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Catalase-Aminotriazole Method for Measuring Secretion of Hydrogen Peroxide by Microorganisms

GERALD COHEN AND NORMAN L. SOMERSON[1]

Departments of Biochemistry and Psychiatry, Columbia University College of Physicians and Surgeons, the New York State Psychiatric Institute, New York, New York 10032, and the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

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A new method for measuring the secretion of H_2O_2 has been based upon an H_2O_2-dependent inhibition of catalase by 3-amino-1,2,4-triazole. The conversion of an H_2O_2-secretion rate into a catalase inhibition rate amplified a relatively small molar concentration of H_2O_2 and provided a highly specific and sensitive method for quantitatively measuring H_2O_2. A major advantage of this approach is that it does not require extensive accumulation of H_2O_2 in the environment. The method was successfully employed to measure H_2O_2 secretion by *Mycoplasma pneumoniae*, which possesses a peroxidase-like activity that limits the accumulation of H_2O_2 in the environment.

Mycoplasma pneumoniae secretes a substance which hemolyzes guinea pig and sheep erythrocytes (3, 14, 15). This hemolysin has been identified as H_2O_2 (8, 16). Other mycoplasmas, including *M. laidlawii*, *M. gallisepticum*, and *M. neurolyticum*, also produce H_2O_2 (15, 16, 17).

In this study, we describe a new method for measuring the secretion of H_2O_2 by microorganisms such as *M. pneumoniae*. It is based upon an H_2O_2-dependent inhibition of catalase by 3-amino-1,2,4-triazole (AT). The reactions leading to enzyme inhibition are

$$\text{catalase} + H_2O_2 \rightleftarrows \text{complex I} \quad \text{(A)}$$

$$\text{complex I} + \text{AT} \rightarrow \text{inhibited catalase} \quad \text{(B)}$$

The H_2O_2 reacts with catalase (reaction A) to form the enzyme-substrate combination designated by Chance (2) as complex I. Complex I reacts with AT (12, 13; reaction B), and, as a result, the enzyme is inhibited. Subsequent measurement of residual catalase activity is a relatively simple and rapid procedure. The rate of inhibition of catalase provides an estimate of the rate of secretion of H_2O_2. In the conversion of an H_2O_2 secretion rate into a catalase inhibition rate, a relatively small molar change in H_2O_2 is amplified; sensitivity and specificity are provided by the high affinity and specificity of the enzyme for its substrate.

Other reactions of complex I include

$$\text{complex I} + H_2O_2 \rightarrow \text{free catalase} + 2H_2O + O_2 \quad \text{(C)}$$

$$\text{complex I} + \text{ethyl alcohol} \rightarrow \text{free catalase} + 2H_2O + \text{acetaldehyde} \quad \text{(D)}$$

Reactions C and D represent, respectively, the catalatic and peroxidatic activities of catalase.

MATERIALS AND METHODS

Reagents. An isotonic solution of sodium chloride and 0.01 M sodium phosphate at *p*H 7.4 (9) was used throughout this investigation. Crystalline beef-liver catalase, horseradish peroxidase, and *o*-dianisidine hydrochloride were obtained from Worthington Biochemical Corp., Freehold, N.J., purified glucose oxidase from Calbiochem, Los Angeles, Calif., and reagent 30% H_2O_2 from Fisher Scientific Co., Pittsburgh, Pa.; the AT (Mann Fine Chemicals, Inc., New York) was recrystallized from ethyl alcohol.

The beef-liver catalase suspension was centrifuged, the supernatant fluid was discarded, and then the isolated crystals were dissolved in buffered saline. Concentration of enzyme protein was adjusted so that when samples were subsequently diluted and assayed for enzyme activity, as described below, roughly 50 to 60% loss in substrate occurred. Most of the experiments were performed with a single lot of catalase (no. 5669), which was diluted to a concentration of 0.25 mg/ml (uncorrected for losses during centrifugation). We also used a second lot (no. 5671) of catalase which was diluted further to compensate for a higher specific activity. Both of these lots were stable when incubated with AT. Two other available catalase lots were not used because they lost activity when incubated with AT; this effect was due, most

[1] Present address: Department of Medical Microbiology, Ohio State University College of Medicine, Columbus, Ohio 43210.

probably, to the presence of trace amounts of reducing agents which reacted with oxygen to form some H_2O_2.

