A Mechanistic Study of the Copper(II)-Peptide-Catalyzed Superoxide Dismutation. A Pulse Radiolysis Study

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Abstract: The reactivity of O_2^- toward Cu(II)-peptides containing glycine and histidine for which $E°_{Cu(III)/Cu(II)} \leq 1.08$ V was investigated by the pulse radiolysis technique. It has been found that the ability of the various complexes to catalyze O_2^- dismutation depends inversely on the redox potential of the couple Cu(III)/Cu(II). The Cu(II)-peptides containing histidine, which have higher redox potentials than that of the couple O_2^-/H_2O_2, do not catalyze the reaction at all. Although no direct evidence was found for the formation of Cu(III)-peptide, the results suggest that the mechanism of the catalysis of O_2^- dismutation proceeds via alternate oxidation and reduction of the metal by O_2^-. Furthermore, this mechanism is supported by indirect observations, which are discussed in detail.

Introduction

The superoxide anion radical (O_2^-) is a highly toxic species in many biological systems, especially in those cases where transition-metal ions are present. It is involved in radiation damage,[1] membrane damage and lipid peroxidation,[2] DNA damage,[3,4] phagocytosis,[5] aging,[6] cancer,[7] etc.[8,9] Superoxide dismutase (SOD) catalyzes O_2^- dismutation very efficiently,[10] and it serves as an important mean of defense against oxygen toxicity.

Many low molecular weight complexes of transition metals, especially those of copper, are efficient catalysts of O_2^- dismutation,[11-16] at least in the test tube. In some cases these same complexes may enhance the biological damage caused by O_2^-.[3,4,17] The suggested mechanism of the reaction of many copper complexes in the catalysis of O_2^- dismutation or in the expression of O_2^- toxicity assumes that the first step in both of these cases is the reduction of the metal by O_2^-.

$$Cu(II) + O_2^- \rightarrow Cu(I) + O_2 \quad (1)$$

In the protective pathway, reoxidation of the metal by O_2^- occurs via

$$Cu(I) + O_2^- + 2H^+ \rightarrow Cu(II) + H_2O_2 \quad (2)$$

In the toxic pathway, reoxidation of the metal by H_2O_2 takes place to form a metal-peroxo complex[18,19]

$$Cu(I) + H_2O_2 \rightarrow CuH_2O_2^+ \quad (3)$$

which can either react with the biological target molecule directly or decompose via

$$CuH_2O_2^+ \rightarrow Cu(II) + OH^- + OH^\bullet \quad (4)$$

or

$$CuH_2O_2^+ \rightarrow Cu(III) + 2OH^- \quad (5)$$

where both $OH^\bullet$ and Cu(III) are highly oxidizing and deleterious species.

However, the concentration of O_2^- in vivo is normally below 10^{-10} M,[20] and $E°_{O_2/O_2^-} = -0.16$ V at 1 M O_2 at pH 7,[21] while other biological reductants, such as glutathione ($E° = -0.23$ V), NAD(P)H ($E° = -0.32$ V), or vitamin C ($E° = -0.058$ V), are present in cells at concentrations exceeding that of O_2^- by several orders of magnitude.[22-24] Therefore, these reductants can easily reduce transition-metal compounds, at least as efficiently as O_2^-; nevertheless, they are not toxic. Thus, it is rather surprising that O_2^- is such a deleterious species in vivo, whereas the other biological reductants are not. Therefore, we have recently suggested an alternative mechanism to explain the unique toxicity of O_2^- as compared to the other biological reductants, where the first stage, for both the protective and the toxic pathways, proceeds via the oxidation rather than the reduction of the metal by O_2^-.[25]

$$Cu(II) + O_2^- + 2H^+ \rightarrow Cu(III) + H_2O_2 \quad (6)$$

In the protective pathway, Cu(III) is reduced by O_2^- via reaction 7, whereas in the toxic pathway it oxidizes the biological target.

