Ascorbate Oxidation Catalyzed by Bis(histidine)copper(II)

Marina Scarpa

Department of Physics, Università di Trento, Via Sommarive 14, 38050 Povo-Trento, Italy

Fabio Vianello, Luca Signor, Lucio Zennaro, and Adelio Rigo*

Department of Biological Chemistry, Università di Padova, Via Trieste 75, 35121 Padova, Italy

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The kinetics of ascorbic acid oxidation by molecular oxygen, catalyzed by bis(histidine)copper(II) (CuL_2^{2+}), was followed in 0.1 M phosphate buffer at pH 7.0. Saturation of the oxidation rate was observed at increasing O_2, ascorbate and CuL_2^{2+} concentrations. The oxidation state of the copper ion during the catalysis and the concentration of the ascorbyl radical were followed by ESR and/or by optical spectroscopy. No significant reduction of Cu(II) was observed under vacuum or in the presence of oxygen at ascorbate concentrations <20 mM. Evidence for the binding of ascorbate to CuL_2^{2+} was found by ESR, and a stability constant of 20 M^{-1} was estimated. We suggested a mechanism which is consistent with our experimental findings and explains some of the contradictory data reported in the past by various authors. The saturation of the reaction rate on increasing [CuL_2^{2+}] is explained in terms of its catalytic effect on ascorbate oxidation and the superoxide dismutase-like activity of this complex. The experimental concentration of the ascorbyl radical, which is an intermediate product, was in good agreement with that calculated on the basis of the proposed mechanism.

Introduction

Much attention has been devoted to one- and two-electron oxidation of ascorbate because of the various roles of this compound in biological systems, which are still a matter of debate. In particular, many studies have been carried out on ascorbate oxidation by molecular oxygen which, at neutral pH, occurs according to the overall reaction:

$$AH^- + O_2 + H^+ \rightarrow A + H_2O_2 \qquad (1)$$

where AH^- and A are the ascorbate monoanion and dehydroascorbate, respectively.[1] In a previous paper we provided evidence for the generation of the ascorbyl ($A^{\bullet -}$) and superoxide radical ($O_2^{\bullet -}$) by ESR and by ^{19}F-NMR, respectively.[2] In this paper we suggest that superoxide acts as an active intermediate, since it contributes to the overall oxidation rate. It is also shown that, in spite of the electrochemical potential of ascorbate (E_0'-(AH^-/A) = 0.054 V at 25 °C and 0.1 M ionic strength),[3] the rate of oxidation by molecular oxygen is negligible in solutions containing low concentrations of heavy metal ions (<1 nM).[2,4] Spin restrictions for ascorbate oxidation were invoked to explain this behavior.[5]

In past years, the ability of various Cu(II) complexes to catalyze oxidation of ascorbate has been investigated, in addition to searching for a model of the enzyme ascorbate oxidase. The catalytic efficiency of Cu(II) complexes was found to be dependent on the nature of the ligands and on the coordination geometry of the metal ion.[6] However, scarce and contradictory results were reported in terms of rate law and reaction mechanism, and therefore further kinetic studies are necessary for understanding the mechanism of ascorbate oxidation. The sensitivity of this reaction to several experimental variables, such as heavy metal ions, pH of the solution, and chelating efficiency of the buffer system,[4,7] makes the proposal of a general mechanism difficult. Questions to be answered are how the electron exchange between ascorbate and oxygen occurs and the mechanism of the interaction between ascorbate or oxygen and metal catalysts. Regarding the latter question, only speculative hypotheses have been reported. In particular, Martell et al.[8,9] and, more recently, Takamura et al.[10] suggested the formation of a ternary complex among the metal, ascorbate, and oxygen, but no experimental evidence has been reported.

In this paper we report a kinetic study performed to clarify the initial stages of the ascorbate oxidation mechanism in the presence of the complex CuL_2^{2+} as catalyst.[11] This reaction could be of biological significance because of the possible presence of CuL_2^{2+} in living systems. On the basis of the kinetic behavior of the system CuL_2^{2+}−ascorbate−molecular oxygen and the spectroscopic evidence for the formation of a complex between ascorbate and CuL_2^{2+} and of the lack of significant formation of Cu(I), we suggest a reaction scheme consistent with our experimental findings. This scheme supports the formation of a ternary intermediate complex between the reagents and the catalyst.

Experimental Section

All chemicals were of the purest grade available. Ascorbic acid was from Fluka; catalase and histidine were from Sigma.

* Corresponding author. Phone: +39-49-8276107. FAX: +39-49-8073310. E-mail: rigo@civ.bio.unipd.it.

