Superoxide Ion as Active Intermediate in the Autoxidation of Ascorbate by Molecular Oxygen

EFFECT OF SUPEROXIDE Dismutase*

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The oxidation of ascorbic acid by molecular oxygen was investigated by optical and magnetic resonance methods in the presence and in the absence of copper and iron complexes. The results show that one superoxide ion is generated for each oxidized molecule of ascorbate. The effect of addition of Cu,Zn superoxide dismutase, which at concentrations higher than $10^{-7}$ M halves the oxidation rate, is discussed and a reaction mechanism is proposed. An upper limit for the kinetic rate constant of the reaction between the ascorbate radical and the superoxide ion is reported.

Increasing evidence indicates that in chemical and biological systems molecular oxygen is reduced to superoxide ion, $O_2^-$, which is an important agent of oxygen toxicity (1). One of the potential sources of $O_2^-$ could be ascorbic acid, an ubiquitous compound in biological systems, which plays an important physiological role. The generation of $O_2^-$ in the autoxidation of ascorbic acid has been discussed in some reports, none of which has demonstrated its presence unequivocally. In fact, although many papers deal with the kinetic and the mechanism of the oxidation of ascorbic acid by molecular oxygen, only few of them suggest $O_2^-$ as a reaction product without any experimental support (2-4). More recent papers, on the basis of the effect of superoxide dismutase, an enzyme which catalyzes $O_2^-$ dismutation very efficiently (5, 6), suggest or deny the formation of superoxide during ascorbic acid autoxidation (7, 8).

In an attempt to get more information on the possible sources of $O_2^-$ and on the role of ascorbic acid in biological systems, we have carried out a careful investigation on the involvement of $O_2^-$ in the autoxidation of ascorbic acid either in highly purified systems or in the presence of copper and iron complexes of biological relevance. In order to investigate the effect of bovine superoxide dismutase on the rate of ascorbate oxidation, we have utilized a new NMR method employing superoxide dismutase as $O_2^-$ scavenger and $^{19}F$ as probe of the oxidation state of the enzyme (9).

MATERIALS AND METHODS

1. (+)-Ascorbic acid, puriss. p.a., was from Fluka all other reagents

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were analytical grade. Triply distilled water, specific conductivity = $9 \times 10^{-5}$ $\Omega^{-1}$ cm$^{-1}$, was used.

Bovine Cu,Zn superoxide dismutase was prepared by the method of McCard and Fridovich (10) while catalase, from Boehringer, was freed of superoxide dismutase as previously described (11).

All solutions, except those containing iron and copper complexes, were passed through a Chelex-100 column (Bio-Rad) to remove traces of heavy metals.

The oxidation of ascorbic acid by molecular oxygen was carried out at 24°C in 0.07 M potassium phosphate brought to pH 7.4 and equilibrated with air. Since the $pK_a$ values of ascorbic acid are 4.2 and 11.8, the ascorbate anion (AH$^-$) is the predominant species at pH 7.4.

The oxidation of ascorbate has been started with the addition of a freshly prepared $10^{-7}$ M ascorbic acid solution into the buffer to which the preformed Cu++, Fe++, or Fe++ complexes were successively added. The initial rates were measured from the decrease of the absorbance at 265 nm, $\varepsilon = 1.5 \times 10^{4}$ M$^{-1}$ cm$^{-1}$, and were corrected for the AH$^-$ spontaneous oxidation, measured before the addition of the metal complex. All kinetics were carried out in the presence of $2 \times 10^{-7}$ M catalase to avoid possible reactions of H$_2$O$_2$ formed in the first step of the oxidation process, with components of the reaction system itself.

The measurements of NMR relaxation of $^{19}F$ nucleus were carried out as previously described (8). EPR spectra were obtained with a Bruker model D 200 spectrometer.

RESULTS

At physiological pH values the oxidation rate of $5 \times 10^{-5}$ M ascorbic acid by molecular oxygen was about $10^{-10}$ M$^{-1}$ s$^{-1}$ in carefully purified solution. This rate is at least two orders of magnitude lower than that reported for similar experimental conditions but without a specific purification of the reaction system (7).

The addition of micromolar concentrations of Cu$^{2+}$-(His)$_2$, Fe$^{3+}$-EDTA, or Fe$^{3+}$-ADP determines a strong increase of the oxidation rate, while superoxide dismutase at concentrations higher than $10^{-7}$ M halves the oxidation rate. The relationship between the oxidation rate of ascorbate and the superoxide dismutase concentration has been reported in Fig. 1 for a series of experiments carried out with Fe$^{3+}$-EDTA as catalyst.

