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Generation of Superoxide Anion in Fe(III)-nta (nta=nitrilotriacetate) and H_2O_2 System. An ESR Study

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The reaction of Fe(III)-nta and H_2O_2 in H_2O/DMSO (v/v=3/2) solution (pH 10) was studied by analyzing ESR spectra. The initial solution of Fe(III)-nta turned dark-brown just after the addition of H_2O_2. After the production of oxygen gas, the solution turned back to brownish-yellow. The initial and final Fe(III)-nta complexes showed high-spin ferric states with maximum rhombicity. In the dark-brown solution before the oxygen production, the majority of the Fe(III)-nta complex was ESR-silent; this appeared to be a binuclear complex. When the binuclear Fe(III)-nta complex was dominant, superoxide anion was detected.

Active oxygen species and free radicals are associated with many diseases that are ascribed to lipid peroxidation, DNA damage, and protein degeneration. It is well known that iron plays a critical role in some free radical injuries.1) In biological system, the iron ion almost always exists in a chelated form and the chemical form of such iron chelate complexes is very important for their catalytic activity. Fe(III)-nta is a biologically important iron complex which induces hemochromatosis, renal tubular necrosis and renal cell carcinoma in animals.2) It is interesting that Fe(III)-nta-induced cell injury and DNA damage can be protected by superoxide dismutase,3) while damage produced by other iron chelate complexes cannot be so protected. Although hydroxyl radical have been suggested to be the offensive species associated with iron, it has been still wondered if other species of active oxygen or free radical are causative of the Fe(III)-nta-induced cell injury, and DNA cleavage and modification in vivo. To elucidate the generation of superoxide anion, we studied the reaction of Fe(III)-nta and H_2O_2 by analyzing ESR spectra. During the reaction, we detected superoxide anion and found different Fe(III)-nta complexes during the reaction. Here we discussed a possible reaction mechanism based on our experimental data.

Fe(III)-nta solution was prepared just before use as follows: Na_2Hnta (0.5878 g) was dissolved in distilled water and 1.0 mol dm^{-3} NaOH (2.0 ml) and dimethyl sulfoxide (DMSO) (20 ml) were added. Iron stock solution (0.10 mol dm^{-3}) of $Fe(ClO_4)_3 \cdot 6H_2O$(2.5 ml) was added and the solution was mixed; the volume was adjusted to 50 ml with distilled water. The final iron concentration was 5.0×10^{-3} mol dm^{-3}, nta 5.0×10^{-2} mol dm^{-3}, and the pH was 10. The reaction was started by adding 30% H_2O_2 (100 ml) to the Fe(III)-nta solution (2.00 ml) at room temperature. Electronic absorption spectra were recorded by a Hitachi 150-20 spectrophotometer. ESR spectra were recorded at 123 K by a JEOL ESR spectrometer, JES-1XG, equipped with 100 kHz magnetic field modulation. 2,5-Cyclohexadiene-Δ 1α,4α'-dimalononitrile(lithium salt) and Mn^{2+} in MgO as supplied by JEOL was used as standard g markers. ESR spectra were analyzed by mononuclear S=5/2 spin Hamiltonian,

$$H = D\left(S_z^2 - \frac{35}{12}\right) + E\left(S_x^2 - S_y^2\right) + g\mu_B \mathbf{H}_0 \mathbf{S}$$

where D and E are zero-field splitting parameters. The spin Hamiltonian matrix was directly diagonalized by NEC MATHLIB for ACOS software and eigenvalues and eigenvectors were obtained. We used D=0.74 cm^{-1}, since the value for Fe(III)-edta (edta=ethylenediaminetetraacetate) was reported to have D=0.83 cm^{-1}, 4) and preliminary calculation indicated that experimental g' values were relatively well simulated at the D value.

The initial solution of Fe(III)-nta complex (**1**) was brownish-yellow. Just after the addition of H_2O_2, the solution turned dark-brown (Fe(III)-nta complex (**2**)) and a few minutes later oxygen gas was produced. After the completion of the reaction, the solution turned back to brownish-yellow (Fe(III)-nta complex (**3**)). In our experiment, broad absorption appeared in the visible region, but no detectable peak was identified during the reaction. In the initial Fe(III)-nta solution, there was another Fe(III)-nta complex (**1'**) which was different from the mononuclear Fe(III)-nta complex (**1**). The Fe(III)-nta complex (**1'**) had three peaks around 470, 490, and 575 nm. Since the Fe(III)-nta complex (**1'**) had not only a peak in μ-oxo dimer region, but also the same visible absorption as Fe(III)-nta complex crystallized by Nishida et al.,5) we inferred that the Fe(III)-nta complex (**1'**) might have a binuclear structure similar to Nishida's complex, which had a μ-oxo, μ-acetato binuclear structure. In our system, one of the three carboxyl groups of nta might ligate the ferric ion in stead of the acetate of Nishida's complex.

