DOI: 10.1002/anie.201305459

Enzyme Models

Superoxide Disproportionation Driven by Zinc Complexes with Various Steric and Electrostatic Properties**

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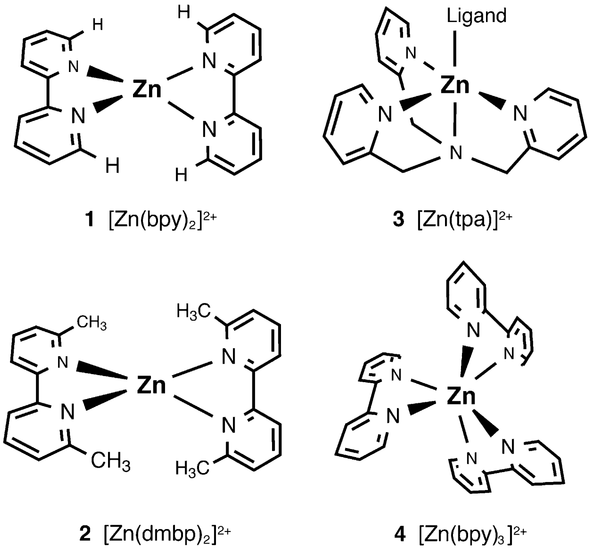
Molecular oxygen (dioxygen) is biochemically converted into reactive oxygen species by higher organisms. Superoxide, $O_2^{\bullet -}$, is a reactive oxygen species which reacts with redox-active metal ions to generate a hydroxo radical, itself a "higher" reactive oxygen species. The hydroxo radical nonspecifically oxidizes proteins, nucleic acids, sugars, and lipids to produce further oxidized biomolecules, which cause various oxidative diseases and cancers.[1] Metal-containing superoxide dismutases (M SODs)[2,3] play an important role in the suppression of superoxide-induced damage by catalyzing the disproportionation of superoxide to hydrogen peroxide and dioxygen under physiological conditions.

Copper–zinc superoxide dismutases (Cu–Zn SODs)[3] were discovered as essential enzymes in natural antioxidant biosystems. The active site forms an imidazolate-bridged heterodinuclear structure containing a copper ion and a zinc ion. Investigations of Cu–Zn SOD have revealed that the copper ion plays an indispensable role as an electron donor and acceptor in the catalytic SOD reaction, and that the function of the zinc ion is to regulate the coordination geometry at the active site.

Investigations of Zn metalloenzymes and synthetic Zn model complexes[4] have provided insight into the relationships between the structure and electronic properties of Zn active sites. These studies led us to investigate the possibility that the Zn site plays additional roles in the superoxide dismutation catalyzed by Cu–Zn SOD. In the present study, to elucidate the effects of the coordination structure and Lewis acidity of Zn^{II} ions on the superoxide-disproportionation reaction, we evaluated the interactions between superoxide and Zn^{II} complexes with various steric and electrostatic properties: $[Zn(bpy)_2](ClO_4)_2$ (**1**), $[Zn(dmbp)_2](ClO_4)_2$ (**2**), $[Zn(tpa)(L)](ClO_4)_2$ (**3**), and $[Zn(bpy)_3](ClO_4)_2$ (**4**); Figure 1).[5] Our study indicates that the Lewis acidity and coordination geometry of the Zn complex regulates the SOD activity and thus demonstrates the unique role of the Zn ion in the operation of SOD. The obtained results provide insight into the mechanism of action of Cu–Zn SOD as well as a useful strategy for the design of Zn complexes as drugs that mimic the action of SOD for antioxidant therapies.



1 $[Zn(bpy)_2]^{2+}$ **3** $[Zn(tpa)]^{2+}$ **2** $[Zn(dmbp)_2]^{2+}$ **4** $[Zn(bpy)_3]^{2+}$

Figure 1. Structures of the synthetic Zn^{II} complexes studied.

The kinetic and thermodynamic parameters for the protonation of superoxide in aqueous solvents were investigated by the use of pulse radiolysis.[6a–c] The data indicated that the protonation of superoxide promotes its rapid disproportionation. In contrast, superoxide is quite stable in aprotic solvents, such as MeCN and dimethyl sulfoxide (DMSO). Superoxide disproportionation to generate the peroxide ion, O_2^{2-}, and molecular oxygen, O_2, is not likely to occur under conditions which inhibit the protonation of superoxide.[6c,d] Therefore, dry aprotic solvents were used in this study to investigate the reactions of various Zn complexes with superoxide and the formation of superoxo–/peroxo–zinc complexes.