$$Cu(III) + O_2^- \rightarrow Cu(II) + O_2 \quad (7)$$

The possible involvement of Cu(III) in the mechanism of the toxicity of O_2^- is not surprising as the redox potential of the couple O_2^-/H_2O_2 at pH 7 is 0.94 V,[26] which implies a strong oxidizing

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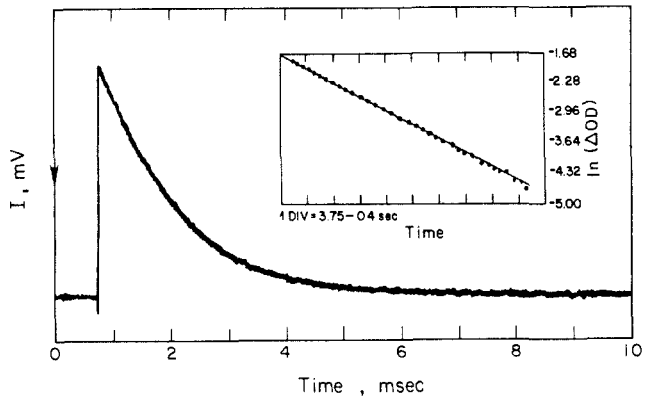

Figure 1. Typical kinetic plot describing the decay of the absorbance of O_2^- at 264 nm in O_2-saturated solutions containing 0.2 mM tetraglycine, 4 μM $CuSO_4$, and 20 mM formate at pH 6.8 (1 mM phosphate buffer). I_0 = -392 mV, ΔI = 152 mV. The inset contains a fit of the decay of O_2^- to a first-order rate law.

capability for O_2^-. The redox potential, $E°_{Cu(III)/Cu(II)}$, of copper complexes, with ligands that are good σ donors, is lower than that of the free ions (which has been found to be 0.2 V less positive than that of the $OH^•/H_2O$ couple at pH 3.65),[27] so that from the thermodynamic point of view reaction 6 can occur.

In this study, the reaction of O_2^- with various Cu(II)–peptides, for which $E°_{Cu(III)/Cu(II)}$ = 0.61 – 1.08 V, was investigated by using the pulse radiolysis technique. The results suggest that the mechanism of O_2^- dismutation catalyzed by these compounds involves alternate oxidation and reduction of Cu(II) by O_2^-.

Materials and Methods

All chemicals were of analytical grade and were used as received: tetraglycine (GGGG), triglycine (GGG) (Sigma), triglycine amide (GGGa) (Vega-Fox Biochemicals), histidylglycylglycine (HGG), glycylhistidylglycine (GHG), glycylglycylhistidine (GGH), glycylglycylhistidylglycine (GGHG) (Bachen Feinchemikalien), cupric sulfate, sodium formate, monosodium and disodium phosphate (Merck). All solutions were prepared with distilled water that was passed through a Millipore ultrapurification system. Unless otherwise stated, solutions for irradiations were prepared by mixing 0.2 mM of the peptide with 1–100 μM copper sulfate in oxygenated solutions containing 20 mM formate at pH 6.8 (1 mM phosphate buffer). Most of the Cu(II)–peptides studied have relatively low stability constants,[28] and therefore high excess of the peptide concentration over the metal was used to assure complete ligation of the metal. This was necessary as Cu(II) ions are known as efficient catalysts of O_2^- dismutation under the experimental conditions,[11,12] and their presence can lead to misinterpretation of the results.

Pulse radiolysis experiments were carried out with a Varian 7715 linear accelerator with a 200-mA current of 5 MeV electrons. Irradiations were done in a 4-cm Spectrosil cell with an optical path length of 12.1 cm. A 150-W Xe–Hg lamp was used as the light source. The detection system included a Bausch & Lomb grating monochromator Model D330/D331 Mk.II and an IP28 photomultiplier. The signal was transferred through a Sony/Textronix 390AD programmable digitizer to a micro PDP-11/24 computer, which operated the whole pulse radiolysis system.

