Rapid Papers

Biochem. J. (1976) **155**, 697–700
Printed in Great Britain

Ascorbic Acid, Metal Ions and the Superoxide Radical

By BARRY HALLIWELL and CHRISTINE H. FOYER
Department of Biochemistry, King's College, Strand, London WC2R 2LS, U.K.

(*Received* 3 *February* 1976)

1. No evidence could be found for production of the superoxide radical, $O_2^{\cdot-}$, during autoxidation of ascorbic acid at alkaline pH values. Indeed, ascorbate may be important in protection against $O_2^{\cdot-}$ generated *in vivo*. 2. Oxidation of ascorbate at pH10.2 was stimulated by metal ions. Stimulation by Fe^{2+} was abolished by superoxide dismutase, probably because of generation of $O_2^{\cdot-}$ during reduction of O_2 by Fe^{2+}, followed by reaction of $O_2^{\cdot-}$ with ascorbate. EDTA changed the mechanism of Fe^{2+}-stimulated ascorbate oxidation. 3. Stimulation of ascorbate oxidation by Cu^{2+} was also decreased by superoxide dismutase, but this appears to be an artifact, since apoenzyme or bovine serum albumin showed similar effects.

The superoxide radical, $O_2^{\cdot-}$, is a highly toxic species that is formed in living organisms (for reviews see Halliwell, 1974; Fridovich, 1975). $O_2^{\cdot-}$ can be produced by autoxidation of some naturally occurring compounds, such as thiols, oxyhaemoglobin, ferredoxins, adrenaline and tetrahydropteridines (Fridovich, 1975). $O_2^{\cdot-}$ generated in this and other ways can be removed *in vivo* by the enzyme superoxide dismutase (superoxide–superoxide oxidoreductase, EC 1.15.1.1), which catalyses the reaction given in eqn. (1):

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

An ancillary protective mechanism in many tissues may be ascorbic acid, which can react with $O_2^{\cdot-}$ (Allen & Hall, 1973; Nishikimi, 1975), as shown in eqn. (2):

$$\text{Ascorbate} + 2H^+ + 2O_2^{\cdot-} \rightarrow 2H_2O_2 + \text{dehydroascorbate} \quad (2)$$

However, ascorbate itself autoxidizes at pH7 or above, especially if metal ions are added. Puget & Michelson (1974) reported that autoxidation of ascorbate at pH8.8 was completely inhibited by superoxide dismutase, suggesting that $O_2^{\cdot-}$ is produced during this reaction. Recent studies (Hirata & Hayaishi, 1975) of the cofactor requirements of indolylamine 2,3-dioxygenase also suggest that ascorbate can generate $O_2^{\cdot-}$. Because of our interest in the role of ascorbate in plant metabolism, we have investigated this possibility further.

Experimental

Materials

Ascorbic acid of AnalaR quality was from BDH Chemicals, Poole, Dorset, U.K. All other reagents were of the highest quality available from BDH or from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Superoxide dismutase (Sigma) was assayed by the method of McCord & Fridovich (1969); 1 unit is defined as that amount which causes a 50% decrease in the rate of cytochrome *c* reduction. The apoenzyme was obtained by dialysing superoxide dismutase against EDTA in 50mM-sodium acetate buffer, pH3.8, for 24h (McCord & Fridovich, 1969), followed by dialysis against phosphate buffer (10mM-KH_2PO_4 adjusted to pH7.2 with KOH) for 24h to remove EDTA. The product had lost 70% of its original enzyme activity. Before use, dialysis tubing had been freed from metal ions by the method of Dalziel & Egan (1972).

Methods

Oxidation of ascorbate was followed by the decrease in E_{265} (Puget & Michelson, 1974) in a final reaction volume of 3.0ml. The same procedure was used to start the reaction in all cases; to an aqueous solution containing any metal ion, EDTA and superoxide dismutase or other protein required in the reaction mixture was added buffer, followed immediately by ascorbic acid (0.15μmol). The buffers used were Na_2CO_3 (300μmol, adjusted to pH10.2 with HCl) or NaH_2PO_4 (300μmol, adjusted to pH8.8 with NaOH). No significant pH change was observed on adding ascorbic acid to either system.