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(11) Abbreviations: CuL_2^{2+}, bis(histidine)copper(II); Cu,Zn SOD, Cu,Zn superoxide dismutase.

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The water used to prepare the solutions was twice-distilled and then passed though a Milli-Q purification system (Millipore). All the glassware was stored in 10 mM HCl, and the solutions were passed through a Chelex 100 column just before use.[12] In this way the concentration of heavy metal ions in the solutions was estimated to be below 1 nM and the Cu(II) impurities were undetectable by atomic absorption even when the solutions were concentrated by a factor of 10^3. A Perkin-Elmer Model 4000 atomic absorption spectrometer was used for these determinations, and the detection limit for copper was 1 μM under the experimental conditions used.

The Cu(II) solutions were prepared by oxidizing copper shot, purity >99.9995% (Aldrich), with 10% HNO_3. A solution of 47 mM Cu(II) was obtained. The complex between Cu(II) and histidine was prepared just before use by adding the Cu(II) solution to a histidine solution and adjusting the pH to 7.0. If not otherwise stated, the ratio [histidine]/[Cu(II)] was 25. According to Kruck and Sarkar,[13] under our experimental conditions the predominant species (>99%) is bis(histidine)copper(II), the stability constant of this complex being $10^{18.5}$ in 0.15 M NaCl, at 25 °C.

ESR Measurements. ESR spectra of the ascorbyl radical and of the Cu(II) complexes were acquired with a Bruker ER 200 D spectrometer. A freshly prepared solution of carboxyproxyl was used as a standard for the calculation of the ascorbyl radical concentration. Carboxyproxyl (Sigma) was recrystallized just before use.

Kinetic Measurements. The oxidation of ascorbic acid by molecular oxygen was followed by spectrophotometry utilizing a Perkin-Elmer Lambda 17 instrument. The kinetic runs were carried out at 25 °C in 0.1 M phosphate at pH 7.0. At this pH value, the predominant species is the ascorbate monoanion.[14] The initial rates were usually measured from the decrease of absorbance at 265 nm, where ascorbate shows a maximum ($\epsilon_{265} = 1.5 \times 10^4$ M^{-1} cm^{-1}). All experiments were carried out in the presence of 50 nM catalase to avoid possible side reactions of H_2O_2 generated in the oxidation process. The spontaneous oxidation of ascorbate was measured for each kinetic run before addition of the CuL_2^{2+} complex. Under our experimental conditions, it was always <1 nM s^{-1}. Solutions of known oxygen concentration were obtained by equilibrating the reaction solution with N_2-O_2 gas mixtures at 25 °C. The final oxygen concentration was measured by voltammetry at the dropping mercury electrode with an Amel 466 apparatus using a saturated calomel electrode as reference.

When necessary, the deoxygenation of solutions was performed under vacuum ($\leq 10^{-5}$ Torr) by freeze−thaw cycles. In this case, the kinetic runs were followed by optical spectroscopy utilizing a quartz cuvette connected to a two-compartment small glass flask. After vacuum-degassing, the system was sealed and the solutions in the glass flask were mixed.

Results

Dependence of the Ascorbate Oxidation Rate on the Concentration of Ascorbate, Oxygen, and CuL_2^{2+}. In Figure 1, curve A, are reported the initial rates of AH^- oxidation in the presence of 3 μM CuL_2^{2+} and 0.24 mM O_2 in the range 0.02−1.20 mM AH^-. Oxidation of ascorbate by molecular oxygen started immediately after addition of CuL_2^{2+}. This behavior is different from that observed in GSH oxidation, where a lag time occurs.[15]

The initial oxidation rate (v_0) showed a saturation effect with respect to the ascorbate concentration. The double-reciprocal plot $1/v_0$ vs $1/[AH^-]$ was linear, and the equation

$$1/v_0\ (M^{-1}\ s) = 1.68 \times 10^3(1/[AH^-]) + 3.06 \times 10^7 \qquad (r = 0.996)$$

was obtained by a least-squares regression procedure.

