Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 329–333

BIOORGANIC & MEDICINAL CHEMISTRY

Models for Superoxide Dismutases: Characterization of Mononuclear Cu(II), Fe(III), and Mn(II) Complexes with 4′,5′-Bis(salicylideneimino)benzo-15-crown-5

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Received 15 June 1998; accepted 2 October 1998

Abstract—Mononuclear Cu(II), Fe(III), and Mn(II) complexes with 4′,5′-bis (salicylideneimino)benzo-15-crown-5, ($SALH_2$), were characterized by elemental analysis, IR and UV–Vis spectroscopy and tested spectrometrically as catalysts for superoxide disproportionation by utilizing xanthine-xanthine oxidase (XXO) assays. The results indicate that the examined mononuclear complexes are speculative potent superoxide dismutase mimics.

Introduction

Superoxide dismutases (SOD) are a family of enzymes that catalytically scavenge superoxide radicals generated by so many spontaneous and enzymatic oxidations in biological systems. These enzymes are widespread among respiring organisms and essential for the survival of aerobic cells since they provide a defense against oxygen toxicity.[1] Therefore, there is a continued interest in understanding both structural and functional aspects of this enzyme. The SOD enzymes from aerobic organisms contain transition metals such as Fe, Mn, or Cu/Zn at the active site of the enzyme.[1] The structure of the metal sites of SOD enzymes is central to understanding the catalytic mechanism. X-ray crystallography[2] and extended X-ray absorption fine structure studies[3] have played an important role in elucidating structural features of the enzyme. Under normal conditions, SODs are very efficient in dismutation of superoxide radicals. However, in the case of an oxygen burst, these enzymes are insufficient in vivo, leading to lipid peroxidation, membrane damage, and cell death. The clinical efficacy of SOD has been disappointing because, as a protein, SOD enzymes have molecular weights too high to cross the cell membranes.[4] Therefore, there is an interest in developing synthetic SOD mimics that should have more favorable pharmaceutical properties such as stability and oral availability. Application of various chelates of manganese, iron, and particularly copper for effective treatment of several diseases in animal models[5,6] stimulated interest especially in designing of transition metal complexes possessing SOD activity. In recent years, the search for SOD models has resulted in a variety of metal chelates which were efficient mimics for superoxide dismutation[7–15] however, most of them loose their activity.[4] SOD activities of salen manganese complexes have been recently examined.[11,16] Consequently, there has been considerable attention in the modelling of low molecular weight, stable and membrane permeable mimics of SOD enzymes as probes to elucidate the physiologic and pathologic significance of intracellular superoxide.[17–19]

Some specific compounds modified with crown ether units have ability to freely diffuse through a membrane. Therefore, incorporation of crown ether moieties into any compound with radical scavenging activity that can cross the cell membrane should protect the cells from radical-dependent oxidative damage. In recent years, considerable interest has been shown in designing stable oximes with these functional groups.[20–23] The synthesis of a salen type of ligand with two benzo-(15-crown-5) rings has been achieved from benzo-15-crown-5, and its electrical and spectroscopic properties have been characterized.[24]

The present paper reports the preparation, and kinetic and spectroscopic characterization of mononuclear Cu(II), Mn(II), and Fe(III) complexes of a Schiff-base ligand with crown ether moiety and investigation of their superoxide scavenging activities.

Key words: Superoxide; dismutases; mononuclear complexes; nitroblue tetrazolium; cytochrome c; radical scavengers.
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0968-0896/99/$ - see front matter
PII: S0968-0896(98)00240-5

Results and Discussion

Superoxide dismutase (SOD) catalyzes dismutation of superoxide very efficiently,[25] and it serves as an important means of defense against oxygen toxicity. The dismutation of superoxide radical by natural SOD enzymes has been investigated in detail and reported to have the following mechanism.[1]

$$2\,O_2^- + 2\,H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Low molecular weight mimics of SOD might be very useful both as antioxidants and as pharmaceutical agents. The beneficial effects of such complexes in protecting biological tissue against oxidative damage have been explained by proposing that these compounds catalyze the dismutation of superoxide.[5–19]

Copper(II), manganese(II), and iron(III) complexes with a tetradentate N_2O_2-donor-site of 4′,5′-bis(salicylideneimino)benzo-15-crown-5, ($SALH_2$) were prepared and characterized by spectrophotometric analyses, and evaluated as superoxide scavengers using xanthine-xanthine oxidase (XXO) assays. All the complexes were prepared from equimolar amounts of $SALH_2{\cdot}H_2O$ and the corresponding metal salts under N_2 atmosphere. In general, the complexes exhibited similar IR features supporting that the complexes are of similar structure. In the IR spectra of all these complexes, (OH) stretching vibrations have disappeared and there is no distinct shift in (C=N) stretches after complex formation. In the Mn(II) complex, a characteristic sharp peak due to (C=O) stretching vibration at 1676 cm^{-1} appeared upon complexation verifying an octahedral geometry of the complex with acetate ions incorporated into axial positions (Fig. 1). All these results are also consistent with the previous information.[26]