The reaction of superoxide with **1** as a structural model of the tetrahedral Zn site in Cu–Zn SOD[3] was performed in a nonaqueous MeCN/DMSO (9:1) solvent mixture at 15 °C and monitored by cyclic voltammetry (CV) and ESR spectroscopy. In the absence of **1**, the redox wave of $O_2^{\bullet -}/O_2$ was observed by cyclic voltammetry (Figure 2 A), and an ESR signal originating from free superoxide was detected (Figure 2 C).[7] The redox wave and ESR signal rapidly disappeared when **1** was added to the superoxide solution. This

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[**] This research was supported in part by Grants-in-Aid for Scientific Research and the JSPS (Japan). The authors thank Dr. Yuko Wasada-Tsutsui for her assistance in DFT calculations.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201305459.

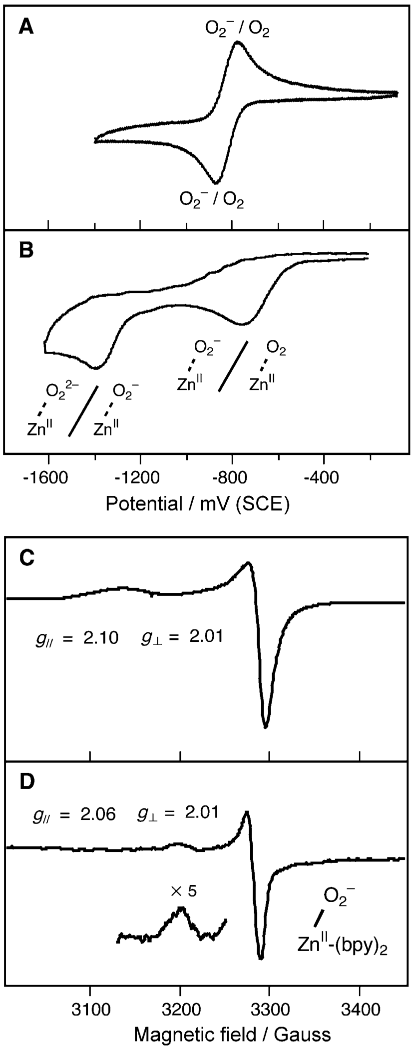

Figure 2. A,B) Cyclic voltammograms of superoxide $O_2^{\bullet -}$ (A) and the reaction intermediates generated by the combination of peroxide, O_2^{2-}, with **1** (B). C,D) X-band ESR spectra of $O_2^{\bullet -}$ (C) and the superoxo–zinc(II) intermediate generated by the reaction of **1** with $O_2^{\bullet -}$ (D). SCE = saturated calomel electrode.

result implies that the Zn complex **1** quenches superoxide in solution. Since the Zn ion is not redox-active and the experiment was conducted in an aprotic solvent, it follows that the Zn^{II} complex acts as a Lewis acid[4a,8] to initiate electron transfer between superoxide affected by the Zn^{II} complex and free superoxide to afford O_2^{2-} and O_2 ($Zn^{II\bullet}\cdots O_2^{\bullet -} + O_2^{\bullet -} \rightarrow Zn^{II}\cdots O_2^{2-} + O_2$; Figure 3 A,B).[6c,9]

To confirm the formation of superoxo–/peroxo–zinc(II) intermediates in this reaction, we performed a CV experiment after the binding of O_2^{2-} to **1**.[10] The characteristic waves were observed (Figure 2 B) and indicated the occurrence of two electrochemically active intermediates in solution. The two cathodic peak potentials at −740 and −1380 mV are derived from the reduction of O_2 to $O_2^{\bullet -}$ and $O_2^{\bullet -}$ to O_2^{2-}, respectively. It is remarkable that these potentials are less negative in the presence of **1** relative to the corresponding reduction potentials of free O_2 and $O_2^{\bullet -}$.[6c,11] The results clearly demonstrate that the electrostatic interaction of the Zn^{II} complex with the dioxygen anions O_2^{2-} and $O_2^{\bullet -}$ contributes to the effective reduction of superoxide/dioxygen. Furthermore, for the reaction of Zn complex **1** with an excess of superoxide (KO_2/**1** molar ratio: 4:1), the ESR spectrum exhibited a signal characteristic of the superoxo–zinc(II) intermediate during the superoxide-disproportionation reaction (Figure 2 D).[12] This observation directly reveals the presence of an interaction between the Zn complex and superoxide during the disproportionation reaction. When the energy gap (Δ) between the $2p\pi_x^*$ and $2p\pi_y^*$ orbitals of the superoxide molecule was evaluated from the ESR parameters, the value for the superoxo–zinc(II) intermediate ($\Delta = 0.48$ V) was significantly larger than that of the metal-free superoxide species ($\Delta = 0.27$ V).[13] On the basis of the positive shift of the reduction potential of $O_2^{\bullet -}$ and the large Δ value, we can conclude that direct interaction between **1** and superoxide should stabilize both π* orbitals energetically to give rise to the favorable one-electron reduction of superoxide. Thus, these observations support the hypothesis that the superoxide-disproportionation reaction in the presence of **1** proceeds by the effective reduction of superoxide, which is electrostatically affected by **1** (Figure 3 A,B).[6c,9b,c]

