A Pulse Radiolysis Study of an Imidazolato-Bridged Asymmetric Dicopper(II) Complex: A Structural and Functional Mimic of Superoxide Dismutase

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The ability of a dicopper(II) complex with a pentadentate, imidazolato-bridged ligand having two different coordination environments to mimic the reactivity of superoxide dismutase has been investigated by pulse radiolysis. Pseudo first-order kinetics were observed for superoxide scavenging, with a very high rate constant of 1.9 ± 0.4 × 10^9 M^{-1} s^{-1}, consistent with catalytic dismutation of superoxide by the complex. This value is essentially constant over the pH range of 5 to 7, and agrees with earlier results obtained with the nitroblue tetrazolium assay.

The superoxide anion radical ($O_2^{\bullet-}$) may be involved in deleterious biological processes, such as lipid peroxidation,[1] aging,[2] and cancer.[3] This anion radical is very efficiently scavenged by the enzyme superoxide dismutase which catalyses the dismutation of superoxide to dioxygen and hydrogen peroxide.[4] The rate constants for reduction and reoxidation of the copper ion of Cu,Zn superoxide dismutase are known:[5] $k_1 = k_2 = 2.4 \cdot 10^9\ \mathrm{M}^{-1}\mathrm{s}^{-1}$. These rate constants indicate that the reactions are nearly diffusion-controlled.[6]

An alternative pathway for reoxidation of copper(I) involves the reaction with the hydrogen peroxide product of the dismutation reaction, with formation of a copper−peroxo complex (Eq. 1)[7][8] which could react directly,[8] or decompose to form the hydroxyl radical[8][9] which in turn would react with the surrounding ligand or biomolecule.[10] The formation of an oxidizing copper(III) species is also possible since four-nitrogen ligands in a square-planar coordination would stabilize copper(III). Theoretical considerations[11] and experimental results[10][12] indicate that the occurrence of a copper(III) oxide(1+) species can be excluded.

$$\mathrm{Cu^I} + \mathrm{H_2O_2} \rightarrow \mathrm{CuH_2O_2^+} \qquad (1)$$

A relative measure of superoxide dismutase activity can easily be obtained from indirect assays in which a very small and stationary amount of superoxide is generated usually by the action of xanthine oxidase on xanthine, and a detector molecule such as nitroblue tetrazolium[13] or cytochrome *c*[14] is used to follow the reaction kinetics spectrophotometrically. In these assays, the rate of reduction of the detector molecule by superoxide is measured, and the reactivity of superoxide dismutase or of superoxide dismutase mimics is inferred from the rate decrease in the presence of these species. This approach cannot provide absolute rate data, since the superoxide dismutation reaction itself is too rapid to be followed directly, and hence no mechanistic information about the reaction can be obtained. Pulse radiolysis is a superior method to investigate superoxide dismutation as one can selectively and at will generate superoxide and hydrogen dioxide in the absence of extraneous and interfering enzyme systems and reporter molecules, and one can make measurements in the microsecond time scale.

Amino acid sequences[15][16] and structures[17][18][19][20] of various Cu,Zn superoxide dismutases are known. The active site is composed of an imidazolato-bridged dinuclear center containing copper(II) and zinc(II). The coordination geometry is distorted square-pyramidal for copper(II) and distorted tetrahedral for zinc(II). For many years efforts have been made to obtain model compounds that show structural, spectroscopic, and catalytic properties similar to that of the active site in Cu,Zn superoxide dismutase.[21][22][23][24][25][26][27][28][29] However, many of the superoxide dismutase mimics prepared to date are unstable in aqueous solution. Further, results obtained with complexes that have been studied in aqueous solution are weakened by the possibility that the observed catalysis is actually carried out by free copper.

