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INDUCTION OF CATALASE IN *ESCHERICHIA COLI* BY ASCORBIC ACID INVOLVES HYDROGEN PEROXIDE

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Summary

Ascorbic acid at concentrations between 0.57 and 5.7 mM in aerated medium caused an eight fold increase in catalase activity in *Escherchia coli*. The hydrogen peroxide concentrations resulting from ascorbate oxidation were between 20 and 120 μM and hydrogen peroxide by itself caused a similar increase in catalase levels in both aerobic and anaerobic media. Three catalase activity bands visualized on polyacrylamide gels were increased. Chloramphenicol which inhibits protein synthesis, anaerobic medium and EDTA, which prevent ascorbate oxidation, and exogenous catalase, which removes hydrogen peroxide from the medium, all prevented the increase in catalase in response to ascorbate. Superoxide dismutase activity was not affected by ascorbate.

Introduction

Ascorbic acid reacts with oxygen in the presence of metal ions to produce dehydroascorbate and H_2O_2 (1,2,3). Inhibition of this oxidation by superoxide dismutase was interpreted to mean that superoxide ion was also a product of the oxidation (4) but the inhibition was subsequently attributed to a general protein effect rather than the involvement of superoxide ion (2). Free radical intermediates from the oxidation of ascorbate have been implicated in the inhibition of catalase by ascorbate (5) and hydroxide radicals have been implicated in the cleavage of DNA by ascorbate (3,6,7). *Escherichia coli* has responded to the presence of both extra- and intracellular H_2O_2 with the production of catalase (8,9) and to the presence of intracellular superoxide ion by producing superoxide dismutase (10,11,12). This report shows that H_2O_2 generated from ascorbate oxidation is responsible

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for the induction of catalase synthesis in *E. coli* following the addition of ascorbate to aerated medium.

Methods

All biochemicals and enzymes were obtained from Sigma while yeast extract and bactotryptone were obtained from Difco. All experiments employed *Escherichia coli* B23 grown at 37°C on a shaker bed in LB medium (10 g bactotryptone, 5 g yeast extract and 5 g NaCl in 1 L of H_2O (13)) following growth with a Klett-Summerson colorimeter using a blue filter (100 Klett units represented 0.14 mg/ml dry cell weight as determined by weighing culture aliquots after drying at 100°C and correcting for medium weight). Anaerobic cultures were grown in sealed flasks flushed with nitrogen prior to use. Ascorbic acid was dissolved in water just prior to use, flushed with nitrogen for anaerobic experiments and added to cultures at a cell density of 70 Klett units. H_2O_2 was assayed using the procedure of Hildebrandt *et al* (14). Because unreacted ascorbate interfered with the assay, it was removed by Norite adsorption prior to the addition of ferroammonium sulfate and KSCN. Superoxide dismutase was assayed according to Buchanan and Lees (15) using sonicated cell extracts and acetylated cytochrome *c* (16). One unit of superoxide dismutase is defined as the amount of enzyme which causes a 50% inhibition in the rate of reduction of acetylated cytochrome *c*. Catalase activity was assayed in chilled culture aliquots without sonication according to Rørth and Jensen (17). One unit of catalase is defined as the amount of enzyme which breaks down 1 µmol of H_2O_2 per minute. Exogenous catalase was removed before the assay by three centrifugations and washings of the cell pellet which did not affect intracellular catalase levels. Enzyme activities are expressed as units per mg dry cell weight where the cell weight was determined from the Klett reading prior to isolation. Catalase and hydroperoxidase activities were visualized on polyacrylamide gels according to Gregory and Fridovich (18) modified to provide better resolution of the catalase-hydroperoxidase isoenzyme pair on 12% gels run as described by Davis (19) and better staining contrast with 3 mM H_2O_2. Protein concentrations were determined by the Lowry method (20). Carbohydrate transport into whole cells was assayed according to Groves and Gronlund (21).

Results and Discussion

Effect of ascorbic acid on catalase levels in *Escherichia coli*. *E. coli* responded to the addition of 5.7 mM ascorbic acid with an eight fold increase in catalase activity within thirty minutes (Figure 1). Chloramphenicol prevented this increase revealing that protein synthesis was necessary for the response. Within fifteen seconds after adding ascorbate the catalase activity had dropped by 50% and it took three or four minutes for the activity to return to normal, coincident with a pause in growth. Subsequent growth rates with and without ascorbate were identical. Because no catalase activity was found in the medium, this drop in activity was not the result of release into the medium, and the direct addition of H_2O_2 did not cause a similar drop