Organisms. The FH strain of *M. pneumoniae* and the S6 strain of *M. gallisepticum* were employed (7).

Catalase-AT system for measuring H_2O_2. Catalase (0.25 mg/ml) and the catalase inhibitor AT (50 μmoles/ml) were prepared together in buffered saline. Glucose (11 μmoles/ml) was added to support the formation of H_2O_2 from the mycoplasmas. Samples (1.8 ml) of buffered catalase-AT-glucose mixture were equilibrated at 37 C for 10 min before the addition of 0.2 ml of mycoplasma suspension. Subsequently, catalase activity was determined on serial samples (0.1 ml), which were first admixed with 0.45 M ethyl alcohol in the *p*H 7.4 saline-phosphate buffer (0.5 ml) to decompose complex I (reaction D) and stop the reaction with AT (6). As a precautionary measure, the samples mixed with ethyl alcohol were incubated for 10 min at room temperature to decompose any complex II, which is an inactive form of the catalase-H_2O_2 combination (2). Samples were diluted to 10 ml with ice-cold distilled water, and then 0.5-ml portions were analyzed in duplicate for residual catalase activity (reaction C) by a permanganate titration method (6). The very great dilution of the samples decreased the concentration of AT to a level where it was no longer inhibitory. The appearance of H_2O_2 in the mycoplasma suspension medium was detected as an irreversible loss of catalase activity. Results are recorded as a percentage of inhibition of catalase as compared to a control sample incubated without mycoplasmas. The catalase activity in the control sample was stable throughout the incubation period.

Calibration of the catalase-AT system. The catalase-AT system was calibrated by measuring the rates of inhibition of catalase when H_2O_2 was generated continuously, at known rates, during the oxidation of glucose by glucose oxidase. The H_2O_2-generation rates for several concentrations of glucose oxidase were independently measured with an adaptation of a standard peroxidase method (1). We omitted the catalase and AT, substituted horseradish peroxidase (40 μg/ml) and *o*-dianisidine (0.4 μmole/ml), and measured the rate of oxidation of the *o*-dianisidine. The sensitivity was increased in the following manner. After 45 min of incubation at 37 C, samples (10 ml) of reaction mixture were acidified with 0.08 ml of 5 N HCl, and then the samples were saturated with NaCl (3 g). The colored product was extracted into 2 ml of ethyl acetate and read at 425 nm. Optical density (OD) readings in the range of 0 to 0.7 OD unit were compared to the straight line obtained by direct addition of reagent H_2O_2 (0 to 0.04 μmole) to the peroxidase-dianisidine mixture, followed by extraction with ethyl acetate.

RESULTS

Calibration of the catalase-AT system for detecting H_2O_2. The catalase-AT system was calibrated with H_2O_2 generated at known rates during the oxidation of glucose by glucose oxidase. A linear relationship was observed between the rates of H_2O_2 generation and the rates of catalase inactivation (Fig. 1). The data of Fig. 1 were used to estimate rates of H_2O_2 secretion by mycoplasma suspensions.

The presence of catalase in either the glucose oxidase or peroxidase preparations would have interfered with the calibration by decomposing H_2O_2 (reaction C), thus decreasing the apparent H_2O_2 generation rates. However, independent assays of these enzyme preparations at concentrations 10-fold higher than those employed in the calibrations indicated no detectable catalase activity.

A striking feature of the catalase-AT system is its insensitivity to the sudden and direct addition of reagent H_2O_2 (Table 1). Inhibition of catalase was negligible when 0.1 μmole of H_2O_2 was added directly. Even the direct addition of up to 1 mmole of H_2O_2 failed to produce as great a response as 0.01 μmole of H_2O_2 generated slowly over the course of 1 hr (Fig. 1).

