MECHANISM OF SUPEROXIDE ANION SCAVENGING REACTION BY BIS-(SALICYLATO)-COPPER(II) COMPLEX

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Summary. The mechanism of the reaction of bis-(salicylato)-copper(II) with superoxide anion has been studied by utilizing electron paramagnetic resonance and polarographic techniques. The proposed reaction sequence is as follows:

$$Cu(II) + O_2^- \longrightarrow Cu(II)O_2^- \longrightarrow Cu(I)O_2 \longrightarrow Cu(I) + O_2$$

$$Cu(I) + O_2^- \longrightarrow Cu(I)O_2^- \longrightarrow Cu(II)O_2^{2-} \xrightarrow{2H^+} Cu(II) + H_2O_2$$

Using the xanthine-xanthine oxidase system as a superoxide generator, it was found that the concentration of this copper complex for 50% inhibition of the xanthine-cytochrome c reductase activity was about 1000 times more per mole of copper than that of erythrocyte superoxide dismutase.

In biological systems superoxide anion radicals are produced upon the oxidation of reduced flavins (1), iron-sulfur proteins (2, 3, 4) and hemoproteins (5) by molecular oxygen. A metalloflavoprotein, xanthine oxidase, is the most studied enzyme among those responsible for its production (6). This radical can serve as either an oxidant or reductant for a variety of organic substances. Because of this reason, superoxide and its related species such as hydroxyl radical have been implicated as toxic for biological organisms. As a protective mechanism, aerobic organisms have a superoxide dismutase which catalyzes the dismutation reaction of superoxide to triplet molecular oxygen and hydrogen peroxide (7, 8, 9). Recently, superoxide and its related species have been implicated to be responsible for the initiation of inflammatory types of arthritis (10). Sorenson (11) described that copper complexes including copper anthranilate and aspirinate have an anti-inflammatory activity stronger than the ligands. We wish to report here the mechanism of the dismutation reaction by copper(II) salicylate.

Materials and Methods

The copper(II) salicylate was prepared as follows: to a sodium salicylate solution (16 g/150 ml water), $CuSO_4$ solution ($CuSO_4 \cdot 5H_2O$, 12.5 g/200 ml water) was added. A deep blue color appeared upon mixing, and after cooling for a few minutes, blue crystals were precipitated. The elemental analysis of this

complex showed: C, 41.0% (calculated 41.0%); H, 4.6% (4.4%); Cu, 15.6% (15.5%); H_2O (17.6%) for $C_{14}H_{18}O_{10}Cu$, being in agreement with bis-(salicylato)-copper(II) $4H_2O$. Dry Me_2SO was prepared by vacuum distillation over barium oxide after standing over molecular sieves (type Linde 3A, Alfa) for a week. Copper-UP83 was the kind gift of Dr. Sorenson, University of Cincinnati. Copper amino acid complexes were prepared by following the method of Folk et al. (12). Potassium superoxide was obtained from ICN•K and K. The superoxide solution in Me_2SO was prepared by adding KO_2 to dry, distilled Me_2SO containing molecular sieves, sealed, and allowed to equilibrate for 5-7 days. The final concentration was determined by the intensity of the epr signal using Cu-EDTA as a standard (1.0 mM).

Electron spin resonance spectroscopy was carried out in a Varian E-4 spectrometer. Polarographic measurements were performed by a Sargent polarograph (model XVI). Xanthine oxidase activity was determined at 22° by following the method described by Komai et al. (13). Optical absorption spectrophotometry was carried out by using a Hitachi spectrophotometer (model-124), and the oxygen consumption was determined by using a rotatory electrode (Gilson, model KM) with a temperature-control device.