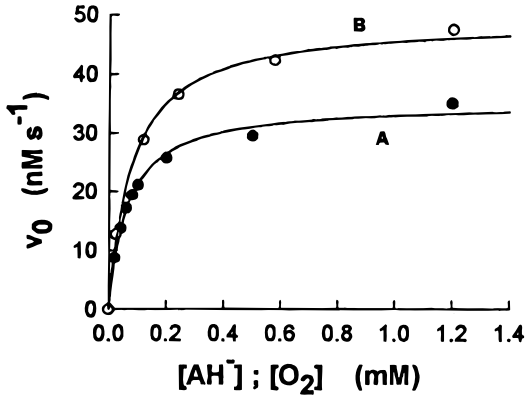

Figure 1. Dependence of the initial rate of oxidation of ascorbate (v_0) on ascorbate and molecular oxygen concentration. The experiments were performed at 25 °C, in 0.1 M phosphate, pH 7.0, containing 50 nM catalase and 3 μM CuL_2^{2+}. The kinetic runs were followed at 265 nm, and the optical path length was varied in the range 0.2−10 mm. Curve A (●): v_0 vs [AH^-]; the oxygen concentration was 0.24 mM and the ascorbate concentration was varied in the range 0.02−1.20 mM. Curve B (○): v_0 vs [O_2]; the ascorbate concentration was 0.6 mM and the oxygen concentration was varied in the range 0.02−1.20 mM.

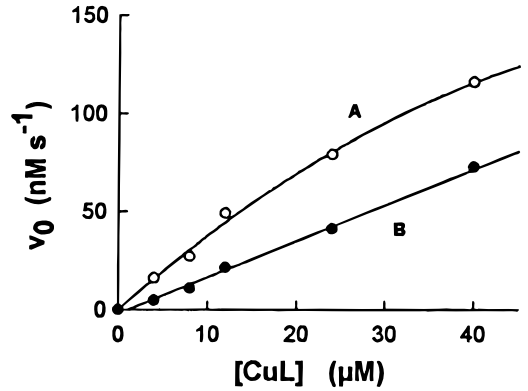

Figure 2. Rate profile of the catalyzed oxidation of ascorbic acid as a function of the CuL_2^{2+} concentration. Curve A (○): the experiments were performed in the presence of 0.20 mM AH^- and 0.24 mM O_2; other experimental conditions as in Figure 1. Curve B (●): as for curve A, except that 0.3 μM Cu,Zn SOD was added.

The dependence of the ascorbate oxidation rate on oxygen concentration, at 0.60 mM AH^- and 3 μM CuL_2^{2+}, showed a saturation effect in the range 0.02−1.20 mM O_2 (see Figure 1, curve B). Also in this case the double-reciprocal plot was linear:

$$1/v_0\ (M^{-1}\ s) = 1.36 \times 10^3(1/[O_2]) + 2.15 \times 10^7 \qquad (r = 0.998)$$

The variation of the oxidation rate with the concentration of CuL_2^{2+}, measured at 0.20 mM AH^- and 0.24 mM O_2, in the range 4−60 μM CuL_2^{2+}, showed unusual catalytic behavior. In fact, we observed a saturation of the oxidation rate with respect to the concentration of the copper complex (see Figure 2, curve A) and the double-reciprocal plot was fitted by the equation

$$1/v_0\ (M^{-1}\ s) = 2.43 \times 10^2(1/[CuL_2^{2+}]) + 2.81 \times 10^6 \qquad (r = 0.995)$$

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Table 1. Kinetic Parameters (V_{max}, k_{cat}, and K_m) of Ascorbate Oxidation under Various Experimental Conditions

experimental conditions[d]			kinetic parameters[a−c]			
[AH⁻] (mM)	[O_2] (mM)	[CuL_2^{2+}] (μM)	V_{max} (nM s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
0.02−1.20	0.24	3.0	32[a]	0.0108[a]	54[a]	200[a]
0.60	0.02−1.20	3.0	46[b]	0.0115[b]	63[b]	182[b]
0.20	0.24	4.0−60.0	380[c]		93[c]	

[a−c] The maximum initial rate (V_{max}), the catalytic constant (k_{cat}), and the apparent Michaelis constant (K_m) for the ascorbate, oxygen, and CuL_2^{2+} were calculated from the reciprocal plots of the initial rates as a function of (*a*) 1/[AH⁻], (*b*) 1/[O_2], and (*c*) 1/[CuL_2^{2+}] [d] For the experimental conditions, see Figures 1 and 2.

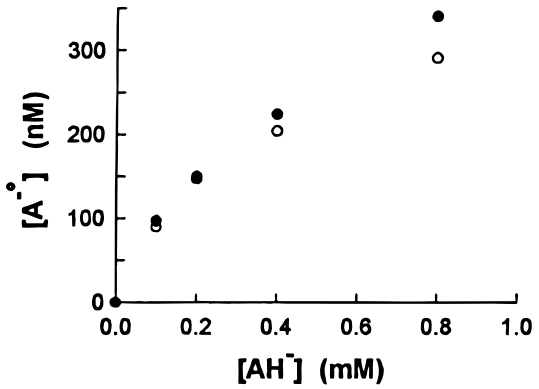

Figure 3. Steady-state ascorbyl radical concentration as a function of ascorbate concentration: (●) concentration measured by ESR; (○) concentration calculated on the basis of eq 14. The experiments were performed at room temperature, in the presence of 0.24 mM O_2 and 10 μM CuL_2^{2+}. Instrumental settings: 3488.5 G, central field; 15 G, sweep width; 9.82 GHz, microwave frequency; 20 mW, microwave power; 0.5 Gpp, modulation amplitude. Other experimental conditions were as for Figure 1.