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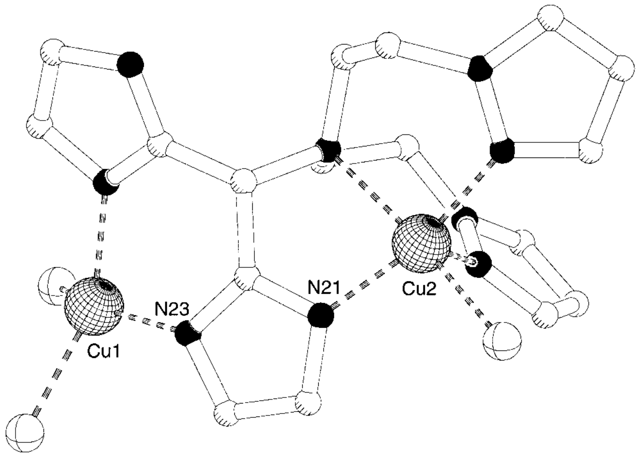
In the present work, the pulse radiolysis technique was used to measure the superoxide dismutase activity of an imidazolato-bridged dicopper(II) complex, 1,5-bis(1-pyrazolyl)-3-[bis(2-imidazolyl)methyl]-3-azapentane ($[Cu_2L]^{3+}$, Figure 1) which has a coordination geometry strikingly similar to that of the active site of native Cu,Zn superoxide dismutase.[30]

Figure 1. Structure of $[Cu_2(L)Cl_3]$ showing the imidazolate bridge



Dismutation of superoxide leads to formation of dioxygen and hydrogen peroxide; interaction of the latter with the Cu^ICu^{II} or Cu^ICu^I complex would produce the hydroxyl radical. For this reason the interaction of this radical with the complex was also briefly investigated.

Results

Superoxide Dismutation Experiments

In Figure 2 a typical decay of the superoxide absorbance as a function of time is shown. Depending on the concentration of copper complex and the presence or absence of phosphate buffer, different rate constants were obtained, see Table 1. The dose-dependent amount of acid produced during the irradiation leads to a proton concentration of approximately 10^{-5} M. The change in pH results in partial protonation of superoxide ($pK_a = 4.7$), therefore reactions of the hydrogen dioxide radical were taken into consideration. The pH under the different experimental conditions was evaluated by calculating the ratio $[HO_2^\bullet]/[O_2^{\bullet-}]$ from the maximum absorbance after the pulse. A correction of smaller than 10% in the rate of dicopper(II) complex dependent dismutation was required on the basis of the rate of dismutation of superoxide in the absence of complex.

The rate constant for dismutation of superoxide by aqueous copper species increases with pH. Values for the rate constant obtained by statistical treatment of the steady-state rate constant, $k_{ss,O_2^{\bullet-}}$, are shown in Table 1. At pH = 7, $k_{ss,O_2^{\bullet-}}$ is $(1.9 \pm 0.4) \times 10^9$ $M^{-1}s^{-1}$. Values for $k_{ss,HO_2^\bullet}$ for the unbuffered solutions were calculated on the basis of equation 15 (see Experimental Section – Methods), with the help of $k_{ss,O_2^{\bullet-}}$ determined in buffered solutions.

Figure 2. Experiment and fit (heavy line) for unbuffered solution at 22°C with a dose of 88 Gy, $[Cu_2L]^{3+}$ = 4 mM, optical pass length of 2 cm, observed at 280 nm; the residual absorption from the fit is also given(lower noisy trace); from the least-square fits it is evident that a second-order fit is systematically wrong

