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Efficient Formation of a Nitrosyl(protoporphyrinato)iron(II) Complex on Magnesium Oxide Powder

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We have investigated the reaction between the system of hydroxo(protoporphyrinato)iron(III) (Heme) complex adsorbed on magnesium oxide (MgO) powder in the presence of cysteine (Cys) (Heme/MgO/Cys) and the nitrite ion (NO_2^-), using a Clark-type oxygen electrode and an ESR method. The nitrite ion acted as an inhibitor on the oxygen consumption by the Heme/MgO/Cys. This inhibition was not observed on the system of Heme aqueous solution ($Heme_{aq}$) in the presence of cysteine ($Heme_{aq}$/Cys) and NO_2^-, where the oxygen consumption was caused. The formation of a nitrosyl(protoporphyrinato)iron(II) complex in the reaction mixture of the Heme/MgO/Cys and NO_2^- was confirmed by the measurement of an ESR spectrum. This result suggests that the formation of the nitrosyl(protoporphyrinato)iron(II) complex during the reduction of NO_2^- to nitrogen monoxide (NO) plays a key role on the inhibitory action of NO_2^-. A possible mechanism for the formation of the nitrosyl(protoporphyrinato)iron(II) complex on the reaction between the Heme/MgO/Cys and NO_2^- is discussed, as a characteristic feature of our heterogeneous system.

We reported recently that some heterogeneous catalysts having porphyrin structure had higher reactivity in chemical and biochemical reactions than homogeneous systems.[1–4] Among these systems, efficient oxygen consumption was observed on the system of hydroxo(protoporphyrinato)iron(III) complex (Heme) adsorbed on magnesium oxide (MgO) powder in the presence of cysteine (Cys) (Heme/MgO/Cys).[4] The oxygen consumption happens in the reaction for a four-electron reduction of oxygen by four molecules of cysteine with the aide of catalytic action of the system of Heme adsorbed on MgO powder (Heme/MgO). Based on the analysis of our results, it is thought that this system can be applicable to some field as a reduction catalyst, the reactivity of which is similar to that of cytochrome P-450.

Minamiyama et al. reported the role of cytochrome P-450 on the release of nitric oxide from organic nitrate derivatives in human heart vessels.[5] In this case, the reduction of nitrate derivatives was induced by cytochrome P-450 to produce nitrogen monoxide (NO). Therefore, the understanding for the reductive action of cytochrome P-450 is also important on the production of NO.

One nitrite reductase consists of two subunits, each of which contains cytochrome *c* and d_1.[6] In the catalytic reaction of this enzyme, porphyrinatoiron complexes act as reaction centers. Especially, the cytochrome d_1 has been identified as the reduction site of NO_2^-.[7–9] The formation of a nitrosyl(porphyrinato)iron(II) complex as a reaction intermediate plays a crucial role during the catalytic cycle of nitrite reductase or its model compounds.[10–14] Some reaction models of nitrite reductase in nonaqueous media were studied using porphrynatoiron complexes.[15–18] However, little study about the reaction model of nitrite reductase has been conducted in aqueous media, even though nitrite reductase acts in aqueous media.

During our study using the Heme/MgO/Cys as a reaction model of nitrite reductase to clarify the reduction of NO_2^- to NO caused by porphyrinatoiron complex in aqueous media, we found efficient formation of a nitrosyl(protoporphyrinato)-iron(II) complex in the system. Its formation can be regarded as a characteristic feature of the Heme/MgO/Cys, because its formation was not observed in the system of Heme aqueous solution ($Heme_{aq}$) in the presence of cysteine ($Heme_{aq}$/Cys). In this paper, we will describe the details of the experimental results and discuss a possible mechanism for its formation.

Experimental

Hydroxo(protoporphyrinato)iron(III) (Heme) was obtained from Aldrich Chem. Co. Inc. Magnesium oxide powder (MgO, 99.9%, 0.01 μm), a standard NO_2^- solution (993 mg dm^{-3}), and L-cysteine (special grade) were purchased from Wako Pure Chemical Industries, Ltd. The Heme adsorbed on MgO (Heme/MgO) system was prepared as follows: Magnesium oxide powder was suspended in water. One mmol dm^{-3} Heme aqueous solution was prepared by adding phosphate buffer solution (PBS, pH 7.4) after the dissolution of Heme in 0.01 mol dm^{-3} NaOH solution. Then the solution was added to the suspension. The amount of powdery MgO used was enough to adsorb all of the Heme addition. The sample suspension was used for experiments after being centrifugally washed with water several times.

