Superoxide Dismutase Activity of Iron(II)TPEN Complex and Its Derivatives

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Superoxide is involved in the pathogenesis of various diseases, such as inflammation, ischemia-reperfusion injury and carcinogenesis. Superoxide dismutases (SODs) catalyze the disproportionation reaction of superoxide to produce oxygen and hydrogen peroxide, and can protect living cells against the toxicity of free radicals derived from oxygen. Thus, SODs and their functional mimics have potential value as pharmaceuticals. We have previously reported that Fe(II)tetrakis-*N,N,N',N'*-(2-pyridylmethyl)ethylenediamine (Fe(II)TPEN) has an excellent SOD activity ($IC_{50}=0.5\ \mu M$) among many iron complexes examined (*J. Biol. Chem.*, 264, 9243—9249 (1989)). Fe(II)TPEN can act like native SOD in living cells, and protect *Escherichia coli* cells from free radical toxicity caused by paraquat.

In order to develop more effective SOD functional mimics, we synthesized Fe(II)TPEN derivatives with electron-donating or electron-withdrawing groups at the 4-position of all pyridines of TPEN, and measured the SOD activities and the redox potentials of these complexes. Fe(II) tetrakis-*N,N,N',N'*-(4-methoxy-2-pyridylmethyl)ethylenediamine (Fe(II)(4MeO)₄TPEN) had the highest SOD activity ($IC_{50}=0.1\ \mu M$) among these iron-based SOD mimics. In addition, a good correlation was found between the redox potential and the SOD activity of 15 Fe(II) complexes, including iron-based SOD mimics reported in the previous paper (*J. Organometal. Chem.*, in press). Iron-based SOD mimics may be clinically applicable, because these complexes are generally tissue-permeable and show low toxicity. Therefore our findings should be significant for the development of clinically useful SOD mimics.

Key words superoxide; Fe-TPEN; Fe complex; superoxide dismutase; SOD mimic; reactive species.

Superoxide (O_2^-) is involved in the pathology of many diseases such as inflammation, ischemic injury and carcinogenesis, and is a mediator of reperfusion diseases, such as those following acute myocardial infarct or stroke. It is also associated with the development and progression of inflammatory processes involved in diseases such as arthritis, and plays a major role in the initiation of neurological disorders such as Parkinson's disease. Superoxide dismutases (SODs), which can destroy superoxide very rapidly, protect the organism from this radical burden. Indeed, native SODs have been shown in many studies to have a protective effect in animal models of inflammatory diseases.^{1,2)} However, there are problems intrinsic to their protein nature (cost, stability, tissue permeability, oral bioavailability, specific tissue targeting, immunogenicity, etc.), which make it difficult to use SODs as pharmaceuticals. Since synthetic SOD mimics may overcome these problems, many low-molecular metal complexes, mainly copper and manganese complexes, have been synthesized^{3—19)} and their SOD activity has been examined *in vitro* and *in vivo*.^{15,17,20—26)} In particular, Fe(II) tetrakis-*N,N,N',N'*-(2-pyridylmethyl)ethylenediamine (Fe(II)TPEN) can act like native SOD in living cells, and protect *Escherichia coli* cells from free radical toxicity caused by paraquat.^{27,28)}

Recently, we showed that Fe(II) *N*-benzyl-*N'*-(6-methyl-2-pyridylmethyl)-*N,N'*-bis(2-pyridylmethyl)ethylenediamine (Fe(II)(*N*'6MeBPEN)), Fe(II)-*N*-(6-methyl-2-pyridylmethyl)-*N,N,N'*-tris(2-pyridylmethyl)trimethylenediamine (Fe(II)-(6MeTPTN)) and Fe(II)-*N*-methyl-*N,N,N'*-tris(2-pyridylmethyl)trimethylenediamine (Fe(II)(*N*MeTPTN)) have lower SOD activity than Fe(II)TPEN and Fe(II) *N*-benzyl-*N,N,N'*-tris(2-pyridylmethyl)ethylenediamine (Fe(II)-

BPEN).²⁹⁾ This may be because the redox potentials of the former three complexes are more positive than those of the latter two. This result suggests that the redox potential influences SOD activity.

We synthesized Fe(II)TPEN analogues bearing electron-donating or electron-withdrawing groups at the 4-position of all pyridines of TPEN in order to examine the correlation between their redox potentials and SOD activity.