The $SALH_2$–metal complexes obtained were analyzed for their ability to scavenge superoxide radicals. The complexes were found to be stable under the experimental conditions. The XXO system was utilized, here, to compare SOD-like activity of the examined mononuclear complexes in vitro with the possible activity in vivo. In these enzymatic assays, cytochrome (cyt.) *c* and nitroblue tetrazolium (NBT) were used as indicator molecules. The results were compared with respect to metal ion incorporated into $SALH_2$ ligand. In the absence of SOD mimic, there was a very slow background rate of oxidation of reduced cyt. *c*. All of the $SALH_2$ complexes investigated in this study inhibited enzymatic reduction of cyt. *c* in a dose-dependent fashion. Among the complexes examined, Mn–$SALH_2$ complex has shown the highest inhibitory pattern (Fig. 2). The IC_{50} value is taken as the concentration of the complex which exerts the SOD activity equal to one unit of dismutase itself and it was found to be about 1.6 μM for Mn–$SALH_2$ complex, being the lowest concentration of all three complexes for dismutation of superoxide radicals in phosphate buffer. The values estimated here are very close to the lowest for salen-type and other SOD mimics reported earlier.[11,19,27,28]

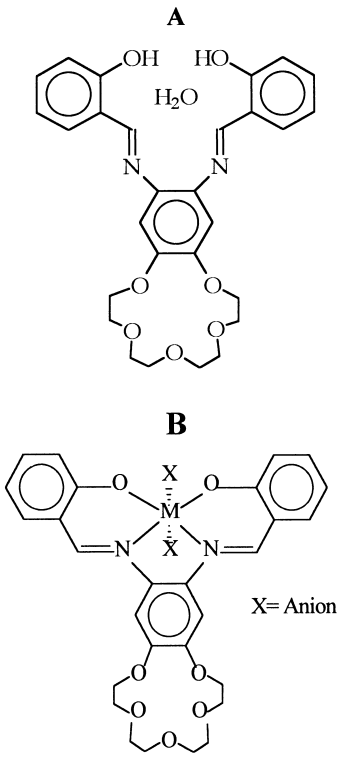

Figure 1. A general formula; (A) of the ligand 4′,5′-bis(salicylideneimino)benzo-15-crown-5, ($SALH_2$), and (B) its mononuclear complexes.

Therefore, it does not seem likely that incorporation of crown ether moieties affects the SOD-like activity. Almost similar inhibitory patterns were obtained for the complexes when using NBT assay (Fig. 3). At concentrations higher than their respective IC_{50} values, the complexes significantly inhibited the enzymatic reduction of either cyt. *c* or NBT indicating that all these complexes were active in both assays as a result of their inhibition of superoxide production. The order of potency of inhibition in both the cyt. *c* and NBT assays (Table 1) was Mn(II) > Fe(III) > Cu(II) in phosphate buffer (pH 7.4). The radical scavenging ability given as percent scavenging efficiency was calculated from the ratio of absorbance changes of the control to sample. A plot of the percent scavenging efficiency index against the time of interaction also reveals that all the complexes exhibit great scavenging ability towards the superoxide radicals (Table 1). The Mn(II) and Fe(III) containing complexes showed the largest catalytic activities, and they possess ability to completely scavenge superoxide radicals within 10 min whereas the Cu(II) complex has shown lesser activity than the other two, and additional periods were needed for Cu(II) complex to completely dismutate superoxide radicals. It is probable that coordination of the metal ions with $SALH_2$ creates their particular geometries which lead to high SOD activities. Metal cavity of all the complexes are surrounded by highly hydrophobic aromatic groups which is consistent with the observation that the presence of aromatic side chains in the active site of the SOD enzymes leads to enhanced SOD activity.[2] The increased catalytic activities of the Mn complex to scavenge superoxide radicals much more efficiently than others