Results

The Cu(II) salicylate complex in ME_2SO solution exhibits an epr* spectrum as seen in Figure 1, curve A. Its g-values are calculated as $g_{\perp} = 2.144$, and $g_{\parallel} = 2.375$ with $|A_{\parallel}| = 131$ gauss, being indicative of axial symmetry, X-ray crystallographic data from Hanic and Michalov (14) show that the copper atom lies in the plane of 4 oxygen atoms: 2 carboxyl oxygen atoms and 2 water oxygen atoms. Superoxide, which is relatively stable in the Me_2SO solution, also exhibits a paramagnetic signal (Figure 1, curve B). It is characterized by its asymmetric absorption with $g_{\perp} = 2.008$ and $g_{\parallel} = 2.108$. As has been studied before (15), this paramagnetic signal is very susceptible to pH changes, where higher pH values create a more axial electron distribution with a shift of the $g_{\parallel}$ absorption. When equimolar amounts of Cu(II) salicylate and superoxide are mixed, no epr signal is detected in a wide magnetic field range (Figure 1, curve C). Addition of the salicylate ligand alone (sodium salt) to superoxide showed no decrease in the superoxide signal intensity. Upon the addition of H_2O or HCl, no paramagnetic species could be detected. However, the addition of an excess amount of superoxide to the epr silent complex gave rise to a new paramagnetic signal which was not identical to that of the original copper(II) complex (Figure 1, curve D). Subsequent addition of HCl generated a paramagnetic signal exactly the same as the original copper(II) salicylate (Figure 1, curve E). Essentially the same results as those with Cu(II) salicylate were obtained when either the

*The abbreviation used: epr, electron paramagnetic resonance.

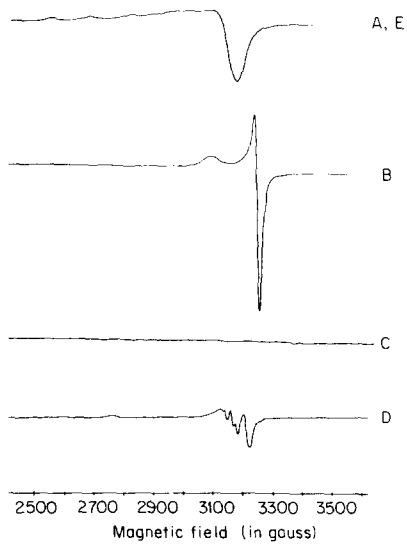


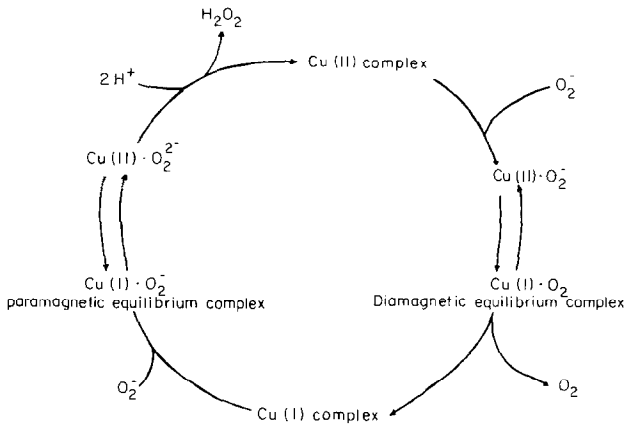
Figure 1. epr Spectral Changes during the Reaction of Copper(II) Salicylate with Superoxide Anion

Curve A: copper salicylate (1.38 mM) in Me_2SO
Curve B: potassium superoxide (1.0 mM) in Me_2SO
Curve C: copper salicylate (0.72 mM) and potassium superoxide (0.72 mM) in Me_2SO
Curve D: copper salicylate (0.77 mM) and potassium superoxide (1.54 mM)
Curve E: sample (D) plus 0.01 N HCl

Conditions for epr spectroscopy: microwave attenuation, 19.0 dB; frequency, 9.171 GHz; modulation amplitude, 3.2 gauss; scanning rate, 125 gauss per minute; time constant, 1.0 sec. Temperature at 77°K

Cu(II) aspirinate, Cu(II) tryptophan, or Cu(II) UP83 complex was used as the scavenger.

In order to ascertain the stoichiometric relationship between the Cu(II) complex and superoxide, we have carried out a titration of the signal intensity of the Cu(II) complex with varying amounts of superoxide. As shown in Figure 2, the signal intensities decrease as a function of the amount of superoxide added. From these results it can be concluded that one mole of the Cu(II) complex reacts with one mole of superoxide, resulting in a diamagnetic complex, presumably a Cu(I) complex. Based upon these observations, we propose the following reaction sequence:



Scheme I. A proposed reaction mechanism

It is important to note here that the above reaction mechanism is similar to that of the proposed dismutation reaction catalyzed by superoxide dismutase