In all kinetic runs, ascorbate and oxygen were in large excess with respect to CuL_2^{2+}, the ratios [ascorbate]/[CuL_2^{2+}] and [O_2]/[CuL_2^{2+}] being usually $\geq$10.

According to the Michaelis−Menten model, the values of the maximum rates and of the apparent Michaelis (K_m) and catalytic (k_{cat}) constants were calculated from the double-reciprocal plots of the data reported in Figures 1 and 2 and are listed in Table 1.

The Ascorbyl Radical as an Intermediate in the Oxidation of Ascorbate. A doublet with a 1.7 G splitting constant was observed, at $g = 2.0052$, by ESR spectroscopy. This signal is assignable to the ascorbyl radical ($A^{\bullet -}$).[16] The ascorbyl radical concentration was constant at constant ascorbate oxidation rate, under various experimental conditions. A saturation of the ESR signal on raising the concentration of ascorbate was observed; see Figure 3, where is shown (curve A) the steady-state ascorbyl radical concentration as a function of ascorbate concentration.

Effect of Cu,Zn Superoxide Dismutase on the Reaction Rate. The inhibitory effect of the Cu,Zn superoxide dismutase (Cu,Zn SOD), which is a scavenger of superoxide ion, on the ascorbate oxidation rate has already been discussed.[2] This effect was explained by the generation of superoxide in ascorbate oxidation and by the reactions of superoxide with ascorbyl radical and with ascorbate itself. The latter reactions double the oxidation rate of ascorbate. To test the possible reaction between CuL_2^{2+} and superoxide, we studied the dependence of the oxidation rate of ascorbate on the CuL_2^{2+} concentration, under the conditions of Figure 2, in the presence of 0.3 μM Cu,Zn SOD. Under these conditions, the reaction rate decreases and the saturation effect disappears. In this case, the plot v_0 vs [CuL_2^{2+}] (see Figure 2, curve B) is linear in the range 4 μM < [CuL_2^{2+}] < 60 μM:

$$v_0\,(\mathrm{M\ s^{-1}}) = 1.85 \times 10^{-3}[CuL_2^{2+}] + 2 \times 10^{-11} \qquad (r = 0.998)$$

Optical and ESR Spectra of the $AH^- - O_2 - CuL_2^{2+}$ System. Experiments were carried out in an attempt to demonstrate the reduction of Cu(II) or the formation of intermediates during the catalysis. To this purpose, we monitored the optical and ESR spectra of the system $AH^- - CuL_2^{2+}$, sealed under high vacuum, before and after the mixing of the reagents. In particular, the optical spectrum of 0.5 mM ascorbate was monitored at 265 nm, before and after the addition of 20 μM CuL_2^{2+}. At this wavelength, the measured absorbance is due to the ascorbate, since the contribution of 20 μM CuL_2^{2+} is negligible. We observed that, in the absence of oxygen, the addition of CuL_2^{2+} to AH^- induced a negligible decrease (<0.2%) of the absorbance, indicating that less than 5% of the total Cu(II) added was reduced.

ESR spectroscopy was used to detect the oxidation state of the copper ion during the ascorbate oxidation. In particular, the spectrum of 0.20 mM CuL_2^{2+} was recorded in 0.1 M phosphate buffer, pH 7.0, before and after the addition of 2 mM ascorbate, both in the presence and in the absence of oxygen. No significant difference was noticed between the spectra in terms of line shape and intensity, except for a resonance at $g = 2.0052$, which appeared when oxygen was present. This signal was ascribed to the ascorbyl radical generated in the ascorbate oxidation which occurs in the presence of oxygen.[16] In particular, the reduction of copper ion was undetectable in the presence of ascorbate, since the Cu(II) signal was constant, within the experimental error, up to 1 h after mixing.

By ESR spectroscopy, we tried to observe the formation of a complex between ascorbate or oxygen and CuL_2^{2+}. We recorded the ESR spectrum of CuL_2^{2+} [17] in 0.1 M phosphate, pH 7.0, at various oxygen pressures and AH^- concentrations.[18] While no significant modification of the spectral features occurred at O_2 pressures up to 10 atm, at increasing AH^- concentrations we observed a progressive change of the CuL_2^{2+} spectrum. In fact, we observed a variation of the relative intensity of the resonances at $g = 1.9515$ and $g = 1.9098$ and a modification of the low-field resonances ($g_{||}$ region), which are well resolved in the absence of ascorbate and progressively

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(18) In these experiments, the ratio [histidine]/[Cu(II)] was 2.0, to ensure that the axial coordination sites of Cu(II) are free. Under these experimental conditions, more than 99% of the Cu(II) is present as bis(histidine)copper(II).

