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Appearance of Electron Spin Resonance Signals in the Interaction of Dithiocarbamate-Fe(II) with Nitrogen Dioxide and Nitrite

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Whether dithiocarbamate-Fe(II) complexes used in the electron spin resonance (ESR) studies are specific for the detection of NO was investigated. It was found that the typical ESR signals of dithiocarbamate-Fe(II)-NO spin adducts appeared in the interaction of *N*-methyl-D-glucaminedithiocarbamate (MGD)-, bis(hydroxyethyl) dithiocarbamate (HED)- and diethyldithiocarbamate (DED)-Fe(II) complexes with the NO-derived nitrogen oxides, nitrogen dioxide (NO_2) and nitrite ion (NO_2^-) in a prolonged incubation. Under the conditions of prolonged incubation, appearance of the ESR signals of the dithiocarbamate-Fe(II)-NO spin adducts may indicate the presence of not only NO but NO_2 and NO_2^-.

Keywords: Dithiocarbamate-Fe(II), dithiocarbamate-Fe(II)-NO spin adduct, nitrogen monooxide, nitrogen dioxide, nitrite

INTRODUCTION

Nitrogen monooxide (NO) is synthesized by NO synthase in various kinds of cells such as endothelial cells,[1] nerve cells[2] and macrophages.[3] NO has activities as an endothelium-derived relaxing factor (EDRF),[1] a signal molecule in nerve system[2] and a cell killing factor of macrophages.[4,5] NO is, however, readily converted in an aqueous solution into various NO-derived nitrogen oxides to exert toxic effects to the cells. NO is converted into ONOONO, nitrogen dioxide (NO_2) and finally nitrite ion (NO_2^-) by interaction with molecular oxygen[6] and into peroxynitrite ($ONOO^-$) and finally nitrate ion (NO_3^-) by interaction with superoxide.[7,8]

Dithiocarbamate-Fe(II) complexes: *N*-methyl-D-glucaminedithiocarbamate (MGD)-Fe(II), bis (hydroxyethyl)dithiocarbamate (HED)-Fe(II) and diethyldithiocarbamate (DED)-Fe(II) (Fig. 1) have been shown to form the dithiocarbamate-Fe(II)-NO spin adducts by interaction with NO.[9,10] MGD-Fe(II) and HED-Fe(II) complexes

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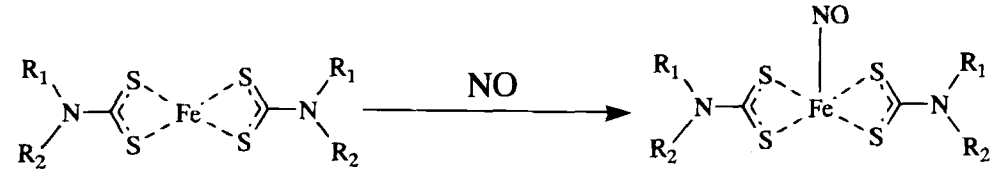

FIGURE 1 Structures of dithiocarbamate-Fe(II) complexes and their NO spin adducts.

are soluble in water, and DED-Fe(II) complex is soluble in organic solvents. The dithiocarbamate-Fe(II)-NO spin adducts give characteristic electron spin resonance (ESR) signals of a triplet. MGD-Fe(II) complex has been often used for detection of NO in biological systems because of its high solubility in water. However, whether the dithiocarbamate-Fe(II) complexes are specific for the detection of NO in the presence of the NO-derived nitrogen oxides is not known.

In the present study, we examined the reactivities of the dithiocarbamate-Fe(II) complexes for the NO-derived nitrogen oxide species: NO_2, NO_2^-, NO_3^- and $ONOO^-$. It was found that the dithiocarbamate-Fe(II) complexes reacted with NO_2 and NO_2^- to give the ESR signals of the dithiocarbamate-Fe(II)-NO spin adducts.

MATERIALS AND METHODS

Materials

N-Methyl-D-glucaminedithiocarbamate (MGD) sodium salt was prepared as reported previously[11] from *N*-methyl-D(–)-glucamine (Wako Pure Chemical Industries, Osaka, Japan) and carbon disulfide (Wako). Diethyldithiocarbamate (DED) sodium salt and bis(2-hydroxyethyl) dithiocarbamate (HED) zinc salt were obtained from Wako and Tokyo Chemical Industry (Tokyo, Japan), respectively. Fe(II) sulfate heptahydrate was from Wako. MGD-Fe(II) complex was prepared by dissolving 75 mM MGD sodium salt and 15 mM Fe(II) sulfate in 0.1 M Tris-HCl buffer (pH 7.4). HED-Fe(II) complex was prepared by dissolving 37.5 mM HED zinc salt and 7.5 mM Fe(II) sulfate in the same buffer. DED-Fe(II) complex was prepared by dissolving 75 mM DED sodium salt and 15 mM Fe(II) sulfate in water followed by extraction with an equal volume of chloroform. The concentration of the dithiocarbamate-Fe(II) complexes was expressed with respect to that of Fe(II).

