Vol. 124, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1984 Pages 75-79

CU(II)-DEPENDENT INACTIVATION OF MN-CATALASE BY HYDROXYLAMINE

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Received August 17, 1984

SUMMARY: Hydroxylamine is a strong inhibitor of the Mn-catalase of *Lactobacillus plantarum* in the presence of hydrogen peroxide [Kono, Y., and Fridovich, I. (1983) J. Biol. Chem. 258, 13646-13648]. In the presence of $CuCl_2$ the Mn-catalase was rapidly inactivated by hydroxylamine without the addition of hydrogen peroxide. $FeSO_4$ and $MnCl_2$ were approximately 10% and 4% as effective as was $CuCl_2$. Under anaerobic conditions, the inactivation did not occur. The chelating agents such as EDTA and histidine completely prevent the inactivation. These results indicate that the hydrogen peroxide produced during the autooxidation of hydroxylamine catalyzed by $CuCl_2$ participates in the $CuCl_2$-dependent inactivation by hydroxylamine.

In contrast to heme-containing catalase, the Mn-catalase, which was recently isolated from *Lactobacillus plantarum* (1), is not inhibited by azide and cyanide, but is irreversibly inactivated when exposed to NH_2OH plus H_2O_2 (2, 3). We proposed that the action of NH_2OH might involve the production of Mn(IV) at the active site (3).

NH_2OH inhibits many enzymatic activities, such as catalase (4), ribulose-1,5-bisphosphate carboxylase (5), cytochrome *c* oxidase (6), urocanase (7), papain (8), urease (9) and alcohol dehydrogenase (10). Cu(II) and O_2 are required for the inactivations of ribonuclease (11) and carboxyesterase (12) by NH_2OH.

The autooxidation of NH_2OH is catalyzed by trace amounts of metal ions, profoundly by Cu(II) (13-15). This autooxidation is suppressed by chelators for Cu(II) or by removal of oxygen. The formation of O_2^- and H_2O_2 during the metal-catalyzed autooxidation of NH_2OH has been reported (16). I now find that the Mn-catalase is rapidly inactivated by NH_2OH in the presence of copper ions without the addition of H_2O_2. In this report

0006-291X/84 $1.50

I describe that the inactivation is dependent upon the H_2O_2 formed during the autooxidation of NH_2OH by copper ions.

MATERIALS AND METHODS

The Mn-catalase was isolated from *L. plantarum* and its concentration was estimated using an absorbance coefficient of 184 mM^{-1} cm^{-1} at 280 nm (1). The manganese-containing superoxide dismutase from *Escherichia coli* B was kindly provided by Dr. I. Fridovich (Duke University). Exposure of the Mn-catalase to NH_2OH plus $CuCl_2$ was performed in test tubes at 25°C with continual shaking at 100 strokes/min, while the incubation mixture was held to 0.1-0.2 ml to insure adequate aeration. Incubation mixture contained 10 mM Tris-HCl at pH 7.0, 0.256 μM Mn-catalase, 50 μM NH_2OH and 0.1 mM $CuCl_2$. At intervals, 10 μl of the incubation mixture was diluted 300-fold with the assay mixture of catalase. Catalase activity was measured as previously described (1), using a Shimadzu Multipurpose MPS-2000 spectrophotometer at 25°C. NH_2OH·HCl was obtained from Nakarai Chemical Co. (Kyoto, Japan). Aqueous solution of NH_2OH was prepared daily and kept in a tight stoppered test tube until use.

