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DETERMINATION OF THE KINETIC PARAMETERS FOR THE "SUICIDE SUBSTRATE" INACTIVATION OF BOVINE LIVER CATALASE BY HYDROGEN PEROXIDE

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The kinetics of the bovine liver catalase inactivation by its suicide substrate, H_2O_2 was investigated in sodium phosphate buffer, 50 mM pH 7.0, at 27°C. By combination of the rate equations of two concurrent reactions, decomposition of H_2O_2 by catalase and suicide inactivation of catalase by H_2O_2, simple, semiempirical kinetic equations were defined and used for the determination of the inactivation rate constant and the partition ratio which were found to be $6.86 \pm 0.19\ M^{-1}\ min^{-1}$ and $1.82 \times 10^7 \pm 5.0 \times 10^5$, respectively. A close match was found between the experimental data and the equations.

KEY WORDS: Enzymes inactivation, suicide substrate, catalase, kinetic equations

INTRODUCTION

Catalase (H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6) is ubiquitously present in aerobic organisms where in part it serves to protect cells from the toxic effects of hydrogen peroxide.[1,2] Catalase is typically a tetramer of identical heminic subunits. Enzymes from various sources exhibit some differences in structure and properties.[3,4] Bovine liver catalase, for example, possesses a molecular mass of 240,000, its amino acid sequence has been determined[5] and its detailed structure elucidated.[6]

Twin reactions occur when catalase reacts with H_2O_2. Firstly, decomposition of H_2O_2 by catalase occurs according to the catalytic cycle:[7