A Clark-type oxygen electrode with 1.1 cm^{-3} volume cell (Central Kagaku Co.) was used for the measurements of oxygen consumption profiles. Oxygen consumption profiles were recorded after the addition of the suspension of the Heme/MgO or an aqueous solution of Heme ($Heme_{aq}$) in the mixed solution of cysteine, nitrite ion, and 50 mmol dm^{-3} phosphate buffer (pH 7.4) at room

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temperature. The oxygen consumption rates were determined by the tangents of kinetic curves at the starting point of oxygen consumption, as described previously.[4] An ESR measurement was conducted using a JEOL RE-2X ESR spectrometer at room temperature. ESR spectra were recorded after adding the suspension of the Heme/MgO or the aqueous solution of Heme ($Heme_{aq}$) in the mixed solution of cysteine, NO_2^-, and 50 mmol dm^{-3} phosphate buffer (pH 7.4).

Results

In our previous study,[4] a small reaction rate was obtained in the case of the $Heme_{aq}$/Cys. As a reason for this, aggregates of Heme may be formed in the $Heme_{aq}$/Cys. In this study, we attempted experiments under a condition that no aggregation was formed in the $Heme_{aq}$/Cys (concentration of PBS over 50 mmol dm^{-3}).

Figure 1 shows the relationship between the oxygen consumption rate [Heme/MgO/Cys (●) and $Heme_{aq}$/Cys (■)] and the concentration of cysteine (Heme, 45 μmol dm^{-3}). The rate for the Heme/MgO/Cys increased with the concentration of cysteine. On the other hand, the rate for the $Heme_{aq}$/Cys increased with the concentration of cysteine up to 30 mmol dm^{-3} and saturated with the concentration of cysteine over 30 mmol dm^{-3}. The maximum rate of the Heme/MgO/Cys was about four times larger than that of the $Heme_{aq}$/Cys under the conditions used in the present study.

Figure 2 shows the relationship between the oxygen consumption rate [Heme/MgO/Cys (●) and the $Heme_{aq}$/Cys (■)] and the concentration of NO_2^- (Heme, 25 μmol dm^{-3}; Cys, 50 mmol dm^{-3}). In Fig. 2, the oxygen consumption rate for the Heme/MgO/Cys of about 0.01 mmol dm^{-3} min^{-1} was the oxygen consumption rate that was caused by the oxidation of cysteine induced by a catalytic action of MgO.[4] An increase in the concentration of NO_2^- led to a nonlinear inhibition of the oxygen consumption for the Heme/MgO/Cys. The oxygen consumption of the Heme/MgO/Cys was almost completely inhibited at the concentration of NO_2^- about 12.5 μmol dm^{-3}, a value that was half of the concentration of Heme (25 μmol dm^{-3}). This result suggests that one molecule of NO_2^- inhibits some (two?) molecules of Heme because the oxygen consumption rate of the Heme/MgO/Cys should linearly decrease with a decrease in the concentration of Heme.[4] This phenomenon was not observed in a homogeneous case for the $Heme_{aq}$/Cys in the presence of NO_2^-, though the oxygen consumption occurred (see Fig. 1). Furthermore, the feature signal in the Soret band of electronic absorption spectrum for nitorosyl(porphyrinato)iron(II)[15–17,19] was not observed in the case for the $Heme_{aq}$/Cys in the presence of NO_2^-. Therefore, this phenomenon can be regarded as a characteristic feature of the Heme/MgO/Cys.

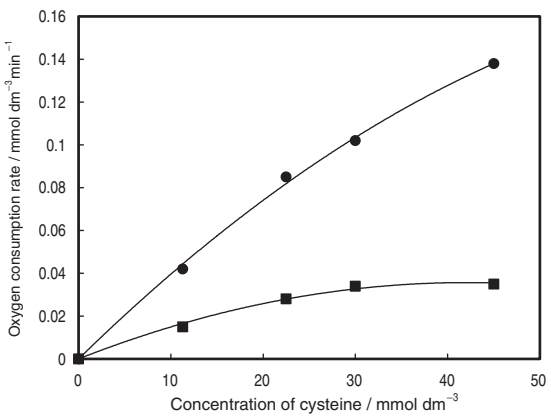

Fig. 1. Relationship between oxygen consumption rate and the concentration of cysteine measured at room temperature. Concentration of Heme, 45 μmol dm^{-3}. Closed circle, Heme/MgO/Cys; closed square, $Heme_{aq}$/Cys.