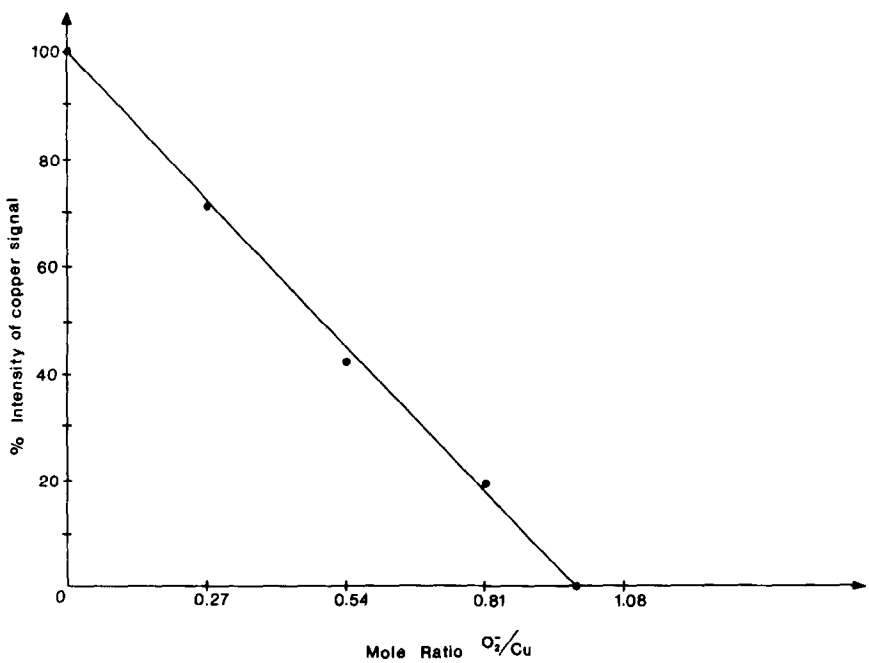


Figure 2. Titration of epr Signal Intensities of Copper(II) Salicylate with Superoxide Anion

The conditions for epr spectroscopy are similar to those of Figure 1. The initial concentration of the copper complex was 1.38×10^{-3} M

(7). From recent 3 Å resolution X-ray crystallographic studies on bovine erythrocyte superoxide dismutase (16), the Cu atom is bonded to three equivalent nitrogen atoms from histidine residues; and one unequivalent nitrogen from the histidine 61 residue, which is shared with the Zn atom. In its coordination sphere, there does exist a rapidly exchangeable water molecule (17). Thus, the coordination structure of the Cu(II) salicylate complex is different from that of the dismutase. Their only common feature is the presence of a water molecule as either ligand or exchangeable solvent molecule.

Some supporting evidence to the scheme was obtained from polarographic experiments. Half-wave potentials of Cu(II) salicylate in Me_2SO gave two waves corresponding to the two possible reduction reactions. The addition of superoxide to the Cu(II) complex reduced the intensity of the Cu(II) wave until it disappeared when a stoichiometric amount of superoxide was introduced. At this time, only one wave was observed. When an excess amount of superoxide and HCl were added to this Cu(I) species, the original Cu(II) wave was regenerated. These results are summarized in Table I.

We have then compared the efficiency of scavenging superoxide by the Cu(II) complex with that of bovine erythrocyte superoxide dismutase by using milk xanthine oxidase as the superoxide generator. The inhibitory effects of the Cu(II) complex on various activities of xanthine oxidase were examined. The results are shown in Table II. The cytochrome c reductase activity was inhibited by the addition of superoxide dismutase; but the other activities including the dichlorophenolindophenol and ferricyanide reductases, as well as the oxidase activity, were not significantly inhibited by the dismutase. This implies that the cytochrome c reduction reaction is mediated by superoxide in agreement with the previous finding (6) and that the other activities do not appear to be mediated by superoxide. These results lead us to believe that at the concentrations used the Cu(II) complex does not inhibit the activity of xanthine oxidase, *per se*. It reacts directly with superoxide which is produced by the enzyme. We found that the Cu(II) complex is about 3000 times less active than the dismutase. The data from Table II indicate that the scavenging action of the copper salicylate complex is not catalytically efficient in aqueous solution, since it is present in ten times greater concentration than that of superoxide to obtain 50% inhibition.

