Effects of Cysteine on the Inactivation of Bovine Liver Catalase

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Bovine liver catalase was exposed to cysteine, as a natural inactivator metabolite, causing autoxidation-generating H_2O_2 continuously. The catalase species concentrations and activity measurement were done by spectrophotometry in phosphate buffer 10 mM, pH 6.5, and 27°C. The activity of catalase decreased continuously due to the conversion of active ferricatalase species, E-Fe (III), to an inactive enzyme species, E-Fe (IV). This conversion is related to the slow production of H_2O_2 generated by autoxidation of cysteine. The free SH-group of cysteine has an essential role in production of H_2O_2 and hence inactivation of catalase. NADPH can protect catalase against inactivation due to the conversion of inactive form of E-Fe (IV) to ferricatalase species. E-Fe (III).

Introduction

Bovine liver catalase (EC 1.11.1.6), such as all catalases, is an antioxidant oligomeric enzyme (MW=240,000) with four identical subunits, tetrahedrally arranged subunits.[1-3] Each subunit consists of a single polypeptide chain that associates with a prosthetic group, ferric protoporphyrin IX.[4] The subunits apparently function independently of one another.[5] It is ubiquitously present in aerobic organisms where in part it serves to protect cells from the toxic effects of hydrogen peroxide.[1-2] Twin reactions occur when catalase reacts with H_2O_2. Firstly, decomposition of H_2O_2 by catalase occurs according to the catalytic cycle:[6]

$$\underset{\text{(Ferricatalase)}}{\text{E-Fe(III)}} + H_2O_2 \longrightarrow \underset{\text{(Compound I)}}{\text{E-Fe(V)}\!=\!O} + H_2O$$

$$\text{E-Fe(V)}\!=\!O + H_2O_2 \longrightarrow \text{E-Fe(III)} + H_2O + O_2 \quad \text{(route 1)}$$

Secondly, inactivation of catalase *via* conversion of compound **I** to compound **II** occurs: [1,7]

$$\underset{\text{(Compound I)}}{\text{E-Fe(V)}\!=\!O} + \text{electron donor} \longrightarrow \underset{\text{(Compound II)}}{\text{E-Fe(IV)}} \longrightarrow \text{etc.} \quad \text{(route 2)}$$

This process occurs *via* endogenous or exogenous electron transfer reaction when catalase exposed to H_2O_2 at high concentration level or continual generation of H_2O_2.[8-9] Compound **II** and its derivatives are catalytically inactive. Thus, during the reaction the active enzyme gradually exits from the catalytic cycle and the process which is termed suicide inactivation.[10-12] Each subunit of catalase contains one molecule of tightly bound NADPH that prevents and reverses the accumulation of compound **II**.[13-15] Between NADPH binding site and haem group in each subunit, there is a β-sheet wall that contain a number of tyrosine residues that some of these act as endogenous electron donor during formation of compound **II** from compound **I**.[16] On the other hands, ferricyanide, a scorbate and semidehydro ascorbate can act as endogenous electron donor during this process.[8,13,17]

Some theories suggested inhibition of catalase during incubation of the enzyme with ascorbate, which is involved in formation of ascorbate-ferric complex, formation of compound **II**,[8] free radical attach of protein[18] and physical degradation of the enzyme into lower molecular weight fragments.[17,19] Univalent electron reduction of molecular oxygen during autoxidation of thiols such as cysteine and some other compounds lead to production of super oxide free radical that able to convert catalytically and uncatalytically to H_2O_2,[20-21] substrate of catalase as follow:

$$O_2^{\cdot} + O_2^{\cdot} + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2$$

This paper describes inactivation of catalase during incubation by cysteine and notatin system. A continuous system for production of H_2O_2 that is including glucose and glucose oxidase is called notatin system. Also, reactivation of the enzyme by NADPH, as a natural metabolite, has been investigated.

Materials and Methods

Materials. Recrystallized bovine liver catalase and lyophilized glucose oxidase were obtained from Sigma. NADPH was obtained from Boehringer Mannheim Chemical Company. Other substances (reagent grade) were obtained from Merck. The buffer utilized throughout the study was sodium phosphate 10 mM, pH 6.5.

Methods. Catalase was dissolved and dialyzed sufficiently against buffer and centrifuged at 3500 rpm for 15 minutes and its concentration estimated by measuring its absorbance at 405 nm, applying the extinction coefficient of 3.24×10^5 lit mol^{-1} cm^{-1} and using a molecular weight of 240,000 for bovine liver catalase. The mean velocity of the decomposi-

tion reaction of H_2O_2 by catalase was measured on a Shimadzu UV-3100 spectrophotometer in kinetic mode at 240 nm and 27 °C. The molar extinction coefficient of H_2O_2 at 240 nm and pH 6.5 was measured and taken as being 39.8 lit mol^{-1} cm^{-1} throughout the remaining experiments.