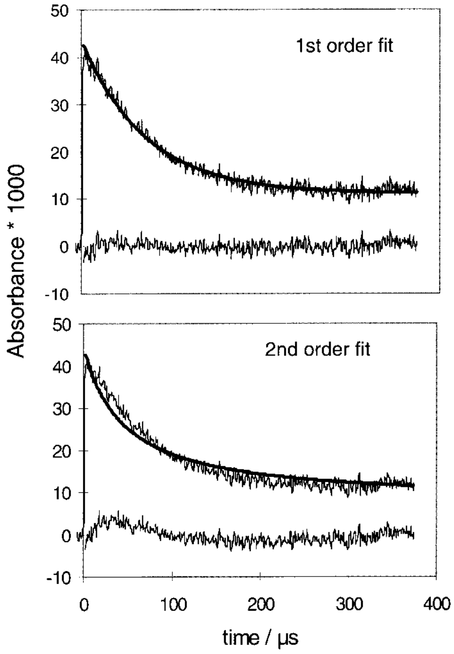
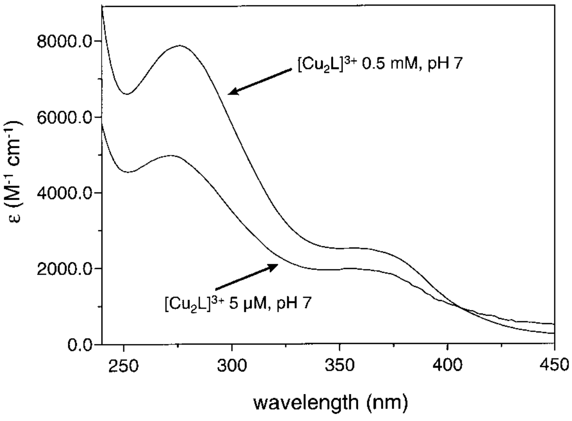


Figure 3. Comparison of the extinction coefficients of $[Cu_2L]^{3+}$ at concentrations of 5 µM and 0.5 mM



The speciation of copper in solutions of the copper–imidazolato complex is not straightforward. The integrity of the dicopper(II) complex was evaluated from the UV/Vis spectrum of a 5 µM solution. On the basis of the absorbance at 375 nm, corresponding to the absorbance maximum of the Cu–Im–Cu chromophore,[30] it was calculated that ca. 80% of the dicopper(II) complex retained the original structure (Figure 3).

We performed a control experiment in which we dermined the catalytic activity of copper ion in a solution that did not contain the imidazole ligand, but was otherwise identical, and found a $k_{ss,O_2^{\bullet-}}$ of $(1.6 \pm 0.2) \times 10^9$ M^{-1} s^{-1}.

No transient spectrum attributable to formation of a copper(II)–superoxide complex was observed. A residual ab-

Table 1. Statistical summary[a] of k_{ss} for $[Cu_2L]^{3+}$

$[Cu^{2+}]$ [μM]	pH after pulse	$[O_2{}^{\bullet -}]_{tot}$ [μM]	$k_{ss,O_2 2}{}^{\bullet -}$obs [$M^{-1}$ s^{-1}]	$k_{ss,HO_2}{}^{\bullet}$ [M^{-1} s^{-1}]
2	4.4 ± 0.3	49 ± 4	$(3.9 \pm 0.4) \times 10^9$	$(4.5 \pm 0.6) \times 10^9$
2	6.83 ± 0.05	3.7 ± 0.8	$(3 \pm 1) \times 10^9$	−
4	5.3 ± 0.1	48 ± 2	$(2.8 \pm 0.2) \times 10^9$	$(6 \pm 1) \times 10^9$
1	7.3[b]	51 ± 4	$(1.9 \pm 0.4) \times 10^9$	−
1[c]	7.3[b]	51 + 4	$(1.6 \pm 0.2) \times 10^9$	

[a] 99% Confidence range. − [b] Phosphate buffer 75 mM, pH = 7.3. − [c] Control experiment: no complexing agent present.

sorbance that decayed over 0.8 ms was always observed in the experiments with 4 μM complex, possibly due to the presence of partially reduced copper complex in the steady state.

Hydroxyl Radical Experiments

The absorbance decay under conditions of $[Cu_2L]^{3+} >> [HO^{\bullet}]$ follows a pseudo first-order kinetics: a rate constant of $1.7 \pm 0.3 \times 10^{10}$ $M^{-1}s^{-1}$ was found. The absorbance decay of the radical species formed was fitted to an apparent second-order reaction with a rate constant of $k/\Delta\varepsilon$ of $(1.3 \pm 0.4) \times 10^7$ $cm^{-1}s^{-1}$. It should, however, be noted that the structure of any putative radical product(s) has not been determined, that many reaction mechanisms are possible, and that it is impossible to report the exact kinetic order at this stage.