Materials and Methods

Materials Catalase, cytochrome *c* (type III), and xanthine sodium salt were purchased from Sigma. Xanthine oxidase was purchased from Boehringer Mannheim GmbH. The other chemicals were all reagent-grade products from Aldrich, Tokyo Kasei Kogyo Ltd., and Wako Ltd.

Assay of SOD Activity SOD activity was measured according to the method of McCord and Fridovich,³⁰⁾ except for omission of EDTA and inclusion of catalase. The reaction mixture was prepared with 50 mM potassium phosphate buffer (pH 7.4) containing 10 μM cytochrome *c*, 50 μM xanthine and catalase (1200 U/ml). The reaction was started by adding xanthine oxidase at 25 °C, and reduction of cytochrome *c* was monitored at 550 nm. The amount of xanthine oxidase added was adjusted so as to give a rate of $\Delta A_{550\text{ nm}}=0.025/\text{min}$ in the absence of SOD mimic. The SOD activity (IC_{50}) was determined as the concentration which halves the rate of reduction of cytochrome *c*.

Cyclic Voltammetry The redox potentials were measured in CH_3CN containing 0.1 M tetraethylammonium perchlorate as the supporting electrolyte under argon. The half wave potentials ($E_{1/2}$) versus a saturated calomel electrode (S.C.E.) were determined by cyclic voltammetry employing a platinum electrode.

Synthesis 4-Nitro-2-picoline *N*-Oxide **2**: To 2-picoline *N*-oxide **1** in c. H_2SO_4 28 ml, fum. HNO_3 21 ml was added dropwise at 0 °C. The reaction mixture was heated at 110—115 °C for 2 h, and poured into ice-water after having cooled to room temperature. The solution was basified with K_2CO_3 , and the product was extracted with $CHCl_3$. The $CHCl_3$ phase was dried over K_2CO_3 and evaporated to afford **2** (83.9%). 1H -NMR ($CDCl_3/TMS$) 2.58 (s, 3H), 8.01 (dd, 1H, $J=7.1, 2.9$ Hz), 8.15 (d, 1H, $J=2.9$ Hz), 8.33 (d, 1H, $J=7.1$ Hz).

4-Chloro-2-picoline *N*-Oxide **3**: **2** was heated in c. HCl in a sealed tube at

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160 °C for 10 h. The HCl was evaporated, and the residue was neutralized with Na₂CO₃ aqueous solution. The product was extracted with CHCl₃. The CHCl₃ phase was dried over K₂CO₃ and evaporated. The residue was chromatographed on alumina (CH₂Cl₂) to afford **3** (77.6%). ¹H-NMR (CDCl₃/TMS) 2.51 (s, 3H), 7.15 (dd, 1H, *J*=6.9, 2.9 Hz), 7.28 (d, 1H, *J*=2.9 Hz), 8.18 (d, 1H, *J*=6.9 Hz).

4-Methoxy-2-picoline *N*-Oxide **4**: NaOMe 1.164 g (29.1 mmol) in MeOH was added dropwise to **2** (4.07 g, 26.5 mmol) in MeOH at 0 °C. The reaction mixture was stirred at room temperature for 1.5 h. The MeOH was evaporated, and the residue was chromatographed on alumina to afford **4** (79.1%). ¹H-NMR (CDCl₃/TMS) 2.53 (s, 3H), 3.85 (s, 3H), 6.71 (dd, 1H, *J*=7.1, 3.3 Hz), 6.79 (d, 1H, *J*=3.3 Hz), 8.19 (d, 1H, *J*=7.1 Hz).