Oxidation of adrenaline was followed by the rise in E_{480} (see Misra & Fridovich, 1972) when adrenaline was included in these reaction mixtures at a final concentration of 1.6mM (in assays at pH8.8) or 0.4mM (at pH10.2). The rate of oxidation of adrenaline was followed both in the absence and in the presence of ascorbate and/or dehydroascorbate.

Reduction of Nitro Blue Tetrazolium was followed by the rise in E_{560} when it was included in these reaction mixtures at a final concentration of 25μM. There was no reduction unless ascorbate was added.

In all experiments, double-distilled water was used to minimize contamination by metal ions. Cuvettes were soaked in dil. HCl between experiments and rinsed immediately before use. $FeSO_4$ was dissolved in water saturated with N_2 at atmospheric pressure, immediately before use.

Results

The rate of autoxidation of freshly prepared solutions of ascorbic acid at pH 8.8 was low. In agreement with the results of Puget & Michelson (1974), it was decreased by addition of superoxide dismutase, although only partial inhibition could be achieved (Table 1). However, exactly the same degree of inhibition was obtained by addition of the apoenzyme of superoxide dismutase or bovine serum albumin. Inhibition cannot therefore be attributed to removal of $O_2^{\cdot-}$, but seems to be a non-specific effect of proteins. Similar results were obtained in studies of the much faster rate of ascorbate autoxidation at pH 10.2 (Table 1).

$O_2^{\cdot-}$ can be detected by its ability to oxidize adrenaline (Misra & Fridovich, 1972) or to reduce Nitro Blue Tetrazolium (Beauchamp & Fridovich, 1971). Neither ascorbate (0.05–0.2 mM) nor dehydroascorbate (up to 0.4 mM) nor a mixture of the two, increased the rate of adrenaline oxidation at pH 8.8 or 10.2 (see the Experimental section). In fact, ascorbate inhibited the oxidation, possibly by reaction with $O_2^{\cdot-}$, which is an intermediate in adrenaline oxidation (Misra & Fridovich, 1972). Dehydroascorbate (0.05–0.2 mM) did not reduce Nitro Blue Tetrazolium at either pH value. Although ascorbate (0.05 mM) reduced Nitro Blue Tetrazolium at both pH values, and this reaction was inhibited by 65% on addition of superoxide dismutase (50 units), it was found that an equal quantity of the apoenzyme or bovine serum albumin (10 μg) inhibited to exactly the same extent.

Table 1. *Effect of proteins on the oxidation of ascorbic acid*

Ascorbate oxidation was followed by the decrease in E_{265} as described in the Experimental section. The rate of fall in absorbance was constant for at least 8 min. The bovine serum albumin used contained no detectable superoxide dismutase activity when assayed by the method of Beauchamp & Fridovich (1971). The superoxide dismutase solution contained 1 unit of enzyme activity/μl: the apoenzyme contained the same protein concentration, but only 0.3 unit of activity/μl (see the Experimental section).

Reagent added	Rate of ascorbate oxidation (ΔE_{265}/min) pH 8.8	pH 10.2
None	0.005	0.037
Superoxide dismutase		
20 μl	0.004	0.033
50 μl	0.002	0.030
100 μl	0.002	0.028
Dismutase apoenzyme		
20 μl	0.002	0.030
50 μl	0.002	0.029
Bovine serum albumin		
10 μg	0.002	0.031
100 μg	0.002	0.028

Effect of metal ions

Metal ions are known to increase the rate of ascorbate oxidation. Table 2 shows that Fe^{2+} had a striking stimulatory effect at pH 10.2. This was abolished by adding superoxide dismutase. In contrast, bovine serum albumin had little effect on Fe^{2+}-stimulated ascorbate oxidation, and the apoenzyme of super-

Table 2. *Effect of iron salts on the oxidation of ascorbic acid at pH 10.2*

Ascorbate oxidation was followed by the decrease in E_{265} as described in the Experimental section; initial rates of absorbance change are presented below. Solutions of iron salts were made up immediately before use. The quantities of reagents added were: EDTA (0.3 μmol); $FeSO_4$ (0.1 μmol); $FeCl_3$ (0.1 μmol); H_2O_2 (0.1 μmol). The superoxide dismutase solution contained 1 unit of enzyme activity/μl; the apoenzyme contained the same protein concentration, but only 0.3 unit of enzyme activity/μl (see the Experimental section).