When O_2-saturated solutions containing formate are pulse-irradiated, the following reactions take place:

$$H_2O \xrightarrow{h\nu} e_{aq}^-, OH^•, H^•, H_2O_2, H_2, H_3O^+ \quad (8)$$

$$e_{aq}^- + O_2 \rightarrow O_2^- \quad k_9 = 2 \times 10^{10}\ M^{-1}\ s^{-1}\ [29] \quad (9)$$

$$OH^• + HCO_2^- \rightarrow H_2O + CO_2^- \quad k_{10} = 3.5 \times 10^9\ M^{-1}\ s^{-1}\ [29] \quad (10)$$

$$CO_2^- + O_2 \rightarrow CO_2 + O_2^- \quad k_{11} = 2.4 \times 10^9\ M^{-1}\ s^{-1}\ [30] \quad (11)$$

$$H^• + O_2 \rightarrow HO_2^• \quad k_{12} = 2 \times 10^{10}\ M^{-1}\ s^{-1}\ [29] \quad (12)$$

$$HO_2^• \rightleftharpoons H^+ + O_2^- \quad pK_a = 4.7 \quad (13)$$

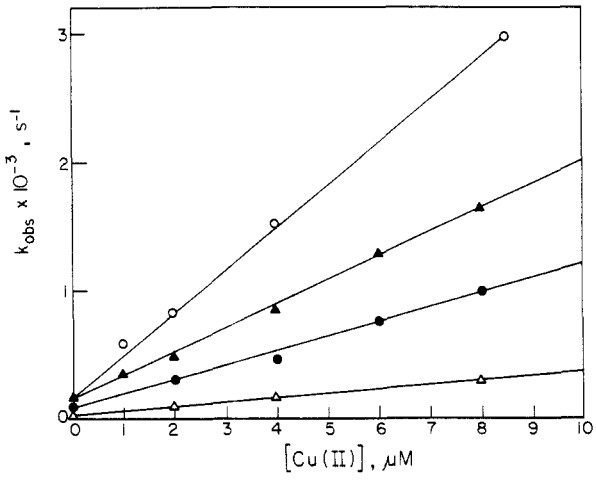

Figure 2. Observed rate constant of the decay of O_2^- as a function of the initial concentration of Cu(II) in O_2-saturated solutions containing 0.2 mM peptide and 20 mM formate at pH 6.8 (1 mM phosphate buffer). ○, GGGa; ▲, GGGG; ●, GGG; △, GG.

Table I. Reactivity of Cu(II)-Peptides Toward O_2^- As Compared to Their $E°_{Cu(III)/Cu(II)}$

peptide	$E°_{Cu(III)/Cu(II)}$,[a,b] V	k_{cat}, $M^{-1}\ s^{-1}$	$k_1(k_6)$, $M^{-1}\ s^{-1}$
GG	nd	3.1×10^7	3.2×10^7[c]
GGG	0.92	1.1×10^8	1.1×10^8
GGGa	0.61	3.8×10^8	3.7×10^8
GGH	0.98		1.1×10^6
HGG	nd	6.0×10^7	1.0×10^8
GHG	nd		$<1 \times 10^5$
GGGG	0.64	1.8×10^8	2.3×10^8
GGHG	1.08		$<1 \times 10^5$

[a] The redox potentials were taken from ref 31. [b] nd, not determined. [c] The literature value taken from ref 12 at pH 6.7 in the presence of 5 mM formate, 10 mM GG, and 100 μM $CuSO_4$ is $1.9 \times 10^7\ M^{-1}\ s^{-1}$. When we repeated the experiments under these conditions a value of $4.3 \times 10^7\ M^{-1}\ s^{-1}$ was obtained.

Because of the high rate constants of these reactions, all the primary radicals are converted into O_2^- within the end of the pulse. The initial concentration of O_2^- thus generated was determinated by using ϵ_{264} = 1725 $M^{-1}\ s^{-1}$ and was ~15 μM under the experimental conditions. The change in the absorbance of O_2^- was followed at 264 nm.

