OH}$ generation and DNA cleavage (Figures 6(a), 6(b), and 6(c)). Similarly with the previous observations, the efficiencies of both $\bullet\text{OH}$ generation and DNA cleavage were higher in phosphate buffer than in bicarbonate, although the reaction rates seemed to be similar. However, in the case of cacodylate buffer, negligible change was observed, and the

addition of 100 μM of ctDNA rapidly increased the $\bullet\text{OH}$ generation (Figure 6(a)). This indicates either that cacodylate buffer is a poor ligand to Fe^{2+} for $\bullet\text{OH}$ generation or that there might be no interaction between Fe^{2+} and cacodylate buffer component.

In the comparison of time-dependent $\bullet\text{OH}$ generation in the presence of ctDNA with various buffer components, H_2O showed the fastest initial rate, while the presence of buffering compounds slowed the generation (Figure 6(a) and (b)), suggesting that DNA itself may be the best chelating agent for Fe^{2+} (DNA cleavage reaction was also performed in H_2O and the cleavage efficiency was comparable with the case in other buffers, data not shown). The presence of buffer components slowed the $\bullet\text{OH}$ generation compared to their absence, indicating that these buffer components may also compete with DNA for Fe^{2+} binding.

DNA Cleavage Mechanism by Fe^{2+} Autoxidation. The rate of $\bullet\text{OH}$ generation decreased with decreasing pH in the absence of H_2O_2 , while the reaction efficiency was higher at lower pHs. This discrepancy can be explained by assuming two different pH-dependent pathways. As discussed above in the section on pH dependency, the autoxidation of iron with the production of superoxide radicals (process 6_a or 6_b in the Scheme 1) and $\bullet\text{OH}$ generation (process 7 and 2 or 8) are considered to be consecutive processes, possibly with different pH dependencies. In the acidic condition, Fe^{2+} complexes with H_2PO_4^- and follows process 6_a. Since this process is, in fact, preferred at high pH, the rate of superoxide radical production can be very low. However, the generated superoxide radicals can be easily converted to $\bullet\text{OH}$ due to low pH preference. On the other hand, Fe^{2+} may form a complex with HPO_4^{2-} at basic condition, and this reaction is preferable at high pH, resulting in the high yield of the superoxide radical. However, the following process 7 requires protons to generate H_2O_2 , and the other process (process 2) also prefers acidic condition because hydroxide ions can be stabilized by association with protons to make H_2O . Of those two processes, the second, which produces $\bullet\text{OH}$, seems to be so fast that pH does not affect the initial reaction rate. This hypothesis is supported by the pH-independent fast reaction rate of $\bullet\text{OH}$ generation in the presence of H_2O_2 (Figure 6(c), insert). Consequently, the first process (process 6) might be responsible for the overall reaction rate.

In the presence of H_2O_2 , the initial reaction rate of $\bullet\text{OH}$ generation was very high at all pHs, whereas DNA cleavage rate was much slower than in its absence. These opposing trends suggest the presence of unknown process or reactive species that is presumably independent of the $\bullet\text{OH}$ generation process. In the action of scavenger effect on DNA cleavage and $\bullet\text{OH}$ generation, it is notable that sodium azide significantly inhibits both reactions, indicating the involvement of a singlet oxygen. The singlet oxygen attacks the guanine base directly and converts it to 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) or induces DNA cleavage, whereas the $\bullet\text{OH}$ shows nonspecific base modification.³⁹⁻⁴² A near-IR chemiluminescence spectroscopic study reported

that singlet oxygen can be produced *via* the Haber-Weiss reaction as depicted as process 8.^{43,44} Another study reported that radiation can convert a triplet oxygen to a singlet oxygen in the presence of metal ions, such as Cu⁺.⁴⁵ Thus, the singlet oxygen is a potential candidate to explain the discrepancy. The unknown process or reactive species seems to act independently of the mechanism proposed in Scheme 1, since the DNA cleavage rate was highest and the [•]OH generation lowest at low pH. This contradiction requires explanation to complete our understanding of the Fe²⁺ autoxidation-mediated DNA cleavage.

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