Discussion

Although Joester *et al.* have reported that low molecular weight copper complexes, such as $Cu(Lys)_2$, $Cu(Tyr)_2$ and $Cu(His)_2$, display suppression of

Table I. Polarographic Measurements of the Reaction of Copper(II) Salicylate with Superoxide Anion

Additions	Halfwave Potentials vs. SCE	
	first reduction	second reduction
copper salicylate, 1.0 mM	-0.570 volts	-1.730 volts
copper salicylate, 0.5 mM plus potassium superoxide, 0.5 mM	ND	-1.751
potassium superoxide, 1.0 mM	ND	ND
copper salicylate, 0.5 mM plus excess potassium superoxide plus HCl, 0.02 N	-0.568	-1.726

The polarographic measurements were carried out in the Me_2SO solution at 22° by using a drop-in mercury electrode in a H cell against a saturated calomel electrode. Tetraethylammonium perchlorate (0.1 M) was used as the supporting electrolyte and 1% agar as maximum suppressor. Typical measurements were made at 0.006 μA/min without damping with a compensation current, 0.5 μA and a drop rate of 2.8 sec/drop. All solutions were purged of oxygen by bubbling nitrogen gas for 5 minutes.

the xanthine-xanthine oxidase-induced cytochrome c reduction and chemiluminescence, they provided no direct evidence for the nature of the reducing species or the mechanism of action of these chelates (18). It is difficult to accept the action of these cupric complexes solely on the intermediary oxygen species, since no attempt was made to study their effect on the flavoprotein itself.

Additionally, the observed chemiluminescence quenching of Weser and his colleague (19), mediated by the removal of singlet oxygen, originating from the decomposition of the tetraperoxochromate (V) complex is in direct contrast to our inability to detect any singlet oxygen quenching effect by either copper(II) salicylate (unpublished) or dismutase. (7)

Even though Cu(II) salicylate is not as catalytically efficient as superoxide dismutase, it is a small molecule relative to the enzyme, which has a molecular weight of 34,000. This factor would aid in its permeability across biological membranes, and thus permit it to serve as an effective scavenger of superoxide anion *in vivo* due to the cyclic nature of the dismutation reaction. When the physiological concentration of superoxide is high in the extracellular fluid that

Table II. Effects of Copper Salicylate and Superoxide Dismutase on the Various Activities of Xanthine Oxidase

Reaction		Addition	Activity μ equiv/min/mg prot	%
xanthine →	cyt. c	none	0.43*	100
		Cu salicylate, 0.48 μM	0.15	34
		dismutase, 0.14 nM	0.17	40
xanthine →	DCIP	none	0.58	100
		Cu salicylate, 0.48 μM	0.59	103
		dismutase, 0.06 nM	0.57	99
xanthine →	$Fe(CN)_6^{3-}$	none	1.78	100
		Cu salicylate, 0.48 μM	1.66	93
		dismutase, 0.14 nM	1.76	99
xanthine →	O_2	none	2.47	100
		Cu salicylate, 0.83 μM	2.36	95
		dismutase, 0.36 nM	2.37	96

*The estimated concentration of superoxide is 4×10^{-8} M during the initial part of the reaction

The reaction mixture contained: xanthine, 5.0×10^{-5} M; cytochrome c, 7.7×10^{-6} M; dichlorophenolindophenol, 4.8×10^{-6} M; ferricyanide, 3.3×10^{-4} M; xanthine oxidase, 4.1×10^{-8} M in 0.05 M phosphate buffer, pH 7.8. The reaction was started by adding the enzyme at 22° under aerobic conditions.

The reaction mixture for xanthine oxidase contained: xanthine, 5.0×10^{-5} M; xanthine oxidase, 1.4×10^{-7} M in 0.05 M phosphate buffer, pH 7.8. The reaction was carried out at 24°, using Grade IV xanthine oxidase from Sigma.

The concentration of dismutase was expressed per copper atom.

contains polysaccharides, superoxide will decrease the viscosity of this fluid by depolymerizing the polysaccharides (10). In this connection we have observed that a bacterial polysaccharide (20) with a molecular weight of 8,000 reacts with superoxide in Me_2SO solution, as judged from the disappearance of the superoxide epr signal (unpublished data). Thus, copper scavengers of superoxide can protect against the initiation of inflammatory types of arthritis of this origin. The anti-inflammatory effect of aspirin is a complex phenomenon. Its action appears to be also associated with prostaglandins since it has been reported to inhibit the prostaglandin synthetase from the seminal vesicles by acetylating the enzyme molecule (21). The superoxide scavenging effect of a copper complex that is structurally related to aspirin observed in this study

might not be its sole mode of action; but this should, nevertheless, be considered as one of the possible functions of anti-inflammatory drugs.

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