RESULTS AND DISCUSSION

When 0.256 μM Mn-catalase was incubated with 10 μM or 50 μM NH_2OH and 0.1 mM Cu(II) at pH 7, the enzymatic activity was rapidly lost in a time-dependent manner (Fig. 1A, lines 3 and 5). The inactivation of the Mn-catalase was first order with respect to the residual activity of Mn-catalase. The degree of inactivation and the protection against the inactivation were evaluated from the pseudo-first order rate constants

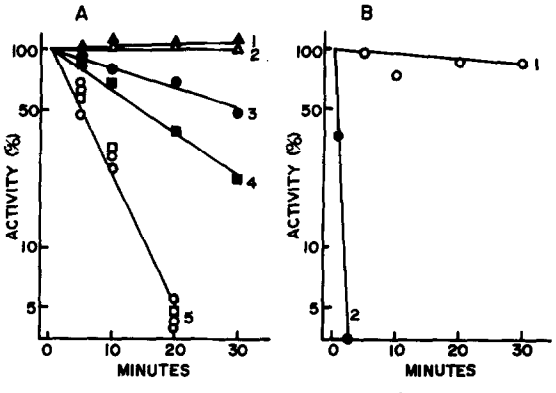

Fig. 1. Inactivation of the Mn-catalase by NH_2OH plus $CuCl_2$. The Mn-catalase was incubated aerobically (A) or anaerobically (B) in 10 mM and 50 mM (line 4 in A) Tris-HCl at pH 7 or 10 mM potassium phosphate at pH 7 (line 5 in A;□). Line 1 in A was obtained when $CuCl_2$ was omitted. Line 2 in A was obtained when NH_2OH was omitted. Line 3 in A was obtained when 10 μM NH_2OH was added. Line 5 in A (○) was control. Line 2 in B was obtained when 0.58 mM H_2O_2 was added.

for the inactivation. Under the same experimental conditions, neither NH_2OH nor Cu(II), when present alone, caused any significant decrease of the enzymatic activity (Fig. 1A, lines 1 and 2). Dialysis of inactivated enzyme against 3000 volumes of Tris buffer at pH 7 did not result in any recovery of the enzymatic activity. Fe(II) and Mn(II) also induced the inactivation by NH_2OH. $CuCl_2$ caused the highest rate of inactivation (k_{app}=0.15 min^{-1}); the rates of the inactivation caused by Fe(II) and Mn(II) were 10% and 4% of that by Cu(II), respectively (Fig. 2). The results are in accord with the ability of Cu(II), but not Fe(II) and Mn(II), to catalyze the autooxidation of NH_2OH (14-16).

In order to verify the need for O_2 in the inactivation of Mn-catalase, the reaction mixture was incubated under N_2. There was only a slight and gradual inactivation of the enzyme (Fig. 1B, line 1).

The effect of chelators, which can inhibit the metal-catalyzed autooxidation of NH_2OH (13-16), was investigated (Fig. 3). The addition of 5 mM EDTA completely suppressed the inactivation of Mn-catalase (line 1). The addition of 0.1 mM and 0.5 mM histidine, which forms a high affinity complex with copper ions (17), resulted in 69% and 98% protection of the enzymatic activity (lines 2 and 3). It has been reported that Tris forms a stable complex with Cu(II), which catalyzes the

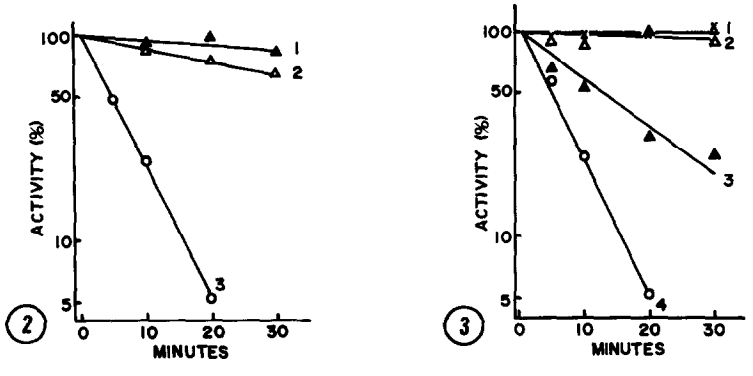

Fig. 2. Inactivation of the Mn-catalase by NH_2OH induced by $CuCl_2$, $FeSO_4$ and $MnCl_2$. Line 1; 0.1 mM $MnCl_2$. Line 2; 0.1 mM $FeSO_4$. Line 3; 0.1 mM $CuCl_2$.