(19) Since ionic strength can affect the spectral features, it was kept constant by suitable addition of NaCl.

(20) At [AH^-] $\gg$ 20 mM, we observed a resonance at $g = 2.0052$, assignable to $A^{\bullet -}$. Under the experimental conditions of Figure 4, the concentration of $A^{\bullet -}$ is of the order of 40 μM. Under these conditions, we observed also a slow reduction of the Cu(II) signal intensity. It is likely that at [AH^-] $\gg$ 20 mM, a slow one-electron oxidation of ascorbate by Cu(II) occurs. A second-order kinetic rate constant of 1.5 mM^{-1} s^{-1} was estimated from the decrease of the Cu(II) signal at 20 mM AH^-. From the kinetic runs performed in the presence of oxygen, we measured an apparent second-order kinetic rate constant

$$k' = \frac{v_0}{[AH^-][O_2]}$$

of the order of 0.5 M^{-1} s^{-1}; as a consequence the reduction of the Cu(II) by AH^- was neglected in the following treatment.

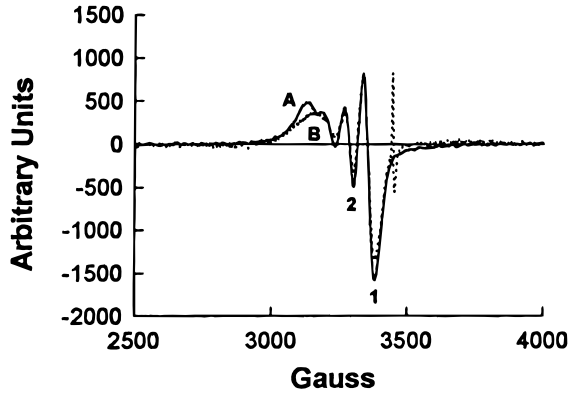

Figure 4. ESR spectra of the CuL_2^{2+} complex obtained under argon atmosphere in the presence or the absence of ascorbate:[23] spectrum A (—), 0.20 mM CuL_2^{2+} in 0.1 M phosphate buffer, pH 7.0; spectrum B (···), as for spectrum A, in the presence of 500 mM ascorbate. The peaks labeled as 1 and 2 were used for the calculation reported in ref 21. The spectra were acquired at room temperature, with a quartz flat cell, under argon. Instrumental settings: 3250 G, central field; 1500 G, sweep width; 1000 s, sweep time; 9.70 GHz, microwave frequency; 63 mW, microwave power; 8 Gpp, modulation amplitude.

disappear on increasing the ascorbate concentration (see Figure 4).[19,20] In particular the ratio (R) between the resonance at $g = 1.9515$ and that at $g = 1.9098$ (peaks 1 and 2 in Figure 4A, respectively) increases with the ascorbate concentration up to an asymptotic limiting value. On the basis of the dependence of the ratio R on the reciprocal of the ascorbate concentration, we calculated a stability constant of 20 M^{-1} for the complex between ascorbate and CuL_2^{2+}.[21]

Discussion

On the basis of the results reported in Figures 1 and 2, the dependence of AH^- oxidation rate on the concentration of AH^-, O_2, and CuL_2^{2+} can be expressed, for each of these compounds, as

$$v_0 = \frac{a[\mathrm{x}]}{b + c[\mathrm{x}]}$$

where v_0 is the initial rate, x is ascorbate, oxygen, or CuL_2^{2+}, and a, b, and c are constants assuming different values for each of these compounds. These relationships fit our kinetic data and the contradictory results reported by various investigators, i.e. the reaction order with respect to molecular oxygen which varies from 1 to 0.5 depending on the initial concentration of the molecular oxygen.[1,6,8]

The limiting reaction rate at increasing AH^- and O_2 concentration is easily understood in terms of catalysis theory, as a result of the saturation of the catalytic center, that is of CuL_2^{2+}.

This catalysis may occur through (i) the alternate reduction and oxidation of the copper ion according to an ordered Ping Pong Bi Bi mechanism or (ii) the formation of mixed-ligand complexes of the type O_2−catalyst−substrate.