Detection of H_2O_2 secreted by the mycoplasmas. The rate of secretion of H_2O_2 by the mycoplasmas was determined by measuring the H_2O_2-dependent rate of inhibition of catalase by AT. In Fig. 2, a

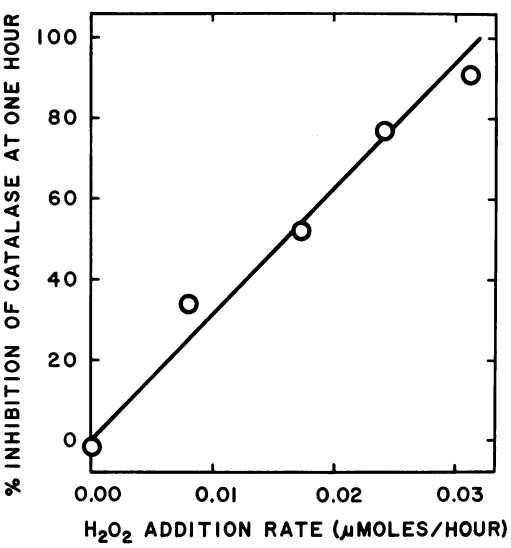

FIG. 1. *Calibration curve: catalase inhibition versus H_2O_2 addition rate. Glucose oxidase concentrations of 0.014, 0.028, 0.042 and 0.056 μg/ml were used. Catalase activity was determined for duplicate vessels after incubation at 37 C for 1 hr. H_2O_2 formation in duplicate vessels was independently measured with horseradish peroxidase and o-dianisidine (in the absence of catalase + AT) during incubation at 37 C for 45 min. After 45 min, some decline in rate of color development was noted; however, the rate of inhibition of catalase by AT in the absence of peroxidase-o-dianisidine remained constant. The declining rate in the peroxidase system may have been due to inactivation of glucose oxidase by the oxidation product derived from o-dianisidine.*

TABLE 1. *Insensitivity of the catalase-AT system to H_2O_2 added directly*[a]

H_2O_2 added	Inhibition of catalase
μmoles	%
10^{-1}	2
1	12
10	14
10^2	17
10^3	20

[a] Samples (0.1 ml) of solutions containing 0.1 μmole to 1 mmole of H_2O_2 were added to 2-ml samples of the buffered catalase-AT system. Samples were incubated for 30 min at 37 C. Catalase activity was compared to a control to which no H_2O_2 had been added.

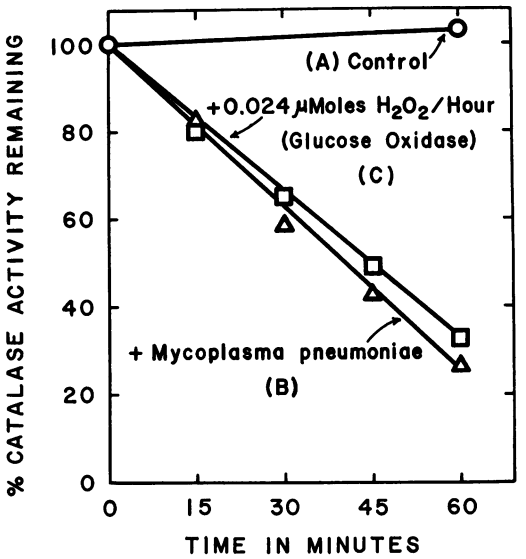

FIG. 2. *Inhibition of catalase by AT in the presence of H_2O_2 generated by Mycoplasma pneumoniae or by glucose oxidase. Glucose (11 μmoles/ml) was present in all samples. The glucose stimulated the formation of H_2O_2 by M. pneumoniae or by glucose oxidase, but had no effect on the control sample.*