Fig. 3. Effects of EDTA and histidine on the inactivation of the Mn-catalase by NH_2OH plus $CuCl_2$. Line 1; plus 5 mM EDTA. Line 2; plus 0.5 mM histidine. Line 3; plus 0.1 mM histidine. Line 4; control.

oxidation of cystein (18). In the present incubation mixture, Tris buffer was used and almost all Cu(II) occurred as a Tris-complex. The inactivation rate of Mn-catalase, however, decreased with increasing concentration of Tris above 10 mM (Fig. 1A, line 4). The inactivation was not affected when 10 mM potassium phosphate (pH 7) was used in place of 10 mM Tris (Fig. 1A, line 5, □). Tris buffer inhibits Cu(II)-catalyzed oxidative loss of NH_2OH (14), but apparently not sufficiently to make H_2O_2 production rate-limiting in the present circumstances.

The addition of 0.58 mM H_2O_2 to the incubation mixture enhanced the rate of the inactivation, causing a complete loss of enzymatic activity below 4 min under both aerobic and anaerobic conditions (Figs. 1B and 4). Omission of Cu(II) from the incubation mixture did not then affect the inactivation rate, nor did the inclusion of EDTA (Fig. 4, line 4). The addition of Cu(II) and H_2O_2 without addition of NH_2OH did not cause any inactivation (Fig. 4, line 1).

The addition of superoxide dismutase to the incubation mixture resulted in a slight increase in the rate of the inactivation of Mn-catalase by NH_2OH plus Cu(II) (Fig. 5, lines 2 and 3); 0.8 μM and 1.6 μM superoxide dismutase enhanced the rate of inactivation by 1.6-

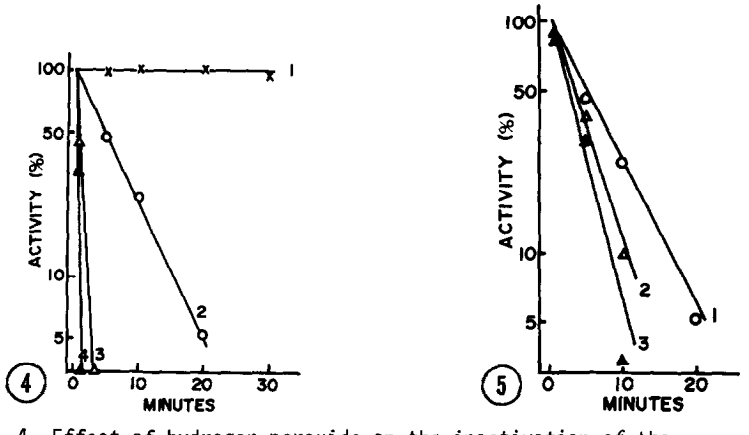

Fig. 4. Effect of hydrogen peroxide on the inactivation of the Mn-catalase by NH_2OH plus $CuCl_2$. Line 1; plus 0.58 mM H_2O_2 without NH_2OH. Line 2; control. Line 3; plus 0.58 mM H_2O_2. Line 4; plus 0.58 mM H_2O_2 and 5 mM EDTA.

Fig. 5. Effect of superoxide dismutase on the inactivation of the Mn-catalase by NH_2OH plus $CuCl_2$. Line 1; control. Line 2; plus 0.8 μM superoxide dismutase. Line 3; plus 1.6 μM superoxide dismutase.

and 2.3-fold. Thus, O_2^- does not participate in the inactivation reaction and that the increased rate of inactivation is due to the increase in the steady state concentration of H_2O_2 produced via enzymatic dismutation of O_2^-, which is formed during the autooxidation of NH_2OH (16).

All of evidence obtained here indicates the involvement of H_2O_2 in Cu(II)-dependent inactivation of Mn-catalase by NH_2OH. Therefore, aerobic NH_2OH solution is a continuous source of H_2O_2 in the presence of Cu(II).

ACKNOWLEDGEMENTS

I would like to thank Drs. K. Asada and I. Fridovich for many helpful discussions and for their constructive comments on the manuscript.

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