ESR spectra of both the initial and the final Fe(III)-nta complexes, **1** and **3**, showed high-spin ferric rhombic states which had two signals around $g' \approx 4.3$ and 9.0 (Fig. 1), which did not split widely. After the addition of H_2O_2 to the Fe(III)-nta solution, the signal intensity decreased to about one-fifth of the initial signal intensity. The majority of Fe(III)-nta (Fe(III)-nta complex (**2**)) might exist in an antiferromagnetically coupled binuclear structure, as discussed later. Figure 2 shows the ESR spectra of the minor component (Fe(III)-nta complex (**2'**)) which is a different one from the Fe(III)-nta complex (**2**). The signals around $g' \approx 4.3$ and 9.0 were split. At the time we found a free radical around $g' \approx 2.0$. The free radical had $g_\perp$=2.009 and $g_\parallel$ =2.084 with axial symmetry and was taken to be a superoxide anion. After the reaction was completed, the ESR signal intensity of the Fe(III)-nta complex was recovered and the splitting around $g' \approx 4.3$ and 9.0 had narrowed (Fig. 1).

The experimental and calculated g' values are summarized in Table 1. As showed by the calculation, when E/D=1/3, the effective g' values around 9.0 and 4.3 do not split widely, but when E/D<1/3, the splitting width of the g' values becomes wide. The experimental g' values indicated that the Fe(III)-nta complexes (**1** and **3**) had E/D=1/3, and that the complex (**2'**) had an E/D value less than 1/3. The Fe(III)-nta complexes (**1** and **3**) were high-spin ferric complexes with maximum rhombicity and the Fe(III)-nta (**2'**) was another high-spin ferric complex with lower rhombicity than the complexes (**1** and **3**).

To elucidate the mechanism of Fe(III)-nta-dependent cell injury, we studied oxygen activation reaction induced by the iron. In aqueous system of physiological pH, superoxide anions rapidly disappear due to the dismutation reaction. When the reaction of Fe(III)-nta and H_2O_2 was performed in a H_2O/DMSO system of high pH, superoxide anion was definitely detected. It was suggested that superoxide anion was involved in Fe(III)-nta induced cell injury and DNA damage.