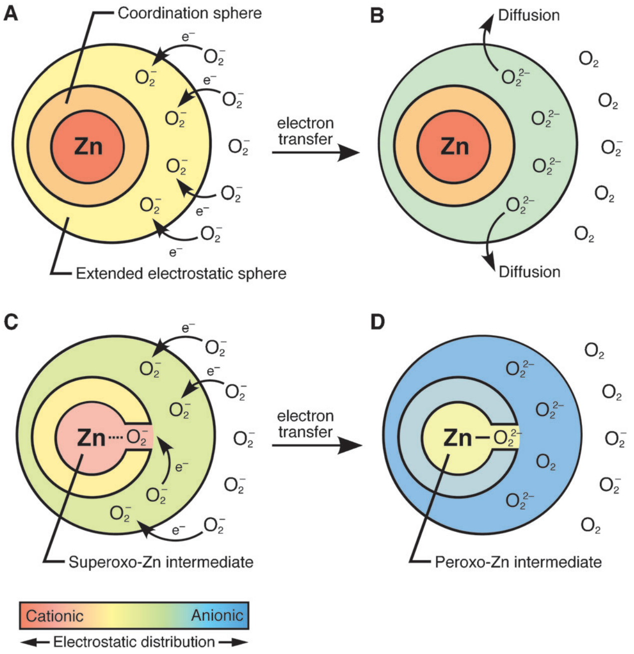

Figure 3. Schematic models for the disproportionation of superoxide $O_2^{\bullet -}$ by Zn^{II} complexes. Superoxides are attracted to the extended electrostatic sphere produced by the Lewis acidity of the Zn^{II} complex (A). Electron transfer from free superoxide to electrostatically affected superoxide affords peroxide O_2^{2-} and dioxygen O_2 (B). A Zn^{II} complex with a ligand-binding site (or with higher Lewis acidity) attracts superoxide and promotes rapid disproportionation (C). However, binding of the peroxide generated by superoxide disproportionation (or higher affinity for the peroxide) inhibits efficient diffusion of the peroxide (D) and subsequent superoxide disproportionation.

To improve our understanding of the effect of the Lewis acidity of the Zn^{II} complex on the superoxide-disproportionation reaction, we assessed the reaction of superoxide with $[Zn(dmbp)_2]^{2+}$ (**2**). The chelation of two dmbp ligands to a Zn ion yields a tetrahedral coordination geometry, and the steric hindrance between the methyl substituents decreases the extent of electron donation from the nitrogen atoms to the Zn center. Therefore, it can be assumed that the Lewis acidity of **2** is higher than that of **1**. To evaluate the electrostatic effect of **2** on dioxygen anions, CV measurements were made for the combination of O_2^{2-} and **2**.[10] As expected, the two reduction potentials observed (-720 and -1300 mV) were less negative than those of **1**. These results demonstrate that the substituent effect of the methyl groups makes the Lewis acidity of the Zn complex **2** higher; the higher Lewis acidity should, in turn, cause an increase in the affinity of **2** for dioxygen anions. Thus, **2** was expected to induce more efficient disproportionation of superoxide than **1**. However, when the molar ratio of superoxide to the Zn complex was increased, the ability of **2** to quench superoxide became weaker than that of **1** (Figure 4).[14] This phenomenon may be rationalized as follows: The Zn complex, which acts as a strong Lewis acid, is advantageous not only for exhibiting its high affinity for superoxide but also for the promotion of a rapid irreversible electron transfer from free superoxide to superoxide in the extended electrostatic sphere (the second coordination sphere; Figure 3 A,B). On the other hand, in the case of a Zn complex with a higher affinity for oxygen anions, the peroxide generated after the disproportionation of superoxide would not be efficiently diffused and/or would bind to the Zn complex. This state would not be suitable for the subsequent approach of superoxide to the Zn complex and would thus prevent the catalytic superoxide-disproportionation reaction (Figure 3 C,D).