Starting from $5 \times 10^{-9}$ M superoxide dismutase, a progressive decrease is noted of the oxidation rate which, at superoxide dismutase concentrations higher than $10^{-7}$ M, is reduced to half of the rate measured in the absence of the enzyme. Parallel EPR measurements show a steady state concentration of ascorbate radical (AH$^-$) of about $5 \times 10^{-8}$ M, which is very little dependent on the experimental conditions. In particular, superoxide dismutase higher than $10^{-7}$ M increases AH$^-$ concentration by an average of about 20%. The addition of bovine serum albumin or apoperoxide dismutase at concentrations of the order of $10^{-7}$ M was without influence on the kinetic and on the steady state AH$^-$ concentration in the presence of iron and copper complexes.

The superoxide dismutase effect was general and an average value of 2 for the ratio $R_{AH^+}/R_{AH^-}$ superoxide dismutase, where $R_{AH^+}$ superoxide dismutase and $R_{AH^-}$ are the initial rates of the oxidation of the ascorbate in the presence and in the absence, respectively, of saturation amounts of superoxide dismutase, was obtained in a variety of experimental conditions and for different types of catalysts (see Table I).

Besides this indirect evidence of the involvement of $O_2^-$ in the ascorbate oxidation, the direct measurement of the rate of $O_2^-$ generation was performed adding superoxide dismutase to the reaction system and following by the $^{19}F$ NMR method.
FIG. 1. Relationship between the concentration of superoxide dismutase and the initial rate of ascorbate oxidation. The oxidation of ascorbate was followed at 265 nm in 0.07 M potassium phosphate, pH 7.4, equilibrated with air and containing 1.2 × 10⁻⁶ M ascorbate, 2 × 10⁻⁶ M catalase, and 4.7 × 10⁻⁵ M Fe³⁺-EDTA. Cu,Zn superoxide dismutase (O); apoperoxidase dismutase (□). SOD, superoxide dismutase.

Effect of superoxide dismutase on the oxidation rate of ascorbate by molecular oxygen

The rates of 10⁻⁶ M superoxide dismutase (Rₐ₈ - superoxide dismutase) and in the absence of superoxide dismutase (Rₐ₈) have been measured in 0.07 M potassium phosphate, pH 7.4, containing 2 × 10⁻⁶ M catalase, 1 × 10⁻⁸-5 × 10⁻⁹ M ascorbate, and 18 × 10⁻⁵-5 × 10⁻⁹ M catalyst. The ratio [AH⁻'/catalyst] was kept ±10.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Number of experiments</th>
<th>Rₐ₈/superoxide dismutase</th>
<th>Rₐ₈</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7</td>
<td>1.95 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>Cu²⁺-(His)₂</td>
<td>13</td>
<td>1.97 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Fe³⁺-EDTA</td>
<td>12</td>
<td>1.96 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Fe³⁺-EDTA</td>
<td>7</td>
<td>2.03 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Fe³⁺-ADP</td>
<td>4</td>
<td>2.02 ± 0.11</td>
<td></td>
</tr>
</tbody>
</table>

TABLE II

Rates of O₂⁻ generation in the ascorbic acid oxidation by molecular oxygen

The rates of O₂⁻ production (Rₒ) have been measured in 0.07 M potassium phosphate, pH 7.4, containing 0.5 M KF, 2 × 10⁻⁶ M superoxide dismutase and 2 × 10⁻⁶ M catalase by the NMR method (9). The rates of oxidation of ascorbate in the presence of superoxide dismutase (Rₐ₈ - superoxide dismutase) and in the absence of the enzyme (Rₐ₈) have been obtained by spectrophotometric measurements at 265 nm. The experiments have been made in duplicate.

<table>
<thead>
<tr>
<th>AH⁻</th>
<th>Catalyst</th>
<th>Rₒ</th>
<th>Rₒ/Rₐ₈</th>
<th>Rₐ₈/superoxide dismutase</th>
</tr>
</thead>
<tbody>
<tr>
<td>× 10⁻⁹ M</td>
<td>Type</td>
<td>× 10⁻⁹ M</td>
<td>s⁻¹</td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>Cu²⁺-(His)₂</td>
<td>0.8</td>
<td>0.50</td>
<td>0.98</td>
</tr>
<tr>
<td>7.6</td>
<td>Fe³⁺-EDTA</td>
<td>3.0</td>
<td>4.9</td>
<td>1.01</td>
</tr>
<tr>
<td>7.6</td>
<td>Fe³⁺-ADP</td>
<td>3.0</td>
<td>5.4</td>
<td>1.03</td>
</tr>
</tbody>
</table>

(9) the rate of approach of the enzyme to steady state condition characterized by a ratio [Cu²⁺]/[Cu⁺] ~ 1 (12). These measurements have been carried out in the presence of 0.5 M KF which is practically without effect on the oxidation rate of ascorbate. The rate of O₂⁻ generation measured for different types of catalysts are reported in Table II together with the corresponding rates of ascorbate oxidation. From these data, it appears that one superoxide ion is generated for each oxidized molecule of ascorbate. It must be remarked that in the same experimental conditions of Table II, but in absence of oxygen, the rate of reduction of Cu²⁺ present in the native superoxide dismutase is orders of magnitude lower than the rate of approach to the enzymic steady state in the presence of O₂, and therefore a possible reduction of superoxide dismutase by single components of the reaction systems is ruled out.