Bovine liver catalase, 1×10^{-6} M, was incubated separately with cysteine (2×10^{-1} M) and notatin system (including 1×10^{-3} M glucose and 30×10^{-9} M glucose oxidase) for three hours. During each incubation, absorbance at 405 and 435 nm measured. The formation and disappearance of compound **II** were measured by change in the absorbance at 435 nm that is a characteristic of compound **II**. The concentration changes of ferricatalase were recorded by changing the absorbance at 405 nm that is characteristic of ferricatalase. After completion of compound **II**, 10 μL of a NADPH solution, 2×10^{-1} M, was added to system and the changes of absorbance were measured.

Reaction between catalase and H_2O_2 for activity measurement was initiated by transferring 10 μL of the enzyme (10^{-8} M) to 990 μL of H_2O_2-buffer solution (15 mM). During each experiment, based on the decrease of the absorbance at 240 nm, catalase was assayed. The assay was repeated 6 times at appropriate intervals. In each assay, the mean velocity of reaction during 25 seconds was recorded.

Results and Discussion

Considering the proposed mechanism of inactivation of catalase by thiol compounds, the catalase solution was incubated with cysteine for conversion of ferricatalase to compound **II**. The absorbance of incubated mixture was measured in appropriate intervals for monitoring the progress of reactions. The measurements were accomplished at 405 nm, the λ_{max} of soret band of ferricatalase, and 435 nm, the λ_{max} of compound **II**. From the known molar extinction coefficients of ferricatalase and compound **II**,[9] the concentrations of these two enzyme species can be estimated. Figure 1 shows the changes of these concentrations during incubation of catalase in the presence of cysteine. The concentrations of ferricatalase and compound **II** were found to be decreased and increased, respectively. Since ferricatalase has an extinction coefficient greater than that of both compound **I** and compound **II** at 405 nm, such variation of A_{405} can be attributed to the decreasing of ferricatalase concentration due to its conversion to its oxidized derivatives by H_2O_2.

The effect of NADPH on incubated mixture of catalase-cysteine was investigated. When the changes in A_{435}, A_{405} and the enzyme activity were found to be relatively low, NADPH was added to the mixture. This led to A_{435}, A_{405} and the enzyme activity attained their original values in start time of incubation (Figure 1A). Since it is proved that NADPH can return compound **II** back to ferricatalase, these results confirm that the changes in absorption spectrum and activity of bovine liver catalase by a thiol compound, such as cysteine, must be due to conversion of catalase to compound **II**. Presumably, the cysteine acts as a continuos source of slow production of H_2O_2 in incubation, like enzymatic production of H_2O_2 in the notatin system. Investigation on catalase incubated with a notatin system gives similar results (Figure 2). This test confirms that the spectrophotometric changes which has been proceeded by NADPH is a consequence of a safely return of inactivated catalase to its native form. Considering the role of NADPH on catalase chemistry, the profile of changes of catalase activity provides further evidence to identification of the product of ferricatalase when it is exposed to cysteine. Different concentrations of NADPH on cysteine-catalase mixture were also used to reduction of compound **II** that are shown in Figure 1B. It can be observed that the elevation of NADPH concentration led to acceleration in conversion of compound **II** to ferricatalase. It is observed that the concentration of NADPH must be 200 μM, at least, for saturation of binding sites of compound **II** for NADPH.

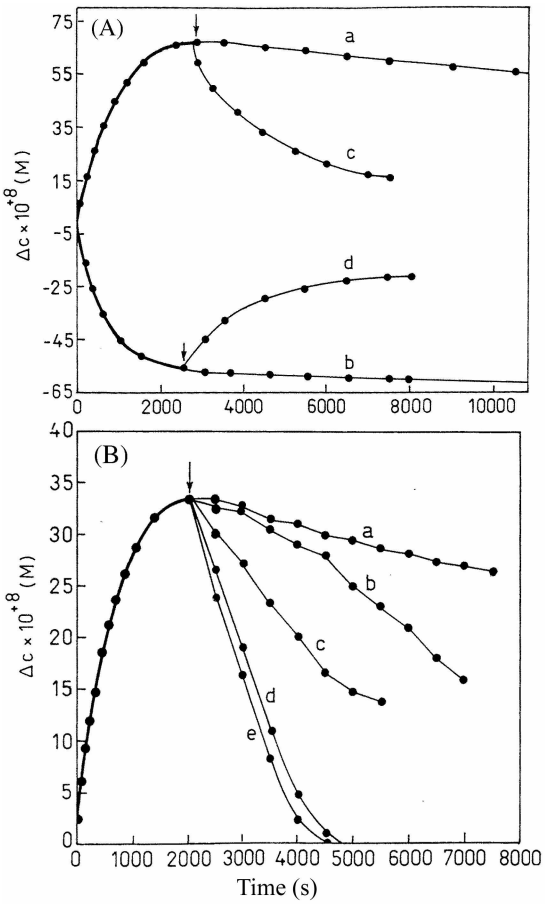

Figure 1. (A): The concentration changes of compound **II** (a) and ferricatalase (b) for a mixture containing 980 μL of a catalase solution (1 μM) and 10 μL of a cysteine solution (0.02 M), which is incubated for three hours. Also, the disappearance of compound **II** (c) and regeneration of ferricatalase (d) have been showed, when 10 μL of NADPH (200 μM) was added to that mixture. Arrows show the time of adding NADPH. (B): Effect of different concentrations of NADPH on the disappearance of compound **II** for a mixture containing 980 μL of a catalase solution (1 μM) and 10 μL of a cysteine solution (0.02 M), which is incubated for a long time. The arrow shows the time of adding 10 μL of NADPH solution at concentration of 0 μM (a), 66 μM (b), 100 μM (c), 200 μM (d) and 400 μM (e).