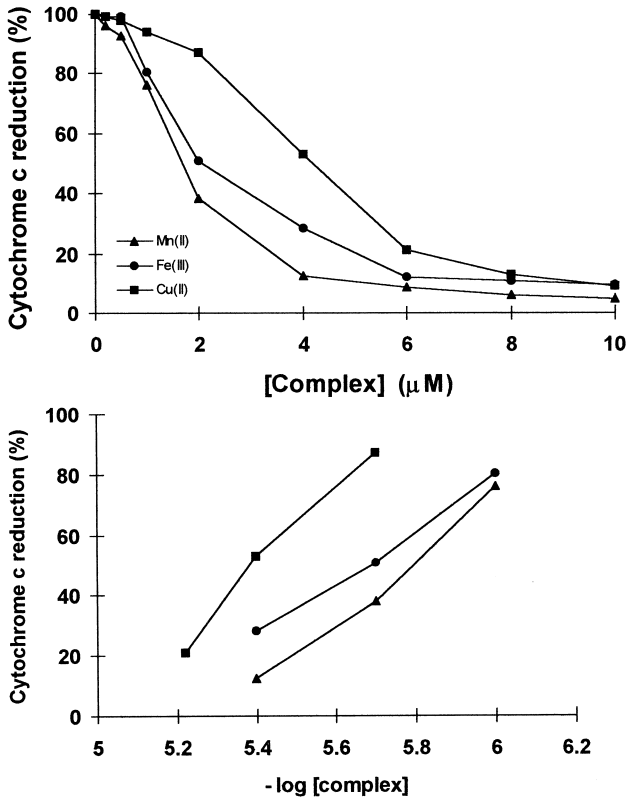

Figure 2. Inhibition of cytochrome *c* reduction by $SALH_2$–metal complexes (▲, Mn(II); ●, Fe(III); ■, Cu(II)). Superoxide radical was generated enzymatically in 50 mM potassium phosphate (pH 7.4) by XXO system in a 2-mL assay mixture containing 35 μM ferricytochrome *c*, 50 μM xanthine, and xanthine oxidase (final concentration of 0.01 unit mL^{-1}). The reduction of cytochrome *c* by superoxide radicals was followed spectrophotometrically at 550 nm. (inset: The data for the inhibition of the cyt. *c* reduction were plotted against −log [complex] values.)

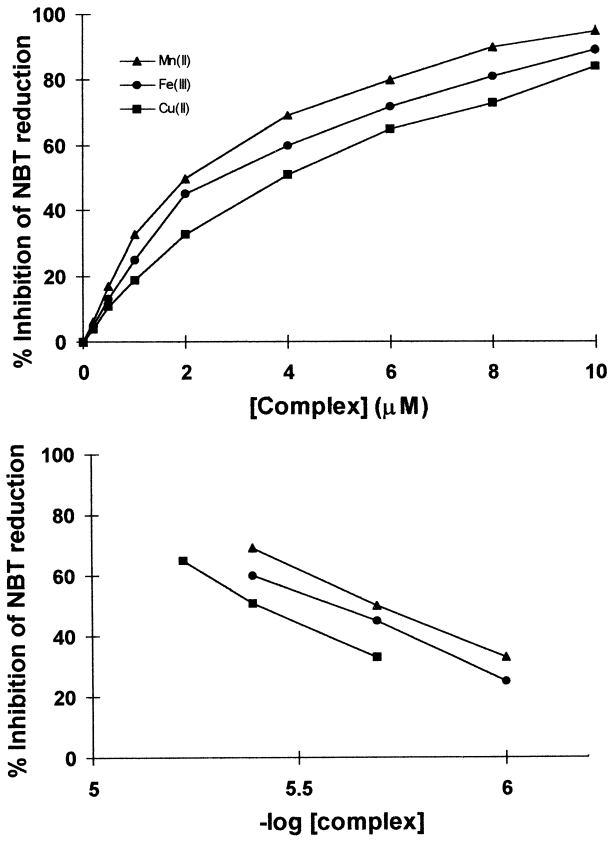

Figure 3. Inhibition of nitroblue tetrazolium reduction by SALH2-metal complexes (▲, Mn(II); ●, Fe(III); ■, Cu(II)). Superoxide radical was generated enzymatically in 50 mM potassium phosphate (pH 7.4) by XXO system in a 2-mL assay mixture containing 100 μM NBT, 50 μM xanthine, and xanthine oxidase (final concentration of 0.01 unit mL^{-1}). The reduction of NBT to blue formazane was followed spectrophotometrically at 560 nm. (inset: The data for the inhibition of the NBT reduction were plotted against −log [complex] values.)

could be attributed to be the electronic nature of manganese and great probability of formation of high-spin complexes in the case of manganese. Moreover, manganese complexes have been found to exhibit quasi-reversible Mn(II)/Mn(III) redox couples at more positive potentials than those in other known metal-ligand systems due to the higher crystal field splitting of the ligand.[29]