Results and Discussion

The SOD-like activity of the various Cu(II)–peptides was studied under the condition where $[Cu(II)]_0 < [O_2^-]_0$, whereas the reaction of O_2^- with these compounds was investigated at $[Cu(II)]_0 \gg [O_2^-]_0$. In cases where the Cu(II)–peptide catalyzed O_2^- dismutation, the decay of the absorbance of O_2^- followed pseudo-first-order kinetics. A typical kinetic plot is given in Figure 1. The second-order rate constants were determined by plotting the observed first-order rate constants as a function of $[Cu(II)]_0$ (Figure 2). All the observed pseudo-first-order rate constants were obtained at high ratio of [L]/[Cu(II)], where the rates were independent of the ligand concentration.

The mechanism of the catalysis of O_2^- dismutation by the copper compounds can be described by either reactions 1 and 2 or 6 and 7. Assuming a steady state for either [Cu(I)] or [Cu(III)], respectively, for both mechanisms, rate eq 14 is obtained.

$$-d[O_2^-]/dt = k_{obs}[O_2^-] \quad (14)$$

where

$$k_{obs} = k_{cat}[Cu(II)]_0 \quad (15)$$

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and

$$k_{cat} = 2k_1k_2/(k_1 + k_2) \text{ or } 2k_6k_7/(k_6 + k_7) \quad (16)$$

Thus, it is impossible to distinguish between both mechanisms kinetically, as already mentioned by Klug-Roth and Rabani in 1976.[12]

Under noncatalytic conditions, where Cu(II) scavenges O_2^-, the decay of O_2^- absorbance also followed pseudo-first-order kinetics. From plots similar to those in Figure 2, either k_1 or k_6 was determined. In Table I the rate constants k_{cat}, k_1, or k_6 are summarized for all Cu(II)-peptides studied. The values of $E°_{Cu(III)/Cu(II)}$ are included when known.

From the results shown in Table I, it can be concluded that as the redox potential of the couple Cu(III)/Cu(II) increases, the catalytic activity decreases, and in the case of GGH and GGHG, no SOD-like activity was observed at all. A similar activity trend had been demonstrated earlier by Kimura et al.[32] with the use of indirect methods for determining the SOD-like activity of these complexes. Although the trend in the activity found by Kimura et al.[32] was the same, their values were orders of magnitude lower than those determined directly with the use of the pulse radiolysis method. Moreover, different results were obtained when these authors used two different indirect assays.[32] The reasons for the discrepancy in the results using direct and indirect methods were discussed by us elsewhere in detail.[33,34]

The results suggest that O_2^- dismutation, catalyzed by the various Cu(II)-peptides, involves oxidation rather than reduction of Cu(II) by O_2^-. This conclusion is further supported by the following observations:

(i) The redox potential of the couple Cu(III)/Cu(II) is very sensitive to the nature of the coordinating groups. Peptide complexes of Cu(II) are formed through coordination with the deprotonated peptide nitrogen atoms,[31,35-37] which stabilize Cu(III) as shown in many studies.[31,32,38,39] However, in contrast to Cu(II) and Cu(III), there is very little evidence of deprotonated peptide nitrogen coordination in Cu(I) complexes, and the bulk of the data in the literature indicate that coordination of deprotonated peptide nitrogen to Cu(I) does not take place readily.[40-43] The report of Osterberg[44] of the species $Cu^ICu^{II}(GGG)_2$ is a notable exception to this rule. However, a cyclic voltammetric study of the reduction of Cu(II) triglycine complexes gives no evidence for a Cu(II,I) redox couple for these complexes.[43]

Histidine coordination increases the potential due to the imidazole nitrogen bond, which stabilizes low-valent transition metals including Cu(I) complexes.[41,42] The observation that Cu(II)-HGG catalyzes O_2^- dismutation whereas Cu(II)-GGH and Cu(II)-GHG do not suggests that Cu(II) binds to HGG through the peptide nitrogen atoms, while it has been demonstrated that it binds to GHG and GGH through the imidazole nitrogen atoms.[45] These results are in accord with the report that Cu(II)-HX (X = Ala, Tyr, Val, Phe) catalyzes O_2^- dismutation very efficiently, while Cu(II)-XH does not.[46] In this case it was also suggested that Cu(II) binds to XH through the peptide nitrogen atoms, and in the case of HX through the imidazole nitrogen atoms. This suggestion has been verified in the case of Cu(II)-GH and Cu(II)-HG with the use of the ESR technique.[47]

(ii) Cu(II)-GGGG, Cu(II)-GGGa and some other Cu(II) complexes of macrocyclic amines are easily oxidized by dissolved oxygen in neutral solutions.[31,39,48] Since the redox potential of the O_2/H_2O couple is lower than that of O_2^-/H_2O_2, the ability of O_2^- to oxidize these compounds is not surprising.