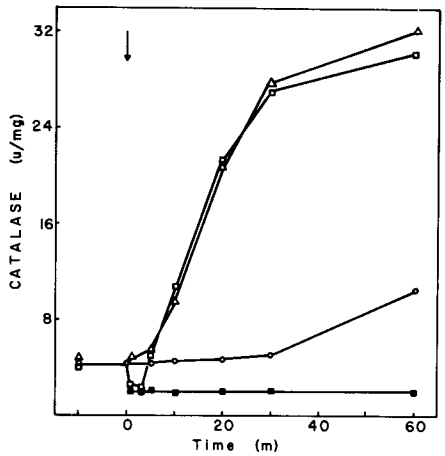

Fig. 1. Effect of ascorbic acid and H_2O_2 on catalase levels in *Escherichia coli* growing in aerated medium. At time 0 the following supplements were added to separate cultures: nothing (○), 5.7 mM ascorbic acid (□), 0.1 mM chloramphenicol with 5.7 mM ascorbic acid (■) and 0.75 mM H_2O_2 (△).

(Figure 1). Sonication of culture aliquots eliminated the drop in activity without affecting either basal or final activities and the addition of 5.7 mM ascorbate to sonicated cell extracts in LB medium did not affect catalase activity. Clearly an interaction between ascorbate or one of its oxidation products and whole cells is responsible for the inhibition of intracellular catalase but the nature of the interaction is unknown. Ascorbic acid was capable of inducing catalase synthesis over a broad range of concentrations with maximal induction occurring between 0.57 and 5.7 mM. The disappearance of catalase activity at 57 mM ascorbate coincided with cell death and a severe inhibition of endogenous catalase (Table 1).

Hydrogen peroxide from ascorbate oxidation induces catalase. It has been shown that catalase synthesis in *E. coli* increased in response to H_2O_2 generated *in situ* from pyocyanine (22) and to H_2O_2 added directly to the medium (8). Therefore, the well characterized oxidation of ascorbate to yield dehydroascorbate and H_2O_2 provided a clear rationale for the induction of catalase by ascorbate. To confirm that H_2O_2 was produced from ascorbate in aerated medium, the H_2O_2 concentrations were determined and found to be

Table 1. Effect of ascorbic acid concentration on catalase levels one hour after the addition of ascorbic acid to *Escherichia coli* in LB medium.

Ascorbic acid concentration (mM)	Catalase (u/mg dry cell weight)
0.000057	4.0
0.00057	4.8
0.0057	5.6
0.057	12.0
0.57	32.0
5.7	28.8
57	0

significantly lower than the ascorbate concentrations (Table 2) suggesting a slow rate of ascorbate oxidation in the growth medium. Despite the earlier report (8) which indicated that it was difficult to achieve direct H_2O_2 induction of catalase, a single addition of either 0.15 mM, 0.75 mM (Figure 1) or 3.3 mM H_2O_2 was sufficient to cause an induction of catalase synthesis with the rate of increase in catalase being the same as the rate of increase following ascorbate addition and very similar to the rate of β-galactosidase synthesis following isopropylthiogalactoside addition (23).

Conditions which removed H_2O_2 before it interacted with the cell or which prevented H_2O_2 formation also prevented catalase induction in response to ascorbate. Increasing concentrations of exogenous catalase added to the medium prior to ascorbate addition progressively reduced the extent of catalase induction until 50 µg/ml prevented any increase in catalase (Figure 2). Chelation of free metal ions by 1.25 mM EDTA also prevented any increase

Table 2. Hydrogen peroxide concentration five minutes after the addition of ascorbic acid to growth medium.

Ascorbic acid mM	H_2O_2 mM
0.057	0.019
0.28	0.037
0.57	0.052
2.8	0.118
5.7	0.122

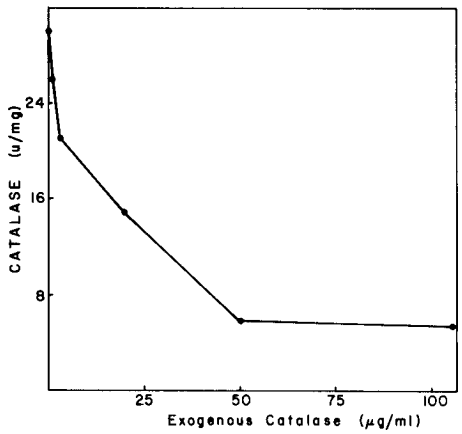

Fig. 2. Effect of exogenous catalase on the extent of catalase induction in response to 5.7 mM ascorbic acid. Catalase activity was assayed one hour after the addition of ascorbic acid to cultures in aerated medium containing different concentrations of bovine liver catalase.

in catalase activity. In anaerobic medium, 5.7 mM ascorbate had no effect on catalase levels whereas 0.75 mM H_2O_2 caused a four fold increase in catalase (Figure 3). This reduced capability for catalase synthesis under anaerobic conditions may reflect the link (9) between the synthesis of catalase and the synthesis of components of the aerobic electron transport chain for which there appears to be reduced synthetic capability under anaerobic conditions (24).

