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BACTERIAL ASCORBIC ACID OXIDASE

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SUMMARY

An ascorbic acid oxidase, somewhat similar to that of plants, has been identified in a strain of *Aerobacter aerogenes*.

Only L-ascorbate and D-isoascorbate were oxidized by the enzyme; catechol, phenol, *p*-cresol, pyrogallol, cysteine, reduced glutathione, hydroquinone, and *p*-phenylenediamine were not oxidized.

It was not inhibited by 50% of CO showing that the oxidation of L-ascorbate is not due to either phenol oxidase or cytochrome oxidase.

The oxidase has maximum activity at pH 6.3; K_m for L-ascorbic acid was $2.36 \cdot 10^{-2}$ M.

INTRODUCTION

It is known that L-ascorbic acid may be used as a carbon source by a variety of bacteria[1-3]. In plants ascorbic acid is oxidized to dehydroascorbic acid by a specific ascorbic acid oxidase. Cytochrome oxidases from a variety of sources, and phenol oxidases from plants, also carry out the same reaction[4].

This paper is concerned with a study of the mechanism of this oxidation by a strain of *Aerobacter aerogenes*. The results show that L-ascorbic acid is oxidized to dehydroascorbic acid by a bacterial ascorbic acid oxidase similar to that occurring in plants.

MATERIALS AND METHODS

Preparation of enzyme

The strain of *A. aerogenes* used was isolated by enrichment culture techniques and grown in Fernbach flasks each containing 2000 ml of medium as previously described[3]. After 24 h of growth, the organisms were collected by continuous centrifugation and washed 3 times with water. The washed organisms were suspended in 0.02 M phosphate buffer (pH 6.5) and disrupted in a 10 kc Raytheon sonic oscillator. Breakage was essentially complete after 10 min. The broken cells were centrifuged at 30 000 × *g* for 15 min, and the small amount of cells and cellular debris which sedimented was discarded. The supernatant solution was then centrifuged at

81 000 × *g* for 30 min in a Spinco Model L centrifuge. The resulting particulate material which sedimented contained almost all of the ascorbic acid oxidase activity. This particulate fraction was washed 3 or 4 times with 0.02 M phosphate buffer (pH 6.5) at which time the wash was essentially free of protein. The particulate enzyme was then suspended in 0.02 M phosphate buffer (pH 6.5) so that 0.2 ml of enzyme suspension would cause the oxidation of approx. 6 μmoles of ascorbate in 20 min under the conditions of the assay. The cells obtained from 13 l of growth medium yielded from 75 to 100 ml of enzyme suspension after dilution of the particulate material to the desired activity.

Enzyme assay

Ascorbic acid oxidase activity was measured at 37° in Warburg vessels of about 5 ml total volume. Each assay flask contained 20 μmoles of phosphate buffer (pH 6.5) 40 μmoles of ascorbate, and enzyme, in a total volume of 0.6 ml. The rate of oxygen uptake remained linear between 5 and 25 min.

In the early phase of this work KOH was added to the center well of the assay flasks. However, it was subsequently found that no CO_2 was evolved, and KOH was therefore no longer used.

RESULTS

Product of the enzymic oxidation of L-ascorbic acid

During the enzymic oxidation of L-ascorbate, 17.9 μmoles of oxygen were taken up and 34.6 μmoles of dehydro-L-ascorbate were formed. Dehydro-L-ascorbate was estimated by the specific enzymic reduction of its hydrolysis product, 2,3 diketo-L-gulonate, to β-keto-L-gulonate[3], followed by decarboxylation to form L-xylulose which was estimated by the cysteine carbazole reaction[5]. A control flask using heated enzyme took up 1.6 μmoles of oxygen during the same period.

Specificity

Of the substrates tested only L-ascorbic acid and D-isoascorbic acid were oxidized by the bacterial enzyme. With D-isoascorbate, 56 μl of oxygen were used compared to 65 μl of oxygen for L-ascorbate in the same period of time. This is similar to the results of DODDS[6] who found that plant ascorbic acid oxidase from cucumber juice oxidized D-isoascorbic acid at 85% of the rate of L-ascorbic acid. The following were not oxidized by the bacterial oxidase: D-glucose, D-fructose, catechol, phenol, *p*-cresol, pyrogallol, cysteine, reduced glutathione, hydroquinone, and *p*-phenylenediamine.

Effect of substrate concentration

Fig. 1 shows the LINEWEAVER–BURK plot from which the K_m for L-ascorbic acid is $2.36 \cdot 10^{-3}$ M. The reaction was carried out with 100% oxygen so that the concentration of ascorbic acid in the Warburg flask would be rate-limiting. Under these conditions there is a considerable oxygen uptake as a result of the auto-

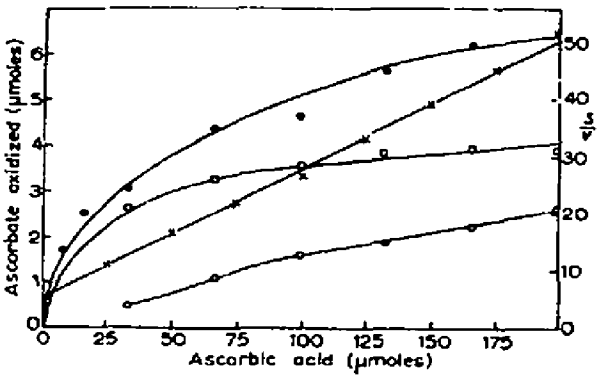

Fig. 1. Reaction velocity as a function of L-ascorbate concentration (see text for details). ●—●, total ascorbate oxidized; ○—○, ascorbate oxidized by autooxidation; □—□, enzymically oxidized ascorbate; ×—×, LINEWEAVER–BURK plot of reciprocal of reaction velocity.

oxidation of ascorbic acid which was subtracted from the total oxygen consumed in order to obtain a true enzymic rate.