NO (100 μl/liter) in nitrogen gas, NO_2 (101 μl/liter) in nitrogen gas and NO_2 (103 μl/liter) in air were produced by Nippon Sanso Company (Tokyo, Japan). The concentrations of NO and NO_2 were determined by the chemiluminescence method[12] using nitrogen oxide analyzer CLA-510SS (Horiba, Tokyo, Japan) at the Yokohama Research Center of Tomoe Company (Yokohama, Japan). The contents of NO in the NO_2 preparations were less than the detection limit of NO (1 μl/liter) as estimated by the chemiluminescence method. The effective concentrations of NO and NO_2 obtained after passage through a transfer tubing in each experiment were determined by the method of Saltzman.[13]

Peroxynitrite ($ONOO^-$) was prepared according to the method previously described.[14] In brief, a solution of $NaNO_2$, a solution of H_2O_2 in HCl and a solution of NaOH were mixed sequentially in a quenched flow reactor with two T-junctions at a flow rate of 40 ml/min. The alkaline $ONOO^-$ solution obtained in a collection chamber was frozen at –20°C to concentrate $ONOO^-$ into a yellow-colored

liquid layer on the top of the ice crystal. The liquid layer contained about 0.5 M $ONOO^-$ when determined using the extinction coefficient of 1670 at 302 nm[14] in 1.0 M NaOH.

ESR Spectroscopy

ESR spectra were obtained on an X-band JES-RE1X spectrometer (JEOL, Tokyo, Japan) at room temperature using a capillary glass tube (1.1 i.d. × 75 mm). The instrumental conditions were: field setting at 330.0 mT, scan range of 10 mT, modulation frequency of 100 kHz, microwave power of 10 mW, modulation amplitude of 0.1 mT, time constant of 1.0 s and scan time of 8 min. Recording the ESR spectra of the reaction mixtures of dithiocarbamate-Fe(II) complexes with NO or NO_2 was started 15–30 min after introduction of each gas.

Determination of Fe(II) Ion

Fe(II) ion was monitored by the method of Emmerie and Engel.[15] Thus, 2 ml of the reaction mixture was mixed with 1.0 ml of 0.5% 2,2′-dipyridyl in ethanol, and the mixture was made up to 20 ml with ethanol. The absorbance at 520 nm of the solution was measured.

RESULTS AND DISCUSSION

ESR spectra of the solutions of dithiocarbamate-Fe(II) complexes were measured after NO in nitrogen gas (less than 100 μl/liter), or NO_2 in nitrogen gas or in air (less than 100 μl/liter) was introduced into the solutions at room temperature for 1 h. In order to minimize unrequired conversion of NO and NO_2 in the solvents, NO or NO_2 gas was directly supplied to the solutions of the complexes.

When NO (1.5 μmol) in nitrogen gas was introduced into a solution of MGD-Fe(II) in Tris-HCl buffer (pH 7.4), the solution showed 3 lines of the typical ESR signals of the MGD-Fe(II)-NO spin adduct with hyperfine splitting constant (hfsc) of a_N = 1.28 mT (Fig. 2. A1). When NO_2 (1.8 or 1.9 μmol) in nitrogen gas or in air was similarly introduced into the solution of MGD-Fe(II), the solution showed the same 3-line ESR signals of the MGD-Fe(II)-NO spin adduct (Fig. 2. A2 and A3). ESR spectra of a solution of HED-Fe(II) complex in Tris-HCl buffer (pH 7.4) exposed to NO (1.4 μmol) in nitrogen gas, NO_2 (2.2 and 1.9 μmol) in nitrogen gas and in air showed the same signals of the HED-Fe(II)-NO spin adduct (data not shown). ESR spectrum of a solution of DED-Fe(II) in chloroform exposed to NO (1.3 μmol) in nitrogen gas (Fig. 2. B1), NO_2 (2.0 or 1.8 μmol) in nitrogen gas (Fig. 2. B2) or in air (Fig. 2. B3) showed the same signals of the DED-Fe(II)-NO spin adduct with hfsc of a_N = 1.28 mT.