Previous studies of the ascorbate oxidation catalyzed by Cu(II), performed in the absence of strong copper ligands, suggested the intermediacy of Cu(I),[1,22] according to mechanism i. If this mechanism holds, under the condition $[O_2] \ll K_m$, the Cu(II) should be reduced to Cu(I) and the dehydroascorbate must be generated, according to the ratio $[A]/[Cu(I)] \approx 1/2$. However, under the experimental conditions of the kinetic runs, we found no ESR or optical evidence for significant formation of Cu(I) or of dehydroascorbate when AH^- and Cu(II) were mixed under vacuum or argon atmosphere.[20] The ascorbate oxidation occurred only when this system was equilibrated with molecular oxygen, but also in this case no evidence of Cu(I) formation was observed.

The formation of mixed-ligand complexes of the type O_2−catalyst−substrate (mechanism ii) has been postulated by some authors, not only to explain ascorbate oxidation[8,9,23,24] but more generally to explain the oxidation chemistry of model compounds mimicking the hydroxylating enzymes.[25] These complexes have been assumed to be redox active and to represent true intermediates of catalyzed oxidation reactions,[5] probably because they may overcome spin restrictions.[5,26] However, no experimental evidence has been reported for the formation of these complexes in the case of copper ion as catalyst.[8,9] The formation of mixed-ligand complexes as an essential step in the catalytic mechanism is strongly supported by our kinetic and spectroscopic data. In fact, a mechanism explaining the saturation with respect to AH^- and O_2 is obtained if we assume the formation of an intermediate ternary complex (AH^-−CuL_2^{2+}−O_2) between the two reagents and the catalyst, according to the scheme

$$AH^- + CuL_2^{2+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AH^- - CuL_2^{2+} \qquad (1)$$

$$AH^- - CuL_2^{2+} + O_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AH^- - CuL_2^{2+} - O_2 \qquad (2)$$

where, on the basis of our experimental data, $K_1 = k_1/k_{-1}$ is 20 M^{-1}.[21]

The formation of the ternary complex should be followed by one-electron transfer from ascorbate to oxygen:

$$AH^- - CuL_2^{2+} - O_2 \xrightarrow{k_3} A^{\bullet -} + O_2^{\bullet -} + CuL_2^{2+} + H^+ \qquad (3)$$

The ascorbyl radical and the superoxide ion generated in this step disappear according to the following reactions:[2]

(21) The calculation of the constant $K_1 = k_1/k_{-1}$ was performed on the basis of the relative intensity of resonances 1 and 2, as a function of ascorbate concentration (see Figure 4). For the two resonance intensities (h_1 and h_2, respectively) the following equations hold:

$$h_1 = \alpha_1^{CuL_2^{2+}}[CuL_2^{2+}] + \alpha_1^{AH^- - CuL_2^{2+}}[AH^- - CuL_2^{2+}]$$

$$h_2 = \alpha_2^{CuL_2^{2+}}[CuL_2^{2+}] + \alpha^{AH^- - CuL_2^{2+}}[AH^- - CuL_2^{2+}]$$

where the coefficients α_1 and α_2 are suitable molar coefficients. The subscripts refer to the resonance peaks 1 and 2 of Figure 4, and the superscripts refer to the species CuL_2^{2+} and AH^-−CuL_2^{2+}. From these equations we obtained

$$\frac{[CuL_2^{2+}]}{[AH^- - CuL_2^{2+}]} = \frac{1}{K_1[AH^-]} = \frac{((h_1/h_2)\alpha_2^{AH^- - CuL_2^{2+}}) - \alpha_1^{AH^- - CuL_2^{2+}}}{\alpha_1^{CuL_2^{2+}} - ((h_1/h_2)\alpha_2^{CuL_2^{2+}})}$$

$1/K_1$ was calculated from the slope of the straight line

$$\frac{((h_1/h_2)\alpha_2^{AH^- - CuL_2^{2+}}) - \alpha_1^{AH^- - CuL_2^{2+}}}{\alpha_1^{CuL_2^{2+}} - ((h_1/h_2)\alpha_2^{CuL_2^{2+}})} \text{ vs } \frac{1}{[AH^-]}$$

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$$AH^- + O_2^{\bullet -} + H^+ \xrightarrow{k_4} A^{\bullet -} + H_2O_2 \quad (4)$$

$$A^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{k_5} A + H_2O_2 \quad (5)$$

$$2A^{\bullet -} + H^+ \xrightarrow{k_6} A + AH^- \quad (6)$$

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{k_7} O_2 + H_2O_2 \quad (7)$$