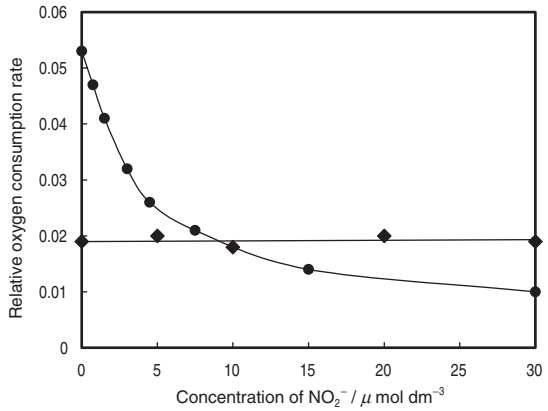

Fig. 2. Relationship between the concentration of NO_2^- and oxygen consumption rates measured at room temperature. Concentration of Heme, 25 μmol dm^{-3}; concentration of cysteine, 45 mmol dm^{-3}. The other conditions are the same as Fig. 1.

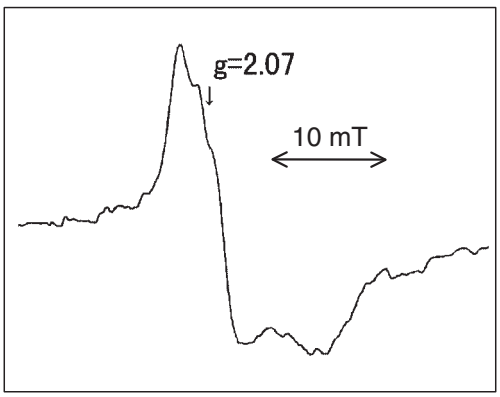

Fig. 3. ESR spectrum of the Heme/MgO/Cys in the presence of 0.25 mmol dm^{-3} NO_2^- measured at room temperature. Concentration of Heme, 0.2 mmol dm^{-3}; concentration of cysteine, 50 mmol dm^{-3}.

In order to clarify the mechanism for the reaction between the Heme/MgO/Cys and NO_2^- in more detail, we conducted the measurements for the ESR spectrum of paramagnetic species formed in the system. Figure 3 shows the ESR spectrum obtained from the Heme/MgO/Cys (Heme, 0.2 mmol dm^{-3}; Cys, 50 mmol dm^{-3}) in the presence of 0.25 mmol dm^{-3} NO_2^- at room temperature. The ESR signal observed can be assigned to a six-coordinated nitrosyl(protoporphyrinato)iron(II) complex.[20] This means that the reduction of NO_2^- to NO is caused by the Heme/MgO/Cys in the presence of NO_2^-. It is thought

that the formation of the nitrosyl(protoporphyrinato)iron(II) complex plays a key role in the inhibitory action of NO_2^- for the Heme/MgO/Cys.

Figure 4 shows the relationship between the concentration of NO_2^- and the ESR signal intensity of the nitrosyl(protoporphyrinato)iron(II) complex produced by the Heme/MgO/Cys (Heme, 0.2 mmol dm^{-3}; Cys, 50 mmol dm^{-3}) measured after 1-min mixing. The ESR signal intensity of the nitrosyl(protoporphyrinato)iron(II) complex increased with the concentration of NO_2^- up to 0.1 mmol dm^{-3} (half of the concentration of Heme). On the other hand, the ESR signal intensity of the nitrosyl(protoporphyrinato)iron(II) complex saturated with the concentration of NO_2^- over 0.1 mmol dm^{-3}. This phenomenon was similar to the case observed in Fig. 2. The above results allow one to presume that one molecule of NO_2^- reacts with two molecules of Heme.

Figure 5 shows the time course for the ESR signal intensity of the nitrosyl(protoporphyrinato)iron(II) complex produced by the Heme/MgO/Cys (Heme, 0.2 mmol dm^{-3}; Cys, 50 mmol dm^{-3}) in the presence of 0.1 (■) and 0.2 (▲) mmol dm^{-3} NO_2^-. No change in the time course of the ESR signal intensity for the nitrosyl(protoporphyrinato)iron(II) complex was observed in the Heme/MgO/Cys in the presence of 0.1 mmol dm^{-3} NO_2^-. This means that the formation of the nitrosyl(protoporphyrinato)iron(II) complex has already finished after 1-min mixing. On the other hand, an increase of reaction time led to an increase in the ESR signal intensity of the nitrosyl(protoporphyrinato)iron(II) complex produced by the Heme/MgO/Cys in the presence of 0.2 mmol dm^{-3} NO_2^-. The maximum ESR signal intensity observed in the case containing 0.2 mmol dm^{-3} NO_2^- after 9-min mixing was two times larger than that observed in the case containing 0.1 mmol dm^{-3} NO_2^- after 9-min mixing. The analysis of these results suggests that the formation of the nitrosyl(protoporphyrinato)iron(II) complex can be performed by two reactions in the system in the presence of 0.2 mmol dm^{-3} NO_2^-. The faster reaction can be conducted by binding one molecule of NO_2^- and two molecules of Heme. The slower reaction can be conducted in such a way that one molecule of NO_2^- combines with one molecule of Heme.