In order to evaluate the kinetics between the complexes and superoxide radicals, cyt. *c* and NBT were chosen as detector molecules since their rate constants for the reaction are well established.[13,24] Preliminary experiments showed that neither ligand nor metal-ligand complexes themselves at the concentrations used in these studies had any effect on xanthine oxidase activity. The superoxide radicals, for which both $SALH_2$-metal complexes and cyt. *c* or NBT compete, were generated enzymatically by xanthine oxidase system when cyt. *c* or NBT concentration was held constant while metal complex concentration was varied. The catalytic activities of the complexes were tested against a great excess of superoxide, and the highest concentration of SOD mimic is kept at least 10-fold below that of the initial concentration of superoxide radicals to prevent any contribution from a stochiometric reaction of the compound with superoxide. The rates of cyt. *c* reduction in the absence and the presence of metal complexes were determined and the data were analyzed by plotting the ratio of the rates versus mimic concentration. Similar assays were also done for the NBT assays. Assuming the second-order rate constants of $2.5\times10^5\,M^{-1}\,s^{-1}$ for the reduction of cyt. *c*[30] and $5.94\times10^4\,M^{-1}\,s^{-1}$ for NBT[13] by superoxide radical at pH 7–7.8, the rate constants for the reaction of $SALH_2$–metal complexes with superoxide radical were estimated as shown in Table 1. The initial rates of superoxide radical dismutation (Table 1) observed by competition kinetics at different catalyst concentrations and in the presence of both detector molecules reveals that the rate of superoxide dismutation has a first-order dependence with respect to $SALH_2$–metal complex concentration. It could be pointed out from the obtained results that $SALH_2$–metal complexes all react with superoxide radical at pH 7.40 on the basis of their ability to competitively inhibit the reduction of cyt. *c* or NBT by superoxide generated by enzymatic system. Thus, Fe–, Cu– and Mn–$SALH_2$ complexes facilitate the disproportionation of superoxide,

Table 1. Scavenging properties of the complexes for superoxide radical in phosphate buffer at pH 7.40 (details are given under Experimental section; all the data are average of triplicates)

SOD-mimic		s.e.i.[a] (%)	IC_{50}[b] (μM)	IC_{50}[c] (μM)	k[b] ($M^{-1}s^{-1}$)	k[c] ($M^{-1}s^{-1}$)
$SALMn(CH_3COO)\cdot H_2O$	(1)	98	1.6	2.0	9.10×10^7	8.06×10^7
$SALFeCl\cdot H_2O$	(2)	93	2.1	2.6	1.10×10^7	1.75×10^7
$SALCuCl_2$	(3)	82	4.2	3.9	0.62×10^7	0.41×10^7

[a]s.e.i. was calculated from the ratio of absorbance changes in the absence and presence of the complexes to that of control.
[b]IC_{50} is defined as the concentration of the complexes produces 50% inhibition of cytochrome *c* reduction.
[c]IC_{50} is defined as the concentration of the complexes produces 50% inhibition of NBT reduction.

which is equivalent to the function of the iron, copper and manganese SOD enzymes.[31] The effect of excess EDTA (3 mM) and bovine serum albumin (1 mg mL^{-1}) on the activity of the complexes was negligible indicating that activity of the complexes was not sensitive to both EDTA and albumin. The greater catalytic activity of the complexes suggests that they may be more efficient mimics of SOD activity. This remains to be elucidated in vivo.

We are currently investigating the generality of such metal complexes that will serve as SOD mimic, and their solubility properties through artificial membranes since crown ether moieties provide permeability through the interior of the membranes.[32] The crown ether moieties also provide an additional cavity for alkaline metal cations. Therefore, the hydrosolubility problems of such metal complexes could be overcome. Finally, the efficieny of these metal complexes to serve as SOD mimic in vivo by utilizing animal model systems for myocardial ischemia-reperfusion injury are also under investigation in our laboratories. It is likely that modification of such complexes will yield other complexes with improved catalytic efficiency and more selective biological activity.

Experimental

Chemicals

Nitro blue tetrazolium, horse heart cytochrome *c* (type III), xanthine, xanthine oxidase, $FeCl_3\cdot6H_2O$, $CuCl_2\cdot2H_2O$, $Mn(CH_3COO)_2\cdot4H_2O$, salicylaldehyde, and hydrazine hydrate (100%) were purchased from Sigma Chem. Co. (St. Louis, USA). Other chemicals were of the highest quality available, obtained from local suppliers, and used as received.