The Cu(III) complexes of GGGG, GGGa, and other peptides have strong absorbance near 365 (ϵ = 7100 M^{-1} cm^{-1} for GGGG and 7500 M^{-1} cm^{-1} for GGGa[31]), and they are moderately stable in neutral aqueous solutions (with a half-life of 5.5 h for GGGG[49]). Therefore, we expected that we would be able to follow the formation of some of the Cu(III) complexes around 365 nm under noncatalytic conditions, where O_2^- reacts only with Cu(II). We were quite surprised that we were unable to observe the formation of any absorption due to the formation of Cu(III)-peptide via the reaction of Cu(II)-peptide with O_2^- at neutral pH as well as in alkaline pH, although it has been reported that Br_2^- oxidizes Cu(II)-tetraglycine[50] and Cu(II)-triglycine[51] to the stable Cu(III) complexes when N_2O-saturated solutions containing 0.1 mM Cu(II)-peptide and 0.1 M NaBr were pulse-irradiated at pH > 7.2. There may be several reasons for the inability to observe the formation of Cu(III)-peptides via the reaction of O_2^- with Cu(II)-peptide: (i) Cu(II) may catalyze Cu(III) decomposition, as under noncatalytic conditions $[Cu(II)]_0 \gg [Cu(III)]_0$. It has previously been proposed[49,50] that $Cu^{II}(H_{-2}GGGG)^-$, which is the predominant form of the Cu(II) complex at pH 6-8,[31] catalyzes $Cu^{III}(H_{-3}GGGG)^{2-}$ decomposition (where H_{-n} refers to the number of deprotonated peptide nitrogens coordinated to copper). The fact that we were unable to observe the formation of Cu(III)-GGGG at alkaline pH, where no catalysis by the Cu(II) complex takes place, rules out this possibility. (ii) Reaction 6 may produce some forms of superoxide-metal complexes [$Cu^{II}O_2^- \rightleftharpoons Cu^{III}O_2^{2-} \rightleftharpoons Cu^{III}HO_2^-$], which have been suggested as intermediates in some other cases.[52-59] These complexes may have low absorbances in the visible region, and their spectra may be more similar to that of O_2^-.[52,54,55,58,59] Thus, the mechanism of the catalysis of O_2^- dismutation by Cu(II)-peptides is described by the following reaction scheme:

$$L\text{-}Cu(II) + O_2^- \rightleftharpoons L\text{-}Cu^{III}O_2^{2-} \quad (17)$$

$$L\text{-}Cu^{III}O_2^{2-} + O_2^- \rightarrow L\text{-}Cu(II) + O_2 + H_2O_2 \quad (18)$$

Moreover, these superoxide-metal complexes may be less stable than the corresponding Cu(III) complexes, and they may decompose via fast ligand oxidation processes. If the latter mechanism is correct, then the catalytic cycle might also proceeds via

$$L\text{-}Cu(II) + O_2^- \rightleftharpoons L\text{-}Cu^{III}O_2^{2-} \quad (17)$$

$$L\text{-}Cu^{III}O_2^{2-} + 2H^+ \rightarrow L'\text{-}Cu(II) + H_2O \quad (19)$$

$$L'\text{-}Cu(II) + L \rightarrow L\text{-}Cu(II) + L' \quad (20)$$

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giving net

$$O_2^- + L + 2H^+ \rightarrow H_2O + L' \quad (21)$$

This last mechanism is completely different from that suggested by reactions 1 and 2, 6 and 7, or 17 and 18, for which the net reaction is the dismutation reaction 22. The sequence of reactions

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (22)$$

17, 19, and 20 describes the catalysis of the oxidation of the ligand by O_2^- by the various Cu(II) complexes. If this mechanism occurs in vivo, and Cu(II) is bound to a biological target, then degradation of the target may take place. In such a case the Cu(II) will enhance the damage caused by O_2^-, rather than protect against the toxicity of this radical in biological systems.