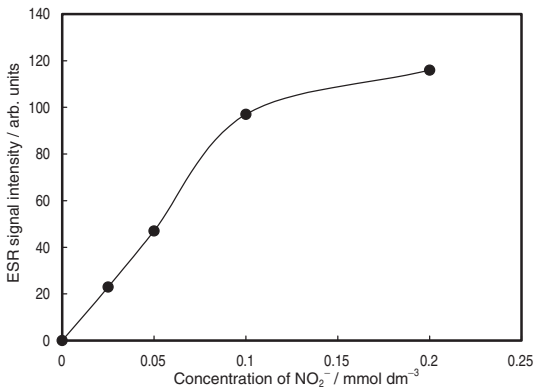

Fig. 4. Relationship between the concentration of NO_2^- and the intensities for the ESR signal of nitrosyl(protoporphyrinato)iron(II) complex observed in the Heme/MgO/Cys. Concentration of Heme, 0.2 mmol dm^{-3}; concentration of cysteine, 50 mmol dm^{-3}.

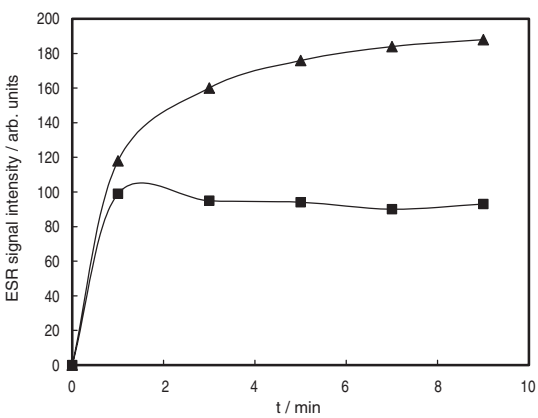

Fig. 5. Time course of the intensities for the ESR signal of nitrosyl(protoporphyrinato)iron(II) complex observed in the Heme/MgO/Cys containing 0.1 mmol dm^{-3} (closed square) and 0.2 mmol dm^{-3} (closed triangle) nitrite ion. The other conditions are the same as Fig. 4.

Discussion

On the basis of the results obtained in the present study, we present here a possible mechanism for the formation of the nitrosyl(protoporphyrinato)iron(II) complex by the reaction between the Heme/MgO/Cys and NO_2^-. The mechanism is described in the following: First, the five-coordinated iron(III) in the Heme/MgO is reduced to the six-coordinated iron(II) ion by the addition of cysteine. The formations of the iron(II) ion and cystine were confirmed from the measurements of Raman spectra and capillary electrophoresis (CE), as described previously.[4] The control of molecular movement by fixing Heme on MgO is thought to be crucial for the occurrence of the following reactions, because no formation of the nitrosyl(protoporphyrinato)iron(II) complex was observed in the $Heme_{aq}$/Cys (see Fig. 2). In the presence of NO_2^-, the reaction between the six-coordinated iron(II) in the Heme/MgO and NO_2^- leads to the formation of an intermediate, such as a porphyrinatoiron(II) dimer–NO_2^- complex. Unfortunately, the formation of this intermediate could not directly be confirmed in our present study. However, the formation of the intermediate can be expected from the result that one molecule of NO_2^- reacts with two molecules of Heme (see Figs. 2, 4, and 5). The six-coordinated nitrosyl(protoporphyrinato)iron(II) complex may be produced by the reaction between the porphyrinatoiron(II) dimer–NO_2^- complex and cysteine (faster reaction, see Fig. 5). The formation of the six-coordinated nitrosyl(protoporphyrinato)iron(II) complex was confirmed from the measurement of an ESR spectrum (see Fig. 3). Further, the six-coordinated nitrosyl(protoporphyrinato)iron(II) complex may be produced by the reaction between the porphyrinatoiron(II)–NO_2^- complex and cysteine in the presence of NO_2^- over a half concentration of Heme (slower reaction, see Fig. 5).

In conclusion, we have demonstrated that nitrosyl(protoporphyrinato)iron(II) complex is formed by the reaction between the Heme/MgO/Cys and NO_2^-. This phenomenon is not observed in the homogeneous case of the $Heme_{aq}$/Cys. Therefore, the production of the nitrosyl(protoporphyrinato)iron(II) complex can be regarded as a characteristic feature of a heter-

ogeneous medium of the Heme/MgO/Cys. Further, the Heme/MgO/Cys is a bifunctional catalyst similar to cytochrome cd_1,[21,22] capable of catalyzing the one-electron reduction of NO_2^- to NO, and the four-electron reduction of oxygen to water.

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