Oxygen affinity

THIMANN *et al.*[7] compared the rates of oxidation by yeast cytochrome oxidase and purified plant ascorbic acid oxidase under different partial pressures of oxygen. Their values show that cytochrome oxidase will give half maximum activity at 0.2–0.5% of oxygen, while plant ascorbic acid oxidase required 16% of oxygen for half maximum activity.

To determine the oxygen affinity of the bacterial oxidase, assays were set up which contained 0.1 ml of 0.1 M phosphate buffer (pH 6.5); 0.1 ml of enzyme; and 0.05 ml of 0.5 M L-ascorbate (pH 6.5). The liquid volume was kept very small so as

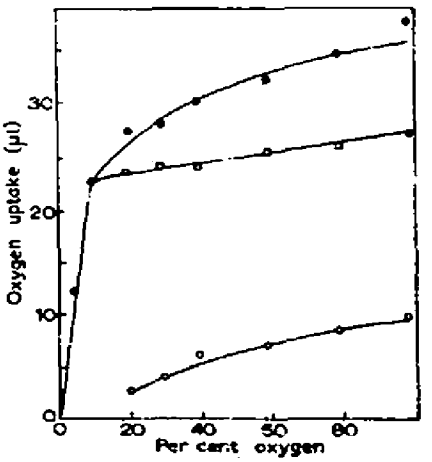

Fig. 2. Reaction velocity as a function of oxygen concentration. ●—●, total oxygen consumed; ○—○, oxygen consumed by the autooxidation of L-ascorbate; □—□, oxygen consumed by enzymic reaction.

to permit a rapid equilibration with the gases in the atmosphere. Each 5-ml Warburg flask was flushed with 1 l of the indicated nitrogen–oxygen mixture. The results are shown in Fig. 2. The concentration of oxygen necessary to give half the maximum rate is approx. 5%.

Inhibitors

A number of selective inhibitors have been used to study various terminal oxidases. Table I lists our results for a number of compounds which are reported to inhibit plant ascorbic acid oxidases, presumably by complexing with the copper of the enzyme[9]. In addition, *p*-chloromercuribenzoate (0.5 μmole/0.6 ml assay volume)

TABLE I

Inhibitor	Range used ($M \times 10^3$)	50% inhibition ($M \times 10^3$)	Concentration giving 95% inhibition ($M \times 10^3$)
Cyanide	0.8 – 33	0.65	8.0
Potassium ethylxanthate	0.8 – 33	0.80	6.5
Sodium sulfide	0.8 – 13	0.8	5.0
Pyridine-KCNS	17 – 500	250*	500
p-Nitrophenol	0.8 – 30	18**	26.7

* Showed 28% stimulation at $80 \cdot 10^{-3}$ M.
** Showed 36% stimulation at $6.5 \cdot 10^{-3}$ M.

resulted in a 50% inhibition of oxidase activity which was restored to 90% of the original activity by the subsequent addition of 5 μmoles of reduced glutathione, but there was no inhibition by iodoacetate (2 μmoles per assay flask).

Gas mixtures containing CO–O_2 (1 : 1) yielded essentially the same activity (72 μl O_2 uptake) as did N_2–O_2 (1 : 1) (73 μl O_2 uptake). This lack of inhibition by CO was the same whether the reaction was carried out in total darkness or whether normal laboratory light was present.

pH optimum

A composite buffer was prepared by mixing equal volumes of 0.21 M concentrations of each of the following constituents: potassium acetate, potassium acid phosphate, and Tris. The pH was adjusted as desired by the addition of either HCl or KOH. Enzyme activities were determined using the standard Warburg assay. At the end of each assay the flask contents were collected and the pH again determined. Maximum activity was obtained at a pH of 6.3, and 58% and 52% of the activity at pH 5.5 and 7.5 respectively.

Occurrence of bacterial oxidase

The oxidase was present in the strain of *A. aerogenes* used when grown with either glucose or ascorbate as a carbon source, whether under aerobic or anaerobic

conditions. The following organisms taken from the departmental culture collection were, however, all unable to oxidize ascorbic acid: *A. aerogenes*, *Escherichia coli*, *Bacillus megaterium*, *Micrococcus lysodeicticus*, *Propionibacterium pentosaceum*, and *Gaffkya tetragena*.

DISCUSSION

These results show that the bacterial enzyme described here is a specific ascorbic acid oxidase which appears to have a limited substrate specificity. The insensitivity to CO as well as the low oxygen affinity serve to differentiate this enzyme from cytochrome oxidase and phenol oxidase.

The affinity of the bacterial oxidase for oxygen appears to be considerably higher than that reported by THIMANN *et al.*[7] for plant ascorbic acid oxidase. However, the latter report states that a 5.0-ml assay volume was used to determine oxygen affinity which would, on the basis of our results, be difficult to keep equilibrated with the gaseous atmosphere except with very low enzyme activities. This may account for the rather low oxygen affinity reported for plant ascorbic acid oxidase.

One major difference between the bacterial oxidase and typical plant ascorbic acid oxidase is the ease with which the latter enzyme is brought into solution. However, there have been a number of reports indicating that in some plants the ascorbic acid oxidase activity is associated with a particulate fraction[9–11]. Because of the particulate nature of the bacterial oxidase, it has not been possible to separate the activity from catalase activity, and hence, it is not yet known whether H_2O_2 is the end product as with plant ascorbic acid oxidase[12], or whether it is a copper-containing enzyme.

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