(4-Chloro-2-pyridyl)methyl Chloride **8**: Compound **3** (1.253 g, 8.73 mmol) was added to acetic anhydride 10 ml preheated at 110–120 °C and stirring was continued at the same temperature for 30 min. EtOH 10 ml was added and refluxed. The solvent was evaporated, and the residue was neutralized with 2 N Na₂CO₃ aqueous solution. The product was extracted with CH₂Cl₂, then the CH₂Cl₂ phase was dried over K₂CO₃ and evaporated. The residue was chromatographed on silica gel (*n*-hexane:CH₂Cl₂=1:1) to afford 2-acetoxymethyl-4-chloropyridine **5** (68.4%). Compound **5** (1.108 g, 5.97 mmol) was hydrolyzed in MeOH 15 ml and 1 N NaOH aqueous solution 8 ml at room temperature for 45 min. The MeOH was evaporated, the residue was diluted with H₂O, and the product was extracted with CH₂Cl₂. The CH₂Cl₂ phase was dried over K₂CO₃ and evaporated to afford 4-chloro-2-pyridinemethanol (69.2%). To 4-chloro-2-pyridinemethanol 1.110 g (7.74 mmol) in CH₂Cl₂, thionyl chloride 1.64 g (13.8 mmol) was added dropwise under reflux. The solvent was evaporated, and the residue was basified with 2 N Na₂CO₃ aqueous solution. The product was extracted with CH₂Cl₂. The CH₂Cl₂ phase was dried over K₂CO₃ and evaporated. The residue was chromatographed on silica gel (CH₂Cl₂) to afford **8** (88.8%). ¹H-NMR (CDCl₃/TMS) 4.65 (s, 2H), 7.25–7.28 (m, 1H), 7.52 (d, 1H, *J*=1.7 Hz), 8.48 (d, 1H, *J*=5.3 Hz).

(4-Nitro-2-pyridyl)methyl Chloride **9**: (4-Nitro-2-pyridyl)methyl chloride **9** was prepared analogously to **8**. ¹H-NMR (CDCl₃/TMS) 4.77 (s, 2H), 7.96 (dd, 1H, *J*=5.0, 2.0 Hz), 8.24 (d, 1H, *J*=2.0 Hz), 8.90 (d, 1H, *J*=5.0 Hz).

(4-Methoxy-2-pyridyl)methyl Chloride **10**: (4-Methoxy-2-pyridyl)methyl chloride **10** was prepared analogously to **8**. ¹H-NMR (CDCl₃/TMS) 3.88 (s, 3H), 4.63 (s, 2H), 6.76 (dd, 1H, *J*=5.7, 2.4 Hz), 7.01 (d, 1H, *J*=2.4 Hz), 8.39 (d, 1H, *J*=5.7 Hz).

2,4-Lutidine *N*-Oxide **12**: To 2,4-lutidine **11** (3.21 g, 30.0 mmol) in CHCl₃, *m*CPBA 5.4 g in CHCl₃ was added dropwise at room temperature. The reaction mixture was stirred at room temperature for 3 h and Na₂SO₃ 2.5 g was added. The reaction mixture was washed with 2 N Na₂CO₃ aqueous solution, dried over K₂CO₃ and evaporated to afford **12** (quant.). ¹H-NMR (CDCl₃/TMS) 2.32 (s, 3H), 2.50 (s, 3H), 6.95 (dd, 1H, *J*=6.6, 1.8 Hz), 7.07 (d, 1H, *J*=1.8 Hz), 8.15 (d, 1H, *J*=6.6 Hz).

(4-Methyl-2-pyridyl)methyl Chloride **14**: (4-Methyl-2-pyridyl)methyl chloride **14** was prepared analogously to **8**. ¹H-NMR (CDCl₃/TMS) 2.38 (s, 3H), 4.64 (s, 2H), 7.06 (d, 1H, *J*=5.0 Hz), 7.29 (d, 1H), 8.43 (d, 1H, *J*=5.0 Hz).

N,N,N',N'-Tetrakis(4-methoxy-2-pyridylmethyl)ethylenediamine ((4MeO)₄-TPEN): (4MeO)₄TPEN was prepared according to Sato *et al.*³¹⁾ from **10**. ¹H-NMR (CDCl₃/TMS) 2.80 (s, 4H), 3.75 (s, 8H), 3.76 (s, 12H), 6.63–6.66 (m, 4H), 7.04 (d, 4H, *J*=2.4 Hz), 8.29 (d, 4H, *J*=6.0 Hz); MS 544 (M⁺).

N,N,N',N'-Tetrakis(4-methyl-2-pyridylmethyl)ethylenediamine ((4Me)₄-TPEN): (4Me)₄TPEN was prepared analogously to (4MeO)₄TPEN from **14**. ¹H-NMR (CDCl₃/TMS) 2.26 (s, 12H), 2.77 (s, 4H), 3.73 (s, 8H), 6.92 (d, 4H, *J*=5.2 Hz), 7.26 (s, 4H), 8.34 (d, 4H, *J*=5.2 Hz); MS 480 (M⁺).