Discussion

As discussed by Costanzo et al.,[31] studies which report catalytic rate constants of copper complexes regrettably hardly ever address the issue of speciation. Sometimes not even the ratio of ligand to metal is stated. This raises the suspicion that the reported catalytic activity was due to free copper, rather than the complex. In our experiments spectrophotometric measurements show that ca. 20% of the complex is hydrolysed. Since the copper(I + II) aquo complex reacts with superoxide with a catalytic rate constant of $k_{ss,O_2}{}^{\bullet -} = 9 \times 10^9$ $M^{-1}s^{-1}$,[32][33][34][35] it was appropriate to consider whether the observed activity is due to undefined complexes of copper in various oxidation states with water and solutes.

The catalytic activity of the hydrolysed copper was determined in control experiments lacking the imidazolato-containing ligand. The activity measured here equals that of solutions in which the complex had hydrolysed completely and in which the copper was bound to formate, phosphate, hydroxide, etc. Had the measured catalytic activity of 1.9×10^9 $M^{-1}s^{-1}$ been due to such copper complexes, and not to the imidazolato-bridged complex, then the catalytic rate constant would have been 5 times higher, namely 9×10^9 $M^{-1}s^{-1}$. If the activity of the copper complex had been found to be clearly lower than that for the control solution, it would not have been possible to ascribe the activity to the complex with certainty.

The decay of superoxide is first order, indicating steady-state kinetics. In phosphate-buffered solution the influence of the hydrogen dioxide radical is negligible, and the steady-state rate constant found for $k_{ss,O_2}{}^{\bullet -} = 1.9 \pm 0.4 \times 10^9$ $M^{-1}s^{-1}$. The rate, determined here by pulse radiolysis, is in good agreement with that determined by the indirect assay[30] and comparable to that of Cu,Zn superoxide dismutase. This may represent the first unequivocal demonstration of superoxide dismutase mimicry by a low molecular weight copper complex.

At lower pH, where $HO_2{}^{\bullet}$ is present at 16−90% and the contribution of $k_{ss,HO_2}{}^{\bullet}$ to the overall observed rate constant is strong, the observed rate constants are higher than those obtained in buffered solution. The lowest single determination of $k_{ss,HO_2}{}^{\bullet}$ was 3.9×10^9 $M^{-1}s^{-1}$, indicating that the dicopper(II) complex is also extremely active in the conversion of hydrogen dioxide.

The absence of a transient spectrum due to a copper(II)−superoxide adduct is understood when one takes into account that the superoxide radical can undergo fast outer-sphere electron-transfer reactions. This interpretation is supported by the exceptionally high rate constants; inner-sphere reactions of copper(II) are expected to be at least one order of magnitude lower.[36] An alternative explanation is that the transient spectrum of the hypothetical adduct has an absorbance too low to be observed.

We cannot exclude an interaction of either the dicopper(II) [= copper(I)−copper(I)] or the copper(II)−copper(I) complex with hydrogen peroxide released after the catalyzed dismutation of superoxide anion. Such an interaction would lead to a degradation of the ligand, probably through the generation of the hydroxyl radical. Thus, the rate of reaction of the hydroxyl radical with the dicopper(II) complex was evaluated. The reaction is apparently pseudo first-order with a rate constant of $1.7 \pm 0.3 \times 10^{10}$ $M^{-1}s^{-1}$, which is about one order of magnitude higher than corresponding rate constants of free imidazole and bis(2-imidazolyl)methane.[33][37]

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Experimental Section

Methods

General: The pulse radiolysis setup with a Febetron 705 2-MeV accelerator (Hewlett-Packard) has been described before.[12][32] All experiments were carried out at room temperature (20−22°C) with an effective 50-ns irradiation pulse and a 2-cm optical path length in a quartz cell. Solutions were prepared from triply distilled water. Uncertainties for rate constants are expressed at the 99% confidence limit.