It was found unexpectedly that dithiocarbamate-Fe(II) complexes gave the typical ESR signals of dithiocarbamate-Fe(II)-NO spin adducts in the interaction with NO_2. Molecular oxygen had

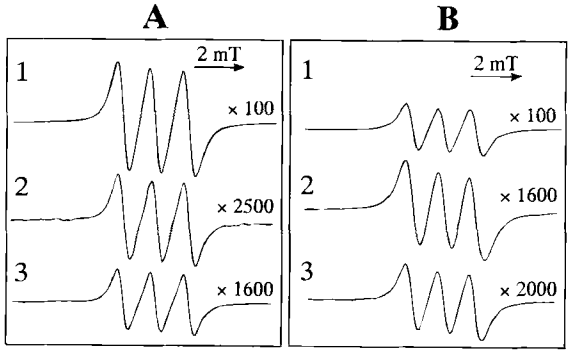

FIGURE 2 ESR spectra of the reaction mixtures of dithiocarbamate-Fe(II) complexes with NO and NO_2. **A:** NO (58–60 μl/liter) in nitrogen gas (total amount of NO 1.5 μmol) (1), NO_2 (66–78 μl/liter) in nitrogen gas (1.8 μmol) (2) or NO_2 (75–77 μl/liter) in air (1.9 μmol) (3) was introduced into 2 ml of a solution of 15 mM MGD-Fe(II) (Fe(II) 30 μmol) in 0.1 M Tris-HCl buffer (pH 7.4) at a flow rate of 10 ml/min for 1 h. The pH value of the aqueous mixture was kept at pH 7.4 during the exposure. **B:** NO (50–55 μl/liter) in nitrogen gas (NO 1.3 μmol) (1), NO_2 (78–85 μl/liter) in nitrogen gas (2.0 μmol) (2) or NO_2 (72–75 μl/liter) in air (1.8 μmol) (3) was introduced into 2 ml of a solution of 15 mM DED-Fe(II) (Fe(II) 30 μmol) in chloroform similarly. The lost amount of chloroform during the period was intermittently supplied. Each spectrum was obtained at a receiver gain mentioned at the right side of each spectrum.

little effect on the formation of the spin adducts from NO_2. Intensities of the ESR signals from NO_2 appeared to be lower than those from NO. The efficiency of the formation of the spin adducts from NO_2 could be roughly estimated to be 3–20% of that from NO based on the intensities of the signals obtained. However, exact comparison of the efficiency of NO_2 to that of NO in the formation of the spin adducts could not be made because the amounts of NO_2 and NO trapped in the solvents containing the complexes may be different.

ESR spectra of the solution of MGD-Fe(II) at pH 7.4 were measured after incubation with NO_2^- (1 or 2 μmol) at room temperature for 2 h. The ESR signals of the MGD-Fe(II)-NO spin adduct with hfsc of a_N = 1.28 mT appeared just after mixing. The intensities of the signals were dependent on the concentration of NO_2^- and on the incubation time. The intensities at both the concentrations linearly increased with an increase of incubation time (Fig. 3. A and B). After 2-h incubation the intensities of the signals from 2 μmol NO_2^- were 2-fold higher than those from 1 μmol NO_2^-. The efficiency of the spin adduct formation from nitrite (2 μmol) during 1 h may be roughly estimated to be about 3% of that from NO (Fig. 2. A1) when compared the intensities of their signals. Nitrate ion at the same concentrations did not give any ESR signals under the same conditions during the period of 2 h (data not shown).

$ONOO^-$ (2 μmol) was added to the solution of MGD-Fe(II) at pH 7.4, and the mixture was incubated at room temperature for up to 1 h. $ONOO^-$ may be partially protonated (pKa = 6.8)[15] to be converted into reactive peroxynitrous acid (ONOOH) under the conditions. The preparation of $ONOO^-$ may contain NO_2^-, NO_3^- and H_2O_2.[14] The ESR signals of the MGD-Fe(II)-NO spin adduct with low intensities appeared just after mixing and their intensities gradually increased with time (Fig. 4. Normal order). Reversed order of addition control,[14] in which $ONOO^-$ was decomposed before the reaction with MGD-Fe(II), also gave the same ESR signals (Fig. 4. Reversed order). Hence, the ESR signals observed in the reaction of ONOOH cannot be attributed to ONOOH but to the contaminated NO_2^-.