N,N,N',N'-Tetrakis(4-chloro-2-pyridylmethyl)ethylenediamine ((4Cl)₄-TPEN): (4Cl)₄TPEN was prepared analogously to (4MeO)₄TPEN from **8**. ¹H-NMR (CDCl₃/TMS) 2.78 (s, 4H), 3.77 (s, 8H), 7.14–7.17 (m, 4H), 7.47 (d, 4H, *J*=1.6 Hz), 8.40 (d, 4H, *J*=5.2 Hz); MS 560 (M⁺): 562 (M⁺+2)=3:4, 434 (M⁺-ClPyCH₂): 436 (M⁺-ClPyCH₂+2): 438 (M⁺-ClPyCH₂+4): 440 (M⁺-ClPyCH₂+6)=27:27:9:1.

N,N,N',N'-Tetrakis(4-nitro-2-pyridylmethyl)ethylenediamine ((4NO₂)₄TPEN): (4NO₂)₄TPEN was prepared analogously to (4MeO)₄TPEN from **9**. ¹H-NMR (CDCl₃/TMS) 2.93 (s, 4H), 4.02 (s, 8H), 7.87–7.90 (m, 4H), 8.18 (d, 4H, *J*=2.0 Hz), 8.82 (d, 4H, *J*=5.6 Hz); MS 604 (M⁺).

Formation of Ferrous Complexes Ferrous complexes were prepared by reacting equimolar FeSO₄·7H₂O (or Fe(ClO₄)₂·6H₂O) and the chelators in MeOH at room temperature for several minutes, followed by evaporation of the solvent and drying *in vacuo*.

Results and Discussion

Synthesis of 4-Substituted TPEN We synthesized four 4-substituted TPEN derivatives bearing electron-donating (MeO, Me) or electron-withdrawing (Cl, NO₂) groups. 4-Substituted-2-pyridylmethyl chloride was prepared according to Chart 1. 4-NO₂-2-picoline *N*-oxide **2** was prepared by the nitration of 2-picoline *N*-oxide **1** with fum. HNO₃ and c. H₂SO₄. 4-Cl-2-picoline *N*-oxide **3** was prepared by reacting **2** with c. HCl and 4-MeO-2-picoline *N*-oxide **4** was prepared by reacting **2** with NaOMe. 4-Me-2-picoline *N*-oxide **12** was prepared by oxidizing 2,4-lutidine **11** with *m*CPBA. 4-Substituted-2-pyridylmethyl chloride was prepared according to Ashimori *et al.*³²⁾ by deoxygenation and 2-acetoxymethylation with acetic anhydride, followed by hydrolysis and chlorination of the alcohol. Finally, 4-substituted TPEN was synthesized by reacting ethylenediamine and 4-substituted-2-pyridylmethyl chloride in alkaline aqueous solution.

Fe(II)(4-substituted TPEN) was prepared by reacting equimolar FeSO₄·7H₂O (or Fe(ClO₄)₂·6H₂O) and 4-substituted TPEN in MeOH at room temperature for several minutes, followed by evaporation of the solvent and drying *in vacuo*. These complexes have strong absorption in the visible region and their wavelengths of maximal absorption and molar extinction coefficients are listed in Table 1. We could not measure the spectrum of Fe(II)(4NO₂)₄TPEN in potassium phosphate buffer because it did not dissolve.

SOD Activity and Redox Potentials of Fe(II)(4-Substituted TPEN) SOD activities of Fe(II)(4-substituted TPEN) were determined by the xanthine–xanthine oxidase–cytochrome *c* method. Table 2 shows the IC₅₀ values of SOD activity. The IC₅₀ means the concentration of the complex which inhibits by 50% the reduction rate of cytochrome *c* and exerts SOD activity equivalent to 1 unit of native SOD. Interestingly, all Fe(II)(4-substituted TPEN) except Fe(II)(4NO₂)₄TPEN had higher SOD activity than Fe(II)TPEN. The IC₅₀ of Fe(II)(4MeO)₄TPEN was 0.1 μM, and this complex is the most active Fe-based SOD mimic so far synthesized.