The conditions for the experiments were carefully chosen: a) the concentration of phosphate was set at 75 μM to buffer the solution at $\mathrm{pH} = 7$ to maximize the concentration of the imidazolato-bridged dicopper(II) complex, since at $\mathrm{pH} < 6.8$ the bridging may be compromised to some extent and at $\mathrm{pH} > 8.0$ hydrolytic species are formed,[30] and to minimize formation of hydrogen dioxide radicals; and b) the concentration of formate was set at 100 μM to maximize the yield of superoxide and to minimize the formation of the copper-formate complexes. Phosphate concentrations were kept at the minimum necessary to maintain the desired pH as phosphate can dramatically influence the rate of reaction of superoxide with copper-containing catalysts.[32] An excess of phosphate can shift the equilibria towards the formation of copper(II) phosphate. The influence of phosphate in experiments with buffered solutions was evaluated by comparison with experiments performed with unbuffered solutions.

Superoxide Experiments: The dicopper(II) complex was dissolved in 100 mM sodium formate (Merck puriss p.a.) and adjusted to $\mathrm{pH} = 7.3$ with sodium hydroxide. Three groups of solutions were prepared: unbuffered solutions having total copper concentrations of 2 and 4 μM, and a buffered solution (75 μM phosphate, $\mathrm{pH} = 7.3$) with 1 μM copper. Solutions were saturated with oxygen (> 99.5%, Pan Gas) for approximately 20 min. A dose of 75−100 Gy was applied to generate superoxide (44−60 μM) in excess over the dicopper(II) complex. Under these conditions a steady state is achieved and the decay of superoxide follows a pseudo first-order kinetics, because the concentrations of reduced and oxidized forms of copper in the complex are constant.

Irradiation of oxygen-saturated solutions containing formate generates primary radicals which are converted to superoxide within 2 μs of the end of the pulse;[38] the corresponding change in absorbance was followed at different wavelengths ranging from 245 to 295 nm. The total concentration of superoxide and hydrogen dioxide was calculated from the dose. The pH after the pulse was calculated from the published $\mathrm{p}K_a$ and extinction coefficients.[39]

Hydroxyl Radical Experiments: Solutions of the dicopper(II) complex at concentrations of 20.7 or 81 μM prepared in water adjusted to $\mathrm{pH} = 7.3$ with sodium hydroxide were saturated with N_2O (> 99.5%, Pan Gas) for approximately 20 min. The more dilute solution was used to follow the absorbance decay at different wavelengths, following radiation doses of about 100 Gy ($[\mathrm{HO}^{\bullet}] = 60$ μM). The kinetics of the reaction was followed at 300 and 350 nm with the 81 μM solution of complex and an irradiation dose of about 20 Gy ($[\mathrm{HO}^{\bullet}] = 12$ μM).

Kinetics: Superoxide dismutation catalyzed by copper aquo complexes is pH-dependent and faster for superoxide than for hydrogen dioxide.

$$\mathrm{Cu^{2+}(aq)} + \mathrm{O_2^{\bullet -}} \rightarrow \mathrm{Cu^{+}(aq)} + \mathrm{O_2} \tag{2}$$

$$\mathrm{Cu^{+}(aq)} + \mathrm{O_2^{\bullet -}} + 2\,\mathrm{H^{+}} \rightarrow \mathrm{Cu^{2+}(aq)} + \mathrm{H_2O_2} \tag{3}$$

$$\mathrm{Cu^{2+}(aq)} + \mathrm{HO_2^{\bullet}} \rightarrow \mathrm{Cu^{+}(aq)} + \mathrm{O_2} + \mathrm{H^{+}} \tag{4}$$

$$\mathrm{Cu^{+}(aq)} + \mathrm{HO_2^{\bullet}} + \mathrm{H^{+}} \rightarrow \mathrm{Cu^{2+}(aq)} + \mathrm{H_2O_2} \tag{5}$$