The kinetic rate constants k_4, k_5, k_6 and k_7 have been measured under conditions similar to those used in the present work: $k_4 = 5.75 \times 10^4$ M^{-1} s^{-1} (as determined from pH dependence in the pH range 3−8 at $I = 0.02$),[27] $k_5 = 2.3 \times 10^8$ M^{-1} s^{-1} (measured in 70 mM phosphate buffer, pH 7.4, containing catalase),[2] $k_6 = 2.4 \times 10^5$ M^{-1} s^{-1} (0.1 ionic strength, pH >3).[28] The kinetic constant of superoxide dismutation at pH 7 is $k_7 = 6 \times 10^5$ M^{-1} s^{-1}.[29] This value increases in the presence of a compound showing superoxide dismutase activity. In particular, in the case of Cu,Zn SOD or CuL_2^{2+}, the kinetic rate constant for the catalyzed process is $k_7 = 2.3 \times 10^9$ M^{-1} s^{-1} (measured in 0.1 M borate, pH 9−10.2)[30] or 3×10^8 M^{-1} s^{-1} (as detected in 0.1 M formate, pH 2−7),[31] respectively.

On the basis of the kinetic constants, the competition between AH^- or $A^{\bullet -}$ and CuL_2^{2+} for the superoxide ion appears responsible for the apparent saturation effect observed at increasing concentrations of CuL_2^{2+}. In fact, the dismutation of $O_2^{\bullet -}$ via reaction 7 decreases the rate of oxidation of AH^- (it decreases the rates of reactions 4 and 5)[2]. CuL_2^{2+} catalyzes the ascorbate oxidation through reactions 1−3 but at high concentrations is active in reaction 7 (superoxide dismutation). The involvement of Cu(II) in this reaction leads to a relative decrease of the overall reaction rate. The balance of the two processes produces the saturation effect showed in Figure 2, curve A. The effect of CuL_2^{2+} on superoxide dismutation was confirmed by addition of Cu,Zn SOD at concentrations ≥0.3 μM. In fact, the addition of Cu,Zn SOD at concentrations that make negligible reactions 4 and 5 with respect to reaction 7 decreases the oxidation rate of AH^- and leads to the disappearance of the saturation effect (see Figure 2, curve B).[32] Although CuL_2^{2+} competes efficiently with the spontaneous dismutation of superoxide, this efficiency is quite low with respect to that of Cu,Zn SOD.

According to the above scheme, assuming that the reaction is rate limited by the formation of the ternary complex (that is reactions 4−6 are faster than reactions 1−3) and introducing the steady-state condition for the intermediate complexes AH^-−CuL_2^{2+} and AH^-−CuL_2^{2+}−O_2, we found that the rate of oxidation of ascorbate is given by the following equation (see Supporting Information for the kinetic treatment) (including eqs 8 and 9):

$$v_0 = \frac{k_1 k_2 k_3 [AH^-][O_2][CuL_2^{2+}]_0}{k_1(k_{-2} + k_3)[AH^-] + k_2 k_3 [O_2] + k_{-1}(k_{-2} + k_3)} \quad (10)$$

Equation 10 can be rearranged as follows:

$$\frac{1}{v_0} = \frac{k_1(k_{-2} + k_3)[AH^-] + k_2 k_3 [O_2] + k_{-1}(k_{-2} + k_3)}{k_1 k_2 k_3 [AH^-][O_2]} \frac{1}{[CuL_2^{2+}]_0} \quad (11)$$

at constant $[AH^-]$ and $[O_2]$

$$\frac{1}{v_0} = \frac{1}{k_1[CuL_2^{2+}]_0}\left\{1 + \frac{k_{-1}(k_{-2} + k_3)}{k_2 k_3 [O_2]}\right\}\frac{1}{[AH^-]} + \frac{(k_{-2} + k_3)}{k_2 k_3 [CuL_2^{2+}]_0 [O_2]} \quad (12)$$

at constant $[CuL_2^{2+}]_0$ and $[O_2]$

$$\frac{1}{v_0} = \frac{k_{-2} + k_3}{k_2 k_3 [CuL_2^{2+}]_0}\left\{1 + \frac{k_{-1}}{k_1[AH^-]}\right\}\frac{1}{[O_2]} + \frac{1}{k_1[CuL_2^{2+}]_0[AH^-]} \quad (13)$$