It was found that NO_2 and NO_2^- reacted with dithiocarbamate-Fe(II) complexes to afford the dithiocarbamate-Fe(II)-NO spin adducts. Introduction of NO_2 (2.0 μmol) in nitrogen gas and NO_2 (2.3 μmol) in air into Tris-HCl buffer (pH 7.4) produced only a limited amount of 0.24 and 0.30 μmol NO_2^-, respectively, when estimated by

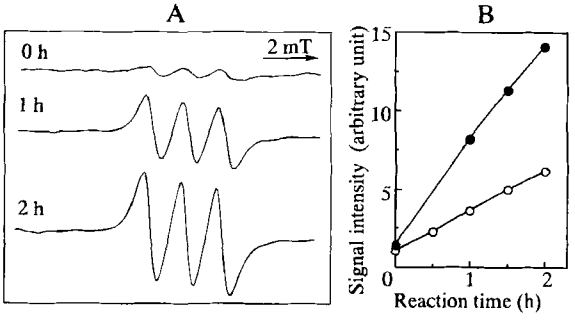

FIGURE 3 ESR spectra of the reaction mixtures of MGD-Fe(II) with NO_2^-. ESR spectra of a 2-ml reaction mixture of 1 mM nitrite (2 μmol) and 15 mM MGD-Fe(II) (Fe(II) 30 μmol) in 0.1 M Tris-HCl buffer (pH 7.4) incubated at room temperature for the indicated period (A), and the time course of the intensity of the central signals of the reaction mixtures with 1 mM (2 μmol) (●) and 0.5 mM (1 μmol) (○) NO_2^- (B). Receiver gain was set at 2500.

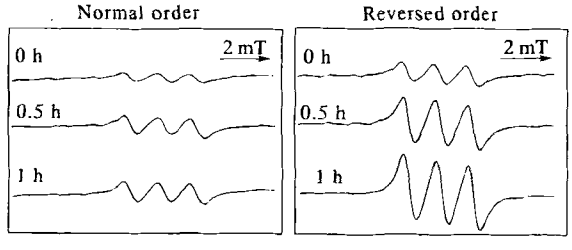

FIGURE 4 ESR spectra of the reaction mixture of MGD-Fe(II) and ONOOH. Normal order: 4 μl of the $ONOO^-$ solution corresponding to 2 μmol $ONOO^-$ was added to 2.2 ml of a solution of 13.6 mM MGD-Fe(II) (Fe(II) 30 μmol) in 0.1 M Tris-HCl buffer (pH 7.4), and the mixture was allowed to stand at room temperature for the indicated period. Reversed order: $ONOO^-$ (2 μmol) was preincubated in 0.2 ml of the buffer for 3 min and mixed with 2 ml of a solution of 15 mM MGD-Fe(II) in the buffer, and the mixture was allowed to stand at room temperature for the indicated period. Receiver gain was set at 2500.

the Saltzman reagent. Furthermore, NO_2 may not be hydrolyzed into NO_2^- in a chloroform solution whereas NO_2 reacted with DED-Fe(II) to form DED-Fe(II)-NO spin adduct in chloroform (Fig. 2. B2 and B3). Conversion of NO_2 into NO_2^- may not be requisite for the spin adduct formation. When NO_2 in nitrogen gas was introduced into the solution of Fe(II) sulfate (2 μmol), the amount of Fe(II) ion was decreased to 1.88, 1.66, 1.16 and 0.40 μmol by introduction of 1.1, 2.2, 4.4 and 6.6 μmol NO_2, respectively. The decrease in the concentration of Fe(II) ion may indicate the oxidation of Fe(II) into Fe(III) and thus the reduction of NO_2 into NO. It has been shown that NO_2^- can be reduced by an excess amount of nitrilotriacetic acid-Fe(II) complex into NO which in turn reacts with the complex to form nitrilotriacetic acid-Fe(II)-NO adduct.[16] It is likely that the interaction of NO_2 and NO_2^- with an excess amount of the dithiocarbamate-Fe(II) complexes generated NO to form the dithiocarbamate-Fe(II)-NO spin adducts. It has been recently shown that a mixture of NO_2^- (100 μM) with MGD-Fe(II) complex gives significant signals of MGD-Fe(II)-NO spin adduct only after prolonged incubation over 90 min.[17] However, in the present study the signals were observed immediately after NO_2^- (1 mM) was mixed with MGD-Fe(II) complex. Appearance of the signals of the spin adduct may depend on the concentration of NO_2^- and incubation time.

In conclusion, dithiocarbamate-Fe(II) complexes reacted slowly with NO_2 and NO_2^- to form the dithiocarbamate-Fe(II)-NO spin adducts. In this reaction, NO_2 and NO_2^- might be reduced into NO to be trapped by the complexes. Hence, the ESR signals of the dithiocarbamate-Fe(II)-NO spin adducts should appear as a result of the presence of not only NO but NO_2 and NO_2^-, especially in the prolonged contact of the sample and the dithiocarbamate-Fe(II) complexes.

Acknowledgments

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