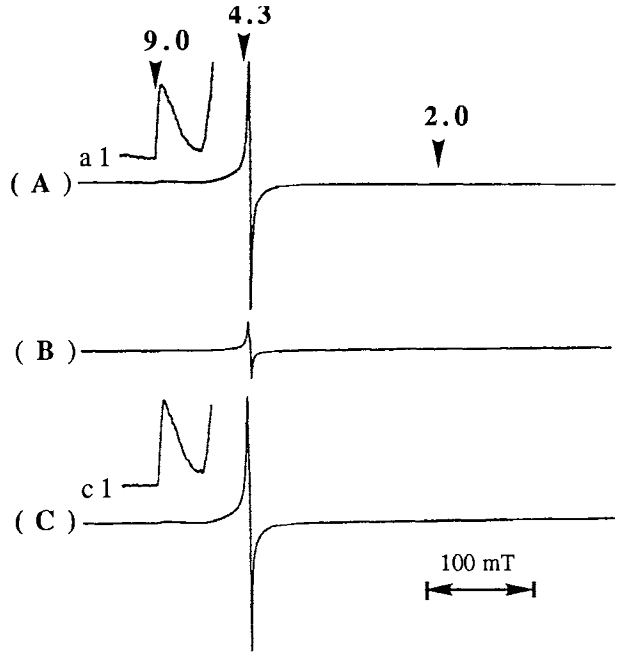


Fig. 1. ESR spectra during the reaction of Fe(III)-nta and H_2O_2. (A) initial Fe(III)-nta solution; (B) spectrum just after the addition of H_2O_2 to Fe(III)-nta solution; Just after the addition of H_2O_2 to the solution, the ESR signal decreased and the signals at $g' \approx 4.3$ split. The mononuclear minor component (Fe(III)-nta complex (**2'**)) was shown in (B); (C) final Fe(III)-nta solution after the oxygen production. Fe(III)-nta complexes, (**1**) and (**3**), showed high-spin ferric state with maximum rhombicity. The a1 and c1 spectra were enlarged around $g' \approx 9.0$. ESR spectra were recorded under the following conditions: microwave power, 10 mW; modulation amplitude, 0.63 mT; scanning field, 250± 250 mT; amplitude, 1.6 x 10; sweep time, 4 min; response, 0.1 s.

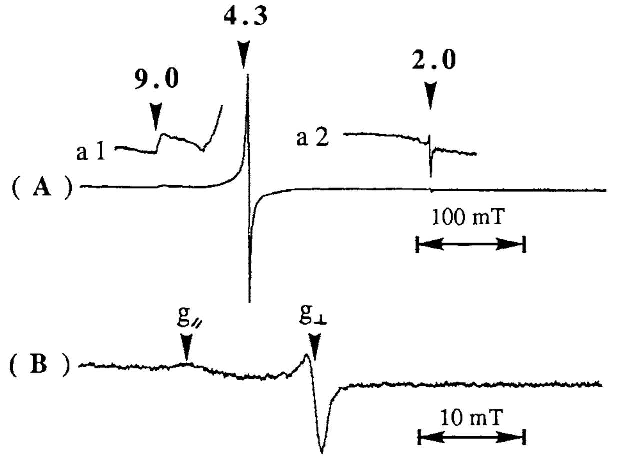


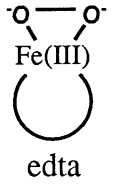
Fig. 2. ESR spectra just after the addition of H_2O_2 to Fe(III)-nta solution. In spectrum (A), a free radical was detected together with a high-spin ferric signal (Fe(III)-nta complex (**2'**)). The a1 and a 2 spectra were enlarged around $g' \approx 9.0$ and 2.0 respectively. As shown in spectrum (B), the free radical had values of $g_{\perp}=2.009$ and $g_{//}=2.084$ with axial symmetry, and was taken to be a superoxide anion. ESR spectra were recorded under the same condition described in Fig.1. The scanning fields were 250 ± 250 mT in spectrum (A) and 330 ± 25 mT in spectrum (B), and the amplitudes were 6.3×10 in spectrum (A) and 2×1,000 in spectrum (B).

Table 1. Experimental and calculated g' values of Fe(III)-nta during the reaction with H_2O_2

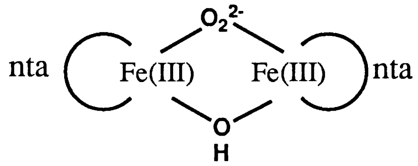
		Experimental		Calculated a)		E/D
Fe(III)-nta	**1**	4.17, 4.22	8.7	4.17, 4.21	9.4, 9.6	1/3
	2'	4.16, 4.25	8.8, 8.1	4.16, 4.29	9.1, 9.7	0.32
	3	4.17, 4.22	8.7	4.17, 4.21	9.4, 9.6	1/3

a) The eigenvalues and eigenvectors of S=5/2 spin Hamiltonian matrix were calculated assuming D=0.74 cm^{-1}, and E/D=1/3 or 0.32. The effective g' values were obtained from Kramers' doublets.

Fujii and et al.[6] suggested a mononuclear Fe(III)-edta peroxo complex (a) as an intermediate. We present a possibility that a binuclear iron-nta complex (b) could be involved in the generation of superoxide anion in our reaction system. Just after the addition of H_2O_2, ESR ferric ion signals decreased markedly, but almost all the Fe-nta complex remained as ferric ion, which was indicated by the finding that the absorbance of 535 nm did not appear after the addition of bathophenanthroline to the mixture. The Fe(III)-nta tends to take a binuclear structure compared with Fe(III)-edta when pH of the system is high and the complex is dissolved in DMSO. We inferred that the Fe(III)-nta complex (**2**) had an antiferromagnetically coupled binuclear structure. The residual Fe(III)-nta complex (**2'**) showed a high-spin ferric state with large rhombicity. Since we have no direct evidence of the binuclear complex, we should do more detailed studies to examine the reaction intermediates.



a



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