Physical measurements

IR spectra were recorded on an ATI Unicam Matson 1000 Model FTIR spectrophotometer as KBr disks and UV–Vis spectra on an ATI Unicam UV2 Model UV–Vis spectrophotometer. C, H, N were analyzed microanalytically on a Hewlett–Packard 85 CHN analyzer, and metal contents of the complexes were estimated by using a Hitachi 180–80 atomic absorption spectrometer in solutions prepared by decomposing the compounds in aqua regia and then subsequently digesting in concentrated HCl.

Preparation of 4′,5′-bis(salicylideneimino)benzo-15-crown-5 and metal complexes

The ligand, 4′,5′-bis(salicylideneimino)benzo-15-crown-5, $SALH_2\cdot H_2O$, was prepared as described before.[26] The orange precipitate formed was filtered, washed and crystallized in ethanol. 1 mmol (0.524 g) of the ligand obtained was dissolved in 50 mL of boiling ethanol and 1 mmol of the metal as its salt ($FeCl_3\cdot6H_2O$, 0.27 g; $CuCl_2\cdot2H_2O$, 0.170 g; $Mn(CH_3COO)_2\cdot4H_2O$, 0.245 g) was added into this solution to obtain the corresponding metal-complexes as described earlier for the preparation of copper complexes.[26] The mixture was refluxed for 30 min, the complexes were precipitated and filtered, washed with ethanol and diethylether, and dried over P_2O_5. The complexes are soluble in dimethylformamide and dimethylsulfoxide. Melting point of the complexes were all above 350 °C. IR (KBr disk)/cm^{-1} for $SALFeCl\cdot H_2O$: 3445 ν(H_2O), 1610 ν(C=N), 1273 ν(Ar-O-C), 1140 ν(C-O-C), 405 ν(Fe-N), 356 ν(Fe-O), and for $SALMn(CH_3COO)\cdot H_2O$: 3454 ν(H_2O), 1615 ν(C=N), 1273 ν(Ar-O-C), 1140 ν(C-O-C), 410 ν(Mn-N), 357 ν(Mn-O). Found: C, 54.50; H, 5.05; N, 4.40; Fe, 8.90. Calcd for $C_{28}H_{30}N_2O_8ClFe$: C, 54.65; H, 4.90; N, 4.55; Fe, 8.60. Found: C, 56.90; H, 5.25; N, 4.50; Mn, 8.80. Calcd for $C_{30}H_{33}N_2O_{10}Mn$: C, 56.60; H, 5.20; N, 4.40; Mn, 8.65.

Superoxide generation and determination of superoxide dimutase-like activity

Scavenging properties of the $SALH_2$–metal complexes were investigated by using two methods generating superoxide radical anions as described earlier.[15] The inhibition of superoxide-dependent reduction of nitro blue tetrazolium (NBT) was measured as previously described with slight modifications.[15]

The ability of the $SALH_2$–metal complexes to inhibit the superoxide-dependent reduction of cytochrome *c* was monitored by measuring the rate of increase in absorbance at 550 nm. Superoxide radical was generated enzymatically in 50 mM potassium phosphate (pH 7.4) by XXO system in a 2-mL assay mixture containing 35 μM ferricytochrome *c* and 50 μM xanthine.[25] The oxidation of ferrocytochrome *c* by the complexes was evaluated by modification of the methods described by Weiss et al.[10] Briefly, the solution of ferricytochrome *c*, xanthine, and xanthine oxidase (final concentration of 0.01 unit mL^{-1}) in 50 mM phosphate, pH 7.4 used for SOD assay was incubated at 25 °C until the 550 nm

absorbance reached a plateau. The complexes at various concentrations were added, and absorbance at 550 nm was monitored for additional periods. A possible interaction between metal complexes and reagents used in XXO system was also tested by monitoring uric acid formation from xanthine at 295 nm in the presence of the complexes, but negligible change was observed indicating that the reagents do not react on the complexes.

The data were treated as described earlier.[15] The radical scavenging ability is given as the percent of scavenging efficiency index (s.e.i.) that was calculated from the ratio of absorbance change of the sample, $\Delta A = A_a - A_p$, to that of control, A_a [s.e.i.% = $(\Delta A/A_a)\times 100$]. Subscripts a and p refer to the experiments in the absence and presence of the examined complexes, respectively.

The SOD-like activity of the complexes has also been defined as the concentration of each complex necessary for the 50% inhibition of NBT and cytchrome *c* at 560 and 550 nm, respectively, by superoxide produced in the XXO system. The more efficient the complex, the lower the concentration that corresponds to 50% inhibiton of NBT or cytochrome *c* reduction. This concentration is termed as IC_{50} value or $-\log IC_{50}$ for comparison.

Acknowledgements

Financial support from The Research Fund of Karadeniz Technical University is greatly acknowledged.

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