DISCUSSION

The production of one molecule of O₂⁻ for each molecule of oxidized ascorbic acid and the halving of the oxidation rate in the presence of 10⁻⁷ M superoxide dismutase clearly indicates that O₂⁻ is not a by-product of the oxidation process but it is directly involved in the oxidation of the ascorbate.

These results obtained in the presence of different catalysts appear quite general since the superoxide dismutase effect on the oxidation rates was still observed in highly purified systems where the direct measurement of O₂⁻ production was not possible because of its low value.

This behavior suggests the following reaction scheme for the oxidation of AH⁻ to dehydroascorbic acid (A) in the presence of catalase:

AH⁻ + O₂⁻ → k₁ₐ₈ AH⁻ + O₂⁻ (rate determining step) (1)

AH⁻ + O₂⁻ → k₂ AH + H₂O₂ (2)

AH⁻ + O₂⁻ → k₃ AH⁻ + H₂O₂ (3)

2AH⁻ → k₄ AH⁻ + A + H₂O₂ (4)

The occurrence of the Reactions 2 and 3 will depend on the competition between AH⁻ and AH⁻ for superoxide ion. However, in both cases, the overall reaction is the same, that is

AH⁻ + O₂⁻ → AH⁻ + H₂O₂.

The addition of superoxide dismutase at concentrations such as to compete efficaciously with AH⁻ and/or AH⁻ for O₂⁻, according to the equation

O₂⁻ → k₅ SOD, H₂O₂ 1/2 O₂ + 1/2 H₂O₂ h₁ = 2.3 × 10⁶ m⁻¹ s⁻¹, (5)

where SOD is superoxide dismutase, halves the rate of ascorbate oxidation (see Refs. 5 and 6).

Taking into account the Reactions 1-5 and considering constant the concentration of AH⁻ radicals, it results:

\[
\frac{Rₐ₈ + SOD}{Rₐ₈} = 1 + \frac{k₅[AH⁻] + k₅[AH⁻]}{k₅[AH⁻] + k₅[SOD]} + 0.5
\]

This equation could be rearranged in the form

\[
\frac{1}{2 \frac{Rₐ₈ + SOD}{Rₐ₈} - 1} = \frac{k₅[SOD]}{k₅[AH⁻] + k₅[AH⁻]}
\]

As in the first approximation, the concentration of AH⁻ could be considered constant, the left hand side of Equation 6 gives a linear function of superoxide dismutase concentration. This equation fits the experimental results of Fig. 1 with a correlation coefficient of 0.987. From the slope of the straight line, a value of 22 s⁻¹ has been calculated for k₅[AH⁻] + k₅[AH⁻].

Since the k₅ values reported in different papers range from 5.7 × 10⁵ m⁻¹ s⁻¹ to 2.7 × 10⁵ m⁻¹ s⁻¹ (13-15) an upper limit of 2.3 × 10⁵ m⁻¹ s⁻¹ can be calculated for k₅.
complete inhibition of the oxidation of ascorbic acid.

However, these authors report a rate of ascorbate oxidation in absence of catalysts of $2 \times 10^3 \text{ M s}^{-1}$, which is about two orders of magnitude higher than the rate we have measured in purified systems in similar conditions. This large difference is due probably to the presence of traces of metal ions. The specific binding of these ions to superoxide dismutase may bring the dramatic decrease of the oxidation rate observed by Puget and Michelson in the presence of this enzyme. According to this hypothesis, we have measured a strong decrease of the oxidation rate when apsuperoxide dismutase was added to unpurified systems in absence of chelating molecules. On the contrary, Halliwell and Foyer infer that, from the slight effect of superoxide dismutase on the rate of ascorbate oxidation, no evidence could be found for the production of the superoxide radical during autoxidation of ascorbic acid at alkaline pH values. However, from a calculation, it appears that the concentrations of superoxide dismutase used by these authors are lower than $10^{-7} \text{ M}$, which, according to the data we have reported in Fig. 1, are not sufficient to halve the rate of ascorbic oxidation.

Though the detailed mechanism of ascorbate oxidation by molecular oxygen appears complicated, our data indicate quite clearly that one of the products of the $\text{AH}^-$ oxidation is $\text{O}_2^-$ which reacts with the ascorbate ion or its radical doubling the oxidation rate. Therefore, in absence of an efficient $\text{O}_2^-$ scavenger, the ascorbate system itself acts as a sink for superoxide.

Furthermore, it appears that superoxide dismutase at micromolar concentration, which is a typical cellular concentration of this enzyme, competes with the ascorbate system for the $\text{O}_2^-$ halving the autoxidation rate of the ascorbic acid.

REFERENCES

Superoxide ion as active intermediate in the autoxidation of ascorbate by molecular oxygen. Effect of superoxide dismutase.
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