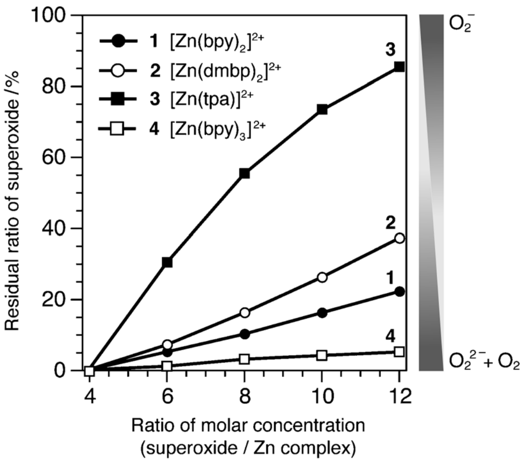

Figure 4. Comparison of the residual ratios of superoxide $O_2^{\bullet-}$ after reactions of Zn^{II} complexes **1–4** with $O_2^{\bullet-}$.

To test the above assumption, we also investigated the reactions of $[Zn(tpa)]^{2+}$ (**3**)[5] and $[Zn(bpy)_3]^{2+}$ (**4**) with superoxide. The Zn complex **3**, which has a trigonal-bipyramidal geometry constrained by the tripodal-type ligand,[4d,15] provides a vacant site at the Zn center which is accessible by the dioxygen anions. The Zn ion in complex **4** has a hexacoordinate octahedral geometry with three bpy ligands. The saturated coordination structure of **4** was expected to inhibit the binding of superoxide and peroxide.

During the reactions of superoxide with the Zn complexes **3** and **4**,[14] it was observed that both **3** and **4** quench superoxide in solution (Figure 4). The rate of superoxide quenching by **3** was much lower than the rates of quenching by **1** and **2**. Furthermore, electrospray ionization mass spectrometry (ESI-MS) after the reaction of KO_2 (1.2 mM) and **3** (KO_2/Zn 4:1) showed a positive-ion peak cluster at *m*/*z* 371.07 corresponding to $[Zn(tpa)(OH)]^+$, and a peak with a very low intensity at *m*/*z* 387.06, which would correspond to $[Zn(tpa)(OOH)]^+$[16a] (see Figure S1(B) in the Supporting Information). This result could be rationalized as follows: According to Figure 3 C,D and Figure 4 (KO_2/Zn 4:1), a peroxide ion produced by superoxide disproportionation binds to the vacant site of **3** and reacts readily with H_2O in the ionization chamber to generate a hydroxide ion and a hydroperoxide ion. In this case, owing to the considerably higher nucleophilicity/basicity of the hydroxide ion ($pK_b = -1.7$) relative to that of the hydroperoxide ion ($pK_b = 2.4$), the hydroxide ion binds predominantly to the Zn ion to afford $[Zn(tpa)(OH)]^+$.[16b] As a result, the ion peak of $[Zn(tpa)(OH)]^+$ was mainly observed in the ESI-MS spectrum. Therefore, in contrast to the other Zn complexes, the binding of dioxygen anions to the vacant site of **3** strongly decreased the Lewis acidity of the Zn ion and thus resulted in the suppression of catalytic disproportionation of superoxide (Figure 4).

Zn complex **4** showed superoxide-quenching ability even though **4** has a coordinatively saturated structure, which inhibits the binding of dioxygen anions to the Zn center (Figure 4). To gain an understanding of the electrostatic effect of the cationic Zn^{II} complex on the superoxide anion, we calculated the disproportionation energy of the superoxide anion around the Zn^{II} complex by using the DFT method (see the Supporting Information). On the basis of the experimental results and the simulation by DFT calculations, we conclude that the electrostatic effect of Zn ions extends beyond the coordination sphere to affect the electronic state of superoxide. This results in the stabilization of the superoxide anion near the Zn^{II} complex. The stabilized superoxide in the extended electrostatic sphere is effectively reduced by one-electron transfer from free superoxide to afford peroxide and dioxygen (Figure 3 A,B). Moreover, according to this mechanism, unless the Lewis acidity of the Zn ions is neutralized by the binding of dioxygen anions, the Zn complexes can facilitate superoxide-disproportionation reactions, as demonstrated by the difference in SOD activity between **3** and **4** (Figure 4).