control specimen consisting of catalase plus AT exhibited stable catalase activity during the 1-hr incubation period at 37 C (curve A). In contrast, a suspension of *M. pneumoniae* produced a steady decline in catalase activity (curve B). The rate of catalase inhibition was equivalent to that produced by H_2O_2 generated at the rate of 0.024 μmole/hr (curve C). In the example shown in curve B, catalase inhibition in the presence of *M. pneumoniae* was linear with time; in other experiments, a decreasing reaction rate was evident. The decrease most probably reflected diminished microbial activity, since the corresponding reaction rate with glucose oxidase was consistently linear. In other experiments, *M. gallisepticum* was also shown to secrete H_2O_2. It was shown that H_2O_2 itself, rather than an H_2O_2-generating agent, was the product of mycoplasma metabolism (7).

DISCUSSION

We present a new method for measuring the secretion of H_2O_2 by microorganisms. The secreted H_2O_2, in conjunction with AT in the suspension medium, causes inhibition of catalase; the rate of inhibition is proportional to the rate of secretion of H_2O_2. The catalase-AT method simultaneously destroys the H_2O_2 (reaction C) and monitors its residual steady-state concentration (reactions A and B).

Actually, with both *M. pneumoniae* and *M. gallisepticum*, the H_2O_2 could not accumulate freely in the medium even in the absence of catalase. This is due to a peroxidase-like activity which is stimulated by glucose (7). As a result, it would be difficult to estimate H_2O_2 secretory activity by studying the accumulation of H_2O_2. The catalase-AT method does not depend upon accumulation of H_2O_2.

The catalase-AT system measures the continuous addition of small amounts of H_2O_2 to the medium (Fig. 2, curve C), but is insensitive to sudden and direct addition of H_2O_2 (Table 1). This unique situation is explainable as follows. The rate of inhibition of catalase (reaction B) is dependent upon the steady-state concentration of complex I. The concentration of complex I is controlled by the concentration of free H_2O_2 in two opposing ways: (i) by accumulation of complex I to satisfy the position of equilibrium in reaction A, and (ii) by the concurrent decomposition of complex I via reaction C. When H_2O_2 is added at once, the relatively high concentration of H_2O_2 strongly favors reaction C and the ensuing, extraordinarily-rapid destruction of H_2O_2 limits the lifetime of complex I. Thus, the exposure time for the slower reaction of complex I with AT is sharply curtailed, and the extent of inhibition of catalase is low. During this brief interval, the concentration of complex I need not be raised proportionately to the amount of added H_2O_2, since a limitation is imposed by the very rapid rate of reaction C. On the other hand, the very slow addition of an identical amount of H_2O_2 results in a lower concentration of complex I, but, thereafter, the reaction with AT, which is present in high concentration, is favored over that of H_2O_2, which is present in low concentration. As a result, the extent of catalase inhibition observed at low H_2O_2 addition rates is greater than that for the same amount of H_2O_2 added all at once. Similar situations, in which a peroxidatic reaction is

favored over the catalatic destruction of H_2O_2, have been described for the competition between H_2O_2 (equation C) and ethyl alcohol (equation D), for reaction with complex I (10), and for the competition between catalase and glutathione peroxidase for reaction with H_2O_2 within intact erythrocytes (5). In our experiments, in which the H_2O_2 was generated continuously in small amounts by the glucose oxidase reaction, the steady-state concentration of complex I appeared to be linearly related to the H_2O_2 generation rate, since the rate of enzyme inactivation was directly proportional to the H_2O_2 generation rate (Fig. 1).

M. pneumoniae contains no catalase (11); however, microorganisms which do contain catalase might provide an opportunity to measure intracellular H_2O_2 as well as the secreted H_2O_2. Measurements employing intracellular catalase have been described for human erythrocytes exposed to H_2O_2-generating drugs (4). Although our experiments were confined to studies with mycoplasmas, the catalase-AT method of determining H_2O_2 secretion should find general application for the study of other microorganisms.

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