Reagents added	Rate of ascorbate oxidation (ΔE_{265}/min)
None	0.029
EDTA	0.027
Superoxide dismutase (50 μl)	0.027
H_2O_2	0.038
$FeSO_4$	0.118
$FeSO_4$+superoxide dismutase	
20 μl	0.074
50 μl	0.026
100 μl	0.025
$FeSO_4$+apoenzyme	
20 μl	0.113
50 μl	0.081
100 μl	0.034
150 μl	0.027
$FeSO_4$+bovine serum albumin (100 μg)	0.112
$FeSO_4$+EDTA	0.103
$FeSO_4$+EDTA+superoxide dismutase (50 μl)	0.090
$FeCl_3$	0.033
$FeCl_3$+EDTA	0.095
$FeCl_3$+EDTA+superoxide dismutase (50 μl)	0.081

oxide dismutase inhibited by only about one-third as much as the holoenzyme, corresponding to its residual enzyme activity (Table 2). It thus seems that Fe^{2+}-stimulated oxidation requires $O_2^{\cdot-}$.

EDTA (0.3 μmol) slightly inhibited ascorbate oxidation in the absence of added metal ion: it also slightly inhibited Fe^{2+}-stimulated oxidation. However, oxidation in the presence of Fe^{2+} and EDTA was almost unaffected by superoxide dismutase (Table 2). Fe^{3+} (100nmol) alone did not increase the rate of ascorbate oxidation, but in the presence of EDTA (0.3 μmol) a marked increase was seen and superoxide dismutase had little effect (Table 2).

Zn^{2+} (100nmol) or Mn^{2+} (100nmol) also increased ascorbate oxidation at pH10.2, but these increases were unaffected by superoxide dismutase (50 units). As expected, Cu^{2+} was a very powerful catalyst of ascorbate oxidation; addition of 50 units of dismutase decreased the rate in the presence of Cu^{2+} (5.0nmol) by 50%. Since the same effect was observed with apoenzyme or bovine serum albumin, it cannot be concluded that $O_2^{\cdot-}$ is involved in Cu^{2+}-catalysed oxidation.

Neither Fe^{2+} (100nmol) nor Fe^{3+} (100nmol) increased ascorbate oxidation at pH8.8.

Discussion

No evidence could be found for production of $O_2^{\cdot-}$ during the autoxidation of ascorbate at alkaline pH values, and consideration of redox potentials rules out the possibility of $O_2^{\cdot-}$ production at pH7 (Nishikimi, 1975). Previous reports of inhibition of autoxidation by superoxide dismutase were probably due to inhibition by the protein rather than removal of $O_2^{\cdot-}$. Why should proteins decrease the rate of ascorbate oxidation? There is evidence that an intermediate formed during oxidation can react with proteins (Orr, 1967*a*,*b*). Also, the capacity of proteins to bind some metal ions, e.g. Cu^{2+} (Brigelius *et al.*, 1974), might contribute to the decreased oxidation rate, since EDTA had a slight inhibitory effect (Table 2).

Fe^{2+} increased oxidation at pH10.2, but not at pH8.8. This can be explained by the observation by Michelson (1973) that Fe^{2+} reduces O_2 to $O_2^{\cdot-}$ at highly alkaline pH values (eqn. 3):

$$Fe^{2+} + O_2 \rightarrow O_2^{\cdot-} + Fe^{3+} \quad (3)$$

$O_2^{\cdot-}$ can then react with ascorbate (eqn. 2), whereas the Fe^{3+} produced does not itself affect oxidation (Table 2). Hence superoxide dismutase inhibits Fe^{2+}-stimulated oxidation.