Conclusions

The observations in the present study suggest that O_2^- oxidizes some Cu(II)-peptides, in which Cu(II) is bound to the peptide nitrogen atoms. This is a probable alternative mechanism for the catalysis of O_2^- destruction by copper in biological systems. In many cases the catalysis proceeds via reactions 1 and 2, especially in the case of the native enzyme as well as in the case of Cu(II)-phenanthroline and Cu(II)-bipyridine, where the formation of Cu(I) as an intermediate has been demonstrated.[16] In the latter complexes the ligands are good π acceptors, and therefore, they stabilize the low-valent oxidation state of the transition-metal complexes.

However, it should be noted that in many biological systems Cu(II) is complexed to the peptide nitrogen atoms, and in these systems O_2^- can oxidize the metal ion as indicated in this study. A similar mechanism is expected for Mn(II) and Fe(III) complexes. Thus, if O_2^- is able to oxidize transition-metal complexes, it is expected that at least in some of the systems biological damage will occur via reactions 17 and 19 or via direct oxidation of biological targets by Cu(III) complexes in addition to the Fenton reaction.

Acknowledgment. This research was supported by the Council for Tobacco Research, by the Israel Academy of Sciences and Humanities, by the Council for Higher Education, and by the Israel Atomic Energy Commission.

Registry No. O_2^-, 11062-77-4.

Chromatographic Band Profiles and Band Separation of Enantiomers at High Concentration

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Abstract: Experimental band profiles for large sample sizes of both single compounds and various binary mixtures of the optical antipodes of *N*-benzoylalanine are obtained on immobilized bovine serum albumin (BSA). The two enantiomers are resolved on this stationary phase. In the case of mixtures, the individual elution profiles are acquired by continuous fraction collection, followed by chromatographic analysis of these fractions. The equilibrium isotherms of each of the two chiral isomers between the two phases of the chromatographic system are determined by frontal analysis on the same column. These isotherms can be accounted for with great precision by a model assuming the existence of two kinds of sites on the stationary phase. For each isomer, the adsorption on each type of site is well described by a Langmuir equation. The first type of site is selective and interacts more fervently with the D isomer. The second type of site is not chiral selective, and the corresponding Langmuir isotherms are identical for the two isomers. The experimental profiles are nearly identical with the profiles calculated by the semi-ideal model of nonlinear chromatography, using the competitive Langmuir isotherms derived from the measured single-component isotherms.

Introduction

Chromatography has become the most general and versatile method for the separation of enantiomers.[1,2] Most work in this area has dealt with analytical separations that require the nearly complete resolution between the bands of the analyte components and their proper detection but not the collection of any purified material. There is a rapidly growing interest in the scaling up of the chromatographic procedures in order to prepare and recover significant amounts of very pure chiral isomers for various applications in pure chemistry or in the pharmaceutical industry.[3] In such preparations, the injection of large-size samples becomes necessary. This permits the collection of more concentrated fractions from which the recovery of the purified isomers is easier. Under such experimental conditions, however, the band profiles become broader and unsymmetrical, the bands overlap, and the separation seems to degrade.

These phenomena are due to the nonlinear behavior of the phase equilibrium isotherms at high concentrations and to the competition between the molecules of the different mixture components for interaction with the stationary phase. In previous papers, a theoretical investigation of the band profiles of pure compounds[4] and of the components of binary mixtures[5,6] has been discussed. This work permits a description of the progressive separation of the sample.[7] The two primary phenomena observed for a binary mixture at high concentrations are the displacement and the

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0002-7863/90/1512-6492$02.50/0