at constant $[CuL_2^{2+}]_0$ and $[AH^-]$

These equations are consistent with the experimental kinetic data, and they explain the dependence of the reaction rate on the concentrations of ascorbate and oxygen and on the metal complex when $[CuL_2^{2+}] \leq 5$ μM, that is when reactions 4 and 5 predominate with respect to reaction 7. According to eq 13, we measured $k_1 = 26$ M^{-1} s^{-1} from the intercept of the $1/v_0$ vs $1/[O_2]$ plot. Finally, since $K_1 = 20$ M^{-1}, $k_{-1} \approx 1$ s^{-1} was calculated. The experimental data suggested that the kinetic behavior of the CuL_2^{2+} complex follows the basic equations of the enzyme kinetic (see Figure 1, curves A and B). However, according to the proposed mechanism, the meaning of the kinetic parameters reported in Table 1 is not that of the characteristic parameters of the enzymatic reactions. In particular k_{cat}/K_M depends on the concentration of one of the reacting substrates, that is

$$\frac{k_{cat}}{K_M} = \frac{k_1 k_2 k_3 [O_2]}{k_2 k_3 [O_2] + k_{-1}(k_{-2} + k_3)} \quad \text{if } AH^- \text{ is the substrate}$$

and

$$\frac{k_{cat}}{K_M} = \frac{k_1 k_2 k_3 [AH^-]}{(k_{-2} + k_3)(k_1[AH^-] + k_{-1})} \quad \text{if } O_2 \text{ is the substrate}$$

Further support for the proposed mechanism was obtained by ESR measurements of the ascorbyl radical. According to our mechanism and as demonstrated by experiments, $A^{\bullet -}$ and $O_2^{\bullet -}$ [2] species are in a steady state. The ascorbyl concentration can be calculated (see Supporting Information) as a function of

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(32) On the basis of the saturation effect at $[CuL_2^{2+}] > 10$ μM (see curve A of Figure 2), due to dismutation of superoxide by CuL_2^{2+}, a value of 0.5×10^6 M^{-1} s^{-1} was found for $k_{7,CuL_2^{2+}}$. This value is about one order of magnitude lower than that reported by Weinstein and Bielski,[31] but it is equal to the value of $k_{7,CuL_2^{2+}} = 0.5 \times 10^6$ M^{-1} s^{-1} we have measured by the cytochrome *c* method,[33] under the experimental conditions of the present paper.
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the reaction rate and of the ascorbate concentration, by the equation

$$[A^{\bullet -}]^3 + \frac{k_4}{k_5}[AH^-][A^{\bullet -}]^2 - \frac{k_3 k_4}{k_5 k_6}[AH^-][AH^- - O_2 - CuL_2^{2+}] = 0 \quad (14)$$

Introducing the experimental values of $v_0 = k_3[AH^- - O_2 - CuL_2^{2+}]$, of $[AH^-]$, and of the catalytic constants k_4, k_5, and k_6 into eq 14, we calculated the $A^{\bullet -}$ concentrations which are very close to the experimental values; see Figure 3.

Since other metals have the ability to mediate electron transfer from ascorbate to oxygen, experiments are in progress in our laboratory with various metal complexes to demonstrate that the mechanism here reported holds for many important low molecular weight complexes of heavy metal ions in biological systems. Finally, it appears that the ascorbate reaction mechanism involves some crucial steps similar to that of glutathione oxidation,[15] thus suggesting that a general reaction mechanism holds for the oxidation of these important reducing agents present in living systems.

In conclusion, a kinetic and spectroscopic study of the oxidation of ascorbate by molecular oxygen catalyzed by the complex bis(histidine)copper(II) showed (i) a saturation effect of the oxidation rate at increasing O_2, ascorbate, and CuL_2^{2+} concentrations, (ii) negligible reduction of the metal catalyst to Cu(I) by ascorbate, under vacuum or in the presence of oxygen or argon, and (iii) the binding of ascorbate to the CuL_2^{2+} complex. These experimental results support a reaction scheme that occurs through the formation of mixed-ligand complexes of the type O_2−catalyst−substrate rather than an ordered Ping-Pong Bi Bi mechanism. On the basis of the experimental data and of the proposed mechanism, we calculated the stability constant $K_1 = 20\ M^{-1}$ of the complex between ascorbate and CuL_2^{2+} and the kinetic constants $k_1 = 26\ M^{-1}\ s^{-1}$ and $k_{-1} \approx 1\ s^{-1}$.

Saturation of the reaction rate with increasing concentration of the CuL_2^{2+} complex was explained in terms of the catalytic effect of Cu(II) on the ascorbate oxidation and of the superoxide dismutase-like activity of this complex. A catalytic constant for superoxide dismutation by CuL_2^{2+} was estimated.

Finally, we calculated the ascorbyl radical concentration expected on the basis of the proposed mechanism. The calculated values were very close to the experimental values obtained by ESR spectroscopy.

Supporting Information Available: Text and equations giving a kinetic treatment of the ascorbate oxidation catalyzed by CuL_2^{2+} (including eqs 8 and 9) (3 pages). Ordering information is given on any current masthead page.

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