Addition of EDTA changes the mechanism of Fe^{2+}-stimulated ascorbate oxidation. Halliwell (1975) proposed that Fe^{2+}–EDTA, but not Fe^{2+}, could react rapidly with $O_2^{\cdot-}$ (eqn. 4):

$$Fe^{2+}\text{–EDTA} + O_2^{\cdot-} + 2H^+ \rightarrow Fe^{3+}\text{–EDTA} + H_2O_2 \quad (4)$$

Hence any $O_2^{\cdot-}$ produced during oxidation of Fe^{2+}–EDTA should be removed by this reaction. The results in Table 2 suggest that it is mainly the Fe^{3+}–EDTA that now oxidizes ascorbate (eqn. 5):

$$2Fe^{3+}\text{–EDTA} + \text{ascorbate} \rightarrow \text{dehydroascorbate} + 2H^+ + 2Fe^{2+}\text{–EDTA} \quad (5)$$

In agreement with this proposal, the redox potential of Fe^{3+} is increased when it complexes with EDTA (Noguchi & Nakano, 1974). Since $O_2^{\cdot-}$ is no longer an essential intermediate, superoxide dismutase does not inhibit significantly.

At pH8.8, where Fe^{2+} reduces O_2 to $O_2^{\cdot-}$ far more slowly (Michelson, 1973) and the rate of non-enzymic $O_2^{\cdot-}$ breakdown, which is dependent on pH, is much faster, Fe^{2+} does not increase ascorbate oxidation.

The mechanism of these reactions may well be far more complicated than this, since we have not taken into account possible interactions of iron salts with H_2O_2 generated during ascorbate oxidation. However, the above proposals seem to account reasonably well for the observations made.

Thus ascorbate does not produce $O_2^{\cdot-}$ and probably helps to protect against it. Unfortunately, other oxidation products of ascorbate are themselves damaging to proteins (see above). Animal cells contain an NADH-dependent semidehydroascorbate reductase (Schulze *et al.*, 1970), and plant cells possess a reduced-glutathione-dependent dehydroascorbate reductase (Mapson, 1958), presumably to help remove such dangerous oxidation products and to regenerate ascorbate.

C. H. F. is very grateful to the Science Research Council for a Research Studentship.

References

Allen, J. F. & Hall, D. O. (1973) *Biochem. Biophys. Res. Commun.* **52**, 856–862
Beauchamp, C. & Fridovich, I. (1971) *Anal. Biochem.* **44**, 276–287
Brigelius, R., Spottl, R., Bors, W., Lengfelder, E., Saran, M. & Weser, U. (1974) *FEBS Lett.* **47**, 72–75
Dalziel, K. & Egan, R. R. (1972) *Biochem. J.* **126**, 975–984
Fridovich, I. (1975) *Annu. Rev. Biochem.* **44**, 147–159
Halliwell, B. (1974) *New Phytol.* **73**, 1075–1086
Halliwell, B. (1975) *FEBS Lett.* **56**, 34–38
Hirata, F. & Hayaishi, O. (1975) *J. Biol. Chem.* **250**, 5960–5966
Mapson, L. W. (1958) *Annu. Rev. Plant Physiol.* **9**, 119–150
McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055
Michelson, A. M. (1973) *Biochimie* **55**, 465–479

Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.* **247**, 3170–3175
Nishikimi, M. (1975) *Biochem. Biophys. Res. Commun.* **63**, 463–468
Noguchi, T. & Nakano, M. (1974) *Biochim. Biophys. Acta* **368**, 446–455
Orr, C. M. W. (1967*a*) *Biochemistry* **6**, 2995–3000
Orr, C. M. W. (1967*b*) *Biochemistry* **6**, 3000–3006
Puget, K. & Michelson, A. M. (1974) *Biochimie* **56**, 1255–1267
Schulze, H. U., Gallenkamp, H. & Staudinger, H. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 809–817