The introduction of electron-donating groups at the 4-position of pyridine was expected to make the redox potentials of Fe(II) complexes less positive. We measured the redox potentials of Fe(II)(4-substituted TPEN) (Table 2). The nature of the substituent groups was reflected in the redox potential: electron-donating groups made the redox potentials less positive and electron-withdrawing groups made them more positive, as expected. The most active SOD mimic, Fe(II)(4MeO)₄TPEN, had the most least-positive redox potential among these complexes.

Correlation between SOD Activity and Redox Potentials To examine the correlation between the redox potential and SOD activity, we plotted the SOD activity of 15 Fe(II) complexes, including the iron-based SOD mimics reported in the previous paper,²⁹⁾ against the redox potential (Table 3, Fig. 1 and Chart 2). The redox potentials of Fe(II)(4-substituted BPEN) and Fe(II)(NMeTPTN) were used instead of those of Fe(II)(4-substituted TPEN) and Fe(II)(6MeTPTN) because the intermediate of Fe(II)(4-substituted TPEN) and Fe(II)(6MeTPTN) during the reaction with O₂²⁻ are considered to be the structures (Form B in Chart 3) in which one pyridine or 6-methylpyridine is replaced and so are the same as Fe(II)(4-substituted BPEN) and

Chart 1. Synthetic Scheme of 4-Substituted TPEN

a) c. H₂SO₄, fum. HNO₃, 110–120 °C, b) c. HCl, 160 °C, c) NaOMe, r.t., d) Ac₂O, 110–120 °C, e) NaOH, r.t., f) SOCl₂, reflux, g) *m*CPBA, h) 8), 9), 10) or 14), NaOH, r.t.

Table 1. Maximal Absorption and Molar Extinction Coefficients of Fe(II) Complexes

Fe(II) complex	$\lambda_{\max}$ (nm)	ϵ (M ⁻¹ cm ⁻¹)
Fe(II)((4MeO) ₄ TPEN)	375	6000
Fe(II)((4Me) ₄ TPEN)	410	9000
Fe(II)TPEN	416	10000
Fe(II)((4Cl) ₄ TPEN)	420	8000
Fe(II)((4NO ₂) ₄ TPEN) ^{a)}	—	—

Fe(II) complexes were measured in 50 mM potassium phosphate buffer (pH 7.4).
 a) The spectra of Fe(II)((4NO₂)₄TPEN) was not measured because Fe(II)((4NO₂)₄-TPEN) could not be dissolved in 50 mM potassium phosphate buffer (pH 7.4).

Table 2. IC₅₀ Values and Redox Potentials of Fe(II) Complexes

Fe(II) complex	IC ₅₀ (μM)	E _{1/2} (mV) (vs. S.C.E.)
Fe(II)((4MeO) ₄ TPEN)	0.1	+580
Fe(II)((4Me) ₄ TPEN)	0.2	+680
Fe(II)TPEN	0.5	+780
Fe(II)((4Cl) ₄ TPEN)	0.25	+910
Fe(II)((4NO ₂) ₄ TPEN)	— ^{a)}	+1180

IC₅₀ value means the concentration of Fe(II) complex required to inhibit 50% of the reduction rate of cytochrome *c* by superoxide. a) SOD activity of Fe(II)((4NO₂)₄-TPEN) was not measured because Fe(II)((4NO₂)₄TPEN) could not be dissolved.