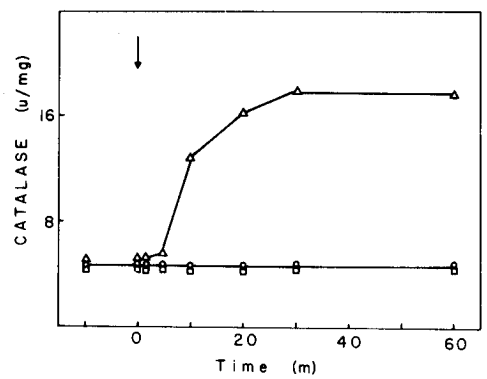

Fig. 3. Effect of ascorbic acid and H_2O_2 on catalase levels in *Escherichia coli* growing in anaerobic medium. At time 0, the following supplements were added to separate cultures: nothing (○), 5.7 mM ascorbic acid (□) and 0.75 mM H_2O_2 (△).

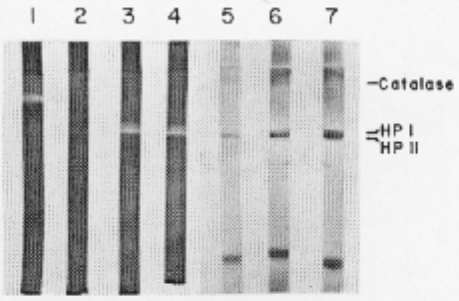

Fig. 4. Visualization of catalase and hydroperoxidase activities from sonicated cell extracts on 12% polyacrylamide gels before and 30 minutes after the addition of 5.7 mM ascorbic acid or 0.75 mM H_2O_2 to aerated cultures. Gels 1-4 were stained for catalase and gels 5-7 were stained for hydroperoxidase. Gel 1: 20 µg bovine liver catalase; Gels 2 and 5: 400 µg of protein from a culture without ascorbate or H_2O_2 added; Gels 3 and 6: 400 µg of protein from a culture with ascorbate added; Gels 4 and 7: 400 µg of protein from a culture with H_2O_2 added. HP; hydroperoxidase.

The catalase and hydroperoxidase activities in sonicated cell extracts were visualized on polyacrylamide gels before and after ascorbate and H_2O_2 addition and there was an identical increase in the predominant isoenzyme pair possessing both catalase and hydroperoxidase activities. A minor catalase activity, not possessing hydroperoxidase activity could also be seen to increase near the top of the gel although it is not as evident in the photograph. Attempts were made to separate the isoenzymes on 4% (25) and 7.5% (18) polyacrylamide gels but the catalase activity band migrated coincident with the bromophenol blue dye. Despite the difference in gel concentration the gel patterns in Figure 4 are similar to those in earlier reports (18,25) and illustrate the increase in catalase activity.

Effect of ascorbic acid on superoxide dismutase levels. The addition of 5.7 mM ascorbic acid to aerated cultures of *E. coli* did not cause any significant change in superoxide dismutase activity (Table 3); this could be interpreted to mean that no superoxide ion was formed during ascorbate oxidation. However, superoxide ion cannot pass through the membrane (26) and if the superoxide ion were formed in the medium, it could not enter the cell to induce superoxide dismutase synthesis. To determine where the oxidation of

Table 3. Superoxide dismutase activity in *Escherichia coli* with and without 5.7 mM ascorbic added at time 0.

Time (m)	Superoxide dismutase (u/mg dry cell weight) - ascorbate	+ ascorbate
-10	14.8	14.8
0	14.7	14.7
1	14.7	13.5
10	13.8	13.4
20	13.7	12.5
30	13.5	12.4
60	14.0	12.2

ascorbate occurred, the rates of uptake of 50 µM glucose, glycerol and ascorbate by whole cells were determined and found to be 350, 1.8 and 0 pmol per minute • mg dry cell weight respectively. Over the range of ascorbate concentrations from 10 µM to 1 mM no detectable uptake of ascorbate occurred and its oxidation must have occurred in the medium. While H_2O_2 can diffuse into the cell to induce catalase synthesis, superoxide ion cannot and, therefore, it is not possible to conclude from this data whether or not superoxide ion was formed during ascorbate oxidation.

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