On the basis of the total number of coordinated electron-donating pyridine groups in the ligands, the Lewis acidity of **4** is considered to be the lowest among the complexes investigated in this study.[8] However, it is interesting that **4** exhibits the highest catalytic activity among these Zn complexes. Figure 4 shows that the superoxide-disproportionation activity increases according to the order **3** < **2** < **1** < **4**, which is in agreement with the order of decreasing Lewis acidity. Therefore, for the catalysis of efficient disproportionation of superoxide by Zn complexes, it should be a priority to design a coordinatively saturated sphere similar to those found in **4** and the Zn site in Cu–Zn SOD.

This study has revealed the following features of Zn complexes: 1) Redox-inactive $\mathrm{Zn^{II}}$ complexes as Zn-site models of Cu–Zn SOD catalyze the disproportionation of superoxide. In particular, **4** accelerated the disproportionation of superoxide even without the direct binding of superoxide to the Zn center. This finding of the catalytic SOD activity of $\mathrm{Zn^{II}}$ complexes might imply that redox-inactive dications (e.g., the $\mathrm{Mg^{II}}$ ion[9c]) present ubiquitously in biological media have an additional role in promoting superoxide disproportionation. 2) The superoxide-disproportionation activity is dependent on both the Lewis acidity of the Zn ion, as governed by the electron-donating ability of the ligands, and the coordination geometry of the Zn ion. Thus, for the development of Zn complexes with SOD-like activity, it is essential to regulate the Lewis acidity to a sufficient extent to promote the interaction with superoxide, and to form a coordinatively saturated structure to inhibit the binding of dioxygen anions, such as peroxide generated in the superoxide-disproportionation reaction (Figure 3 A,B). This mechanism will provide insight into the development of new Zn complexes as redox-inactive SOD analogues that might contribute to antioxidant therapies.[1]

Received: June 25, 2013
Revised: September 12, 2013
Published online: October 7, 2013

Keywords: disproportionation · enzyme models · peroxide · superoxide · zinc complexes

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[7] To prepare the superoxide solution, we mixed potassium superoxide (KO_2; 0.5 mg, 7.2 μmol) and [18]crown-6 (17.2 mg, 65 μmol) in MeCN/DMSO (9:1; 6.5 mL). The mixture was stirred for 10 min, and the remaining small amount of undissolved KO_2 was removed by filtration. The resulting solution with a 1 mM concentration of superoxide was used for CV measurements. Further details of electrochemical measurements are included in the Supporting Information. A 2 mM solution of superoxide in MeCN/DMSO (9:1) was prepared by the same method and characterized by ESR spectrometry under the following conditions: magnetic field: 3210 ± 250 G, microwave power 1 mW, modulation frequency: 100 kHz, modulation amplitude: 10 G, sweep time: 40 G/min, response time: 0.03 s, temperature: 77 K.

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[12] A solution of superoxide (2 mM) in MeCN/DMSO (9:1) was prepared by the method described in Ref. [7]. The reaction of superoxide with **1** was carried out by the addition of a solution of **1** (1.26 μmol) in MeCN/DMSO (9:1; 20 μL) to the solution of superoxide (2.5 mL) with stirring at 15 °C under an Ar atmosphere. The reaction mixture was frozen rapidly in a sample tube and then subjected to ESR spectroscopy under the same conditions. The $g_{\parallel}$ value observed (see Figure 2 D) was similar to those of $O_2^{\bullet-}$ adsorbed on ZnO (2.04–2.05): J. H. Lunsford, *Catal. Rev.* **1974**, *8*, 135.

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[14] Superoxide-disproportionation experiments were initiated as follows: A solution of the $\mathrm{Zn^{II}}$ complex **1**, **2**, **3**, or **4** (0.42–1.26 μmol) in MeCN/DMSO (9:1; 20 μL) was added all at once to a 2 mM solution of superoxide (2.5 mL) at 15 °C with stirring

under an Ar atmosphere. The reaction mixture was frozen 10 s after initiation of the reaction, and then the samples were subjected to ESR spectroscopy under the conditions described in Ref. [7]. The consumption of superoxide was determined by monitoring the peak intensity of the ESR signal for superoxide in the solutions. The intensity of the ESR signal for superoxide was measured as a ratio to the intensity of the signal for Mn^{2+} used as an internal standard. The yields given are an average for six experiments.

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