If superoxide radicals are in large excess, the steady-state condition for $\mathrm{Cu^{2+}(aq)}$ is obtained where

$$k_{ss} = (2\,k_2k_3)/(k_2 + k_3) = 8.7 \cdot 10^{9}\ \mathrm{M}^{-1}\mathrm{s}^{-1\,[32]} \tag{6}$$

which is ca. four times higher than k_{ss} of Cu/Zn superoxide dismutase. Hydrogen dioxide radicals react with aqueous copper much more slowly:

$$k_{ss} = (2\,k_4k_5)/(k_4 + k_5) = 1.6 \cdot 10^{8}\ \mathrm{M}^{-1}\mathrm{s}^{-1\,[32]} \tag{7}$$

In the case of the dicopper(II) imidazolato complex investigated here we investigated only the overall (i.e. steady-state) reaction rate as is commonly reported for superoxide dismutases. Microscopically, several reactions may be involved (formulated for deprotonated species only):

$$[\mathrm{Cu^{II}Cu^{II}L}]^{3+} + \mathrm{O_2^{\bullet -}} \rightarrow [\mathrm{Cu^{I}Cu^{II}L}]^{2+} + \mathrm{O_2} \tag{8}$$

$$[\mathrm{Cu^{I}Cu^{II}L}]^{2+} + \mathrm{O_2^{\bullet -}} \rightarrow [\mathrm{Cu^{I}Cu^{I}L}]^{+} + \mathrm{O_2} \tag{9}$$

$$[\mathrm{Cu^{I}Cu^{I}L}]^{+} + \mathrm{O_2^{\bullet -}} + 2\mathrm{H^{+}} \rightarrow [\mathrm{Cu^{I}Cu^{II}L}]^{2+} + \mathrm{H_2O_2} \tag{10}$$

$$[\mathrm{Cu^{I}Cu^{II}L}]^{2+} + \mathrm{O_2^{\bullet -}} + 2\mathrm{H^{+}} \rightarrow [\mathrm{Cu^{II}Cu^{II}L}]^{3+} + \mathrm{H_2O_2} \tag{11}$$

the sum of which is:

$$4\ \mathrm{O_2^{\bullet -}} + 4\ \mathrm{H^{+}} \rightarrow 2\mathrm{O_2} + 2\ \mathrm{H_2O_2} \tag{12}$$

with a steady-state rate law of:

$$\mathrm{d}[\mathrm{O_2^{\bullet -}}]/\mathrm{d}t = k_{ss,\mathrm{O_2}^{\bullet -}}\,[\mathrm{Cu_2L}][\mathrm{O_2^{\bullet -}}],\ k_{ss,\mathrm{O_2}^{\bullet -}} = (1.9 \pm 0.4) \times 10^{9}\ \mathrm{M}^{-1}\mathrm{s}^{-1} \tag{13}$$

$$\mathrm{d}[\mathrm{HO_2^{\bullet}}]/\mathrm{d}t = k_{ss,\mathrm{HO_2}^{\bullet}}\,[\mathrm{Cu_2L}][\mathrm{HO_2^{\bullet}}],\ k_{ss,\mathrm{HO_2}^{\bullet}} > 3 \times 10^{9}\ \mathrm{M}^{-1}\mathrm{s}^{-1} \tag{14}$$

Under conditions where superoxide/hydroperoxyl radical is present in great excess (15- to 60-fold) over the dicopper complex, the steady-state condition is reached where the kinetics obey a pseudo first-order rate law.

Near $\mathrm{pH} = 4.7$, the $\mathrm{p}K_a$ of hydrogen dioxide, both Eq. 13 and 14 must be considered. The apparent rate constant k'_{ss} is

$$k'_{ss} = 1/(1 + \beta) \times k_{ss,\mathrm{HO2}} + 1/(1 + \beta) \times k_{ss,\mathrm{O2}^{-}} \tag{15}$$

where

$$\beta = [\mathrm{O_2^{\bullet -}}]/[\mathrm{HO_2^{\bullet}}] = 10^{(\mathrm{pH}\ -\ \mathrm{p}K\mathrm{HO2})}$$

and at $\mathrm{pH} = 4.7$ reduces to

$$k'_{ss} = 0.5 \times k_{ss,\mathrm{HO_2}} + 0.5 \times k_{ss,\mathrm{O_2}^{-}} \tag{16}$$

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