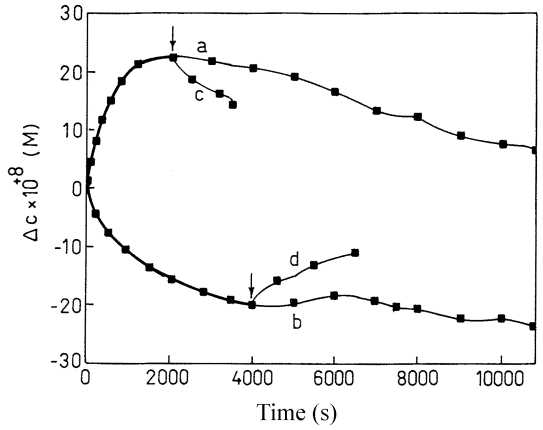

Figure 2. The concentration changes of compound **II** (a) and ferricatalase (b) for a mixture containing 965 μL of a catalase solution (1 μM) and 25 μL of a notatin solution (including 10 μL of a glucose solution, 0.1 M and 15 μL of a glucose oxidase solution, 2 μM), which is incubated for three hours. Also, the disappearance of compound **II** (c) and regeneration of ferricatalase (b) have been shown, when 10 μL of NADPH (200 μM) was added to that mixture. Arrows show the time of adding NADPH.

Catalase was also incubated with blocked thiol compounds, such as methionine (R-S-CH_3) and cystine (R-S-S-R). The concentration changes of compound **II** in incubated mixture were tested as discussed for cysteine. The results are shown in Figure 3. The changes in these variables were not significant in comparison with the corresponding results obtained on cysteine. These results suggest that the free SH-group of thiol compounds has an essential role in the formation of compound **II**.

In the other hand, the activity of incubated catalase was monitored in parallel with the spectrophotometric works. The small volumes of incubated mixture were taken to assay of the residual activity of catalase. Figure 4 shows the progressive decreasing in the catalase activity during the incubation time. The correlation between spectrophotometric changes and measured enzyme activities confirms the generation of

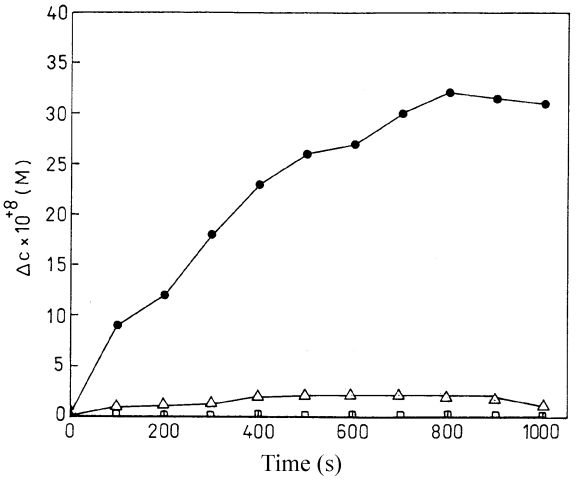

Figure 3. Increasing of the concentration of compound **II** in the presence of cysteine (●), cystine (△) and methionine (□).

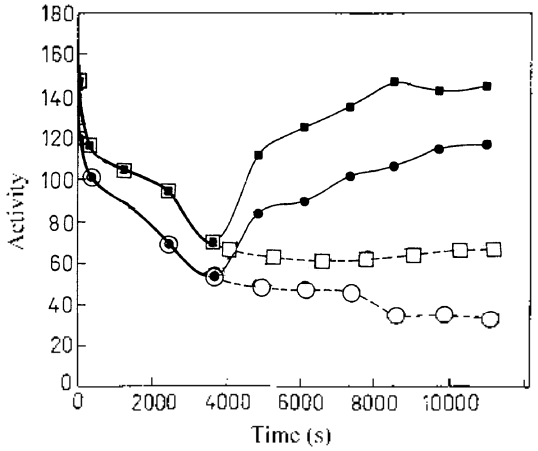

Figure 4. Inactivation of catalase in the presence of cysteine (○) and notatin system (□) and reactivation of enzyme by adding 10 μL of NADPH (200 μM) to the mixture reaction after 1 hour, in the presence of cysteine (●) and notatin system (■).

the compound **II** of catalase as its inactive species in the incubated mixture.

Thus, autoxidation of cysteine provides a slow and continuous source of H_2O_2 production for inactivation of catalase. The free SH-group has an essential role in the autoxidation of cysteine. Here, it is confirmed NADPH induced the catalase from the inactivated enzyme species, compound **II**, to active ferricatalase species. Therefore, it can be conclude NADPH as a natural reducing agent protects catalase against inactivatory effect of H_2O_2 that generated with autoxidation of cysteine as a natural inactivator metabolite.

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