Table 3. Apparent Rate Constants and Redox Potentials of 15 Fe(II) Complexes

No.	Fe(II) complex	k _{cat} (M ⁻¹ s ⁻¹)	E _{1/2} (mV) (vs. S.C.E.) ^{a)}
1	Fe(II)((4MeO) ₄ TPEN)	7.0 × 10 ⁷	+425 ^{a)}
2	Fe(II)((4Me) ₄ TPEN)	3.5 × 10 ⁷	+510 ^{a)}
3	Fe(II)(<i>N,N'</i> -(3Me) ₂ TPEN)	8.8 × 10 ⁶	+520
4	Fe(II)(<i>N,N'</i> -(5Me) ₂ TPEN)	1.8 × 10 ⁷	+540
5	Fe(II)PeTPEN	1.4 × 10 ⁷	+570
6	Fe(II)BPEN	1.8 × 10 ⁷	+590
7	Fe(II)TPEN	1.4 × 10 ⁷	+590 ^{a)}
8	Fe(II)(6MeTPEN)	1.8 × 10 ⁷	+595
9	Fe(II)(<i>N</i> MeTPTN)	4.7 × 10 ⁶	+640
10	Fe(II)(6MeTPTN)	3.5 × 10 ⁶	+640 ^{a)}
11	Fe(II)(<i>N,N'</i> -(6Me) ₂ TPEN)	2.3 × 10 ⁶	+640
12	Fe(II)(<i>N'</i> 6MeBPEN)	2.0 × 10 ⁶	+640
13	Fe(II)(<i>N</i> 6MeBPEN)	2.3 × 10 ⁶	+645
14	Fe(II)(<i>N,N'</i> -(6Me) ₂ TPEN)	2.3 × 10 ⁶	+655
15	Fe(II)((4Cl) ₄ TPEN)	2.8 × 10 ⁷	+685 ^{a)}

a) The redox potentials mean those of the proposed intermediates (Form C in Chart 3). The redox potentials of Fe(II)(4-substituted BPEN) and Fe(II)(*N*MeTPTN) are used instead of those of corresponding Fe(II)(4-substituted TPEN) and Fe(II)(6MeTPTN) respectively.

Fe(II)(NMeTPTN) (Chart 3). In other words, the first step in the reaction with O_2^- is the conversion of Form A to Form B, as reported in the previous paper.²⁹⁾

The SOD activities, IC_{50} s, were converted to the apparent rate constant (k_{cat}) by the use of Eq. (1);

$$k_{cat} = k_{cyt.c} \times \frac{[cyt.c^{III}]}{2 \times IC_{50}} \quad (1)$$

$$k_{cat} = 1.4 \times 10^6 \text{ (M}^{-1} \text{s}^{-1})^{33}$$

Fig. 1. Correlation between Redox Potential and SOD Activity

The structures of the chelators in the Fe complexes are shown in Chart 2.
 1. Fe(II)((4MeO)₄TPEN). 2. Fe(II)((4Me)₄TPEN). 3. Fe(II)(N,N'(3Me)₂TPEN).
 4. Fe(II)(N,N'(5Me)₂TPEN). 5. Fe(II)PeTPEN. 6. Fe(II)BPEN. 7. Fe(II)TPEN.
 8. Fe(II)(6MeTPEN). 9. Fe(II)(NMeTPTN). 10. Fe(II)(6MeTPTN). 11.
 Fe(II)(N,N'(6Me)₂TPEN). 12. Fe(II)(N'6MeBPEN). 13. Fe(II)(N6MeBPEN). 14.
 Fe(II)(N,N'(6Me)₂TPEN). 15. Fe(II)((4Cl)₄TPEN).

Chart 2. Chelators of Fe Complexes with SOD Activity

Figure 1 shows that SOD activity is correlated with redox potential for Fe(II) complexes. It is suggested that SOD catalyses O_2^- through the redox cycle of the metal center as represented by the Eqs. (2) and (3).³⁴⁾ These Fe(II) complexes inhibited the reduction cytochrome *c* by O_2^- at the less concentration than O_2^- produced in this assay and therefore it is considered that they quenched O_2^- catalytically through the redox cycle of Fe(II)/Fe(III) like SOD. To take this mechanism into consideration, these data suggests that the oxidation process of Fe(II) by O_2^- (Eq. (2)) is the rate-determining step.

The catalase activity of Mn-complexes has been reported to correlate with redox potential,³⁵⁾ though there has been no report yet about the correlation between SOD activity and redox potential. Our findings should be helpful in developing more active SOD mimics.

In summary, we have established the relationship between redox potential and SOD activity, and developed the most active iron-based SOD mimic, Fe(II)(4MeO)₄TPEN, currently known. Iron-based SOD mimics may have potential for clinical application, because these complexes are generally tissue-permeable and have low toxicity.²⁷⁾ Further studies on the biological application of these SOD mimics are in progress.

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Chart 3. First Step in the Reaction of Fe Complex with Superoxide

Fe complex (Form A) reacts with superoxide by ligand substitution to produce the intermediate (Form B), which is similar to the structure of Fe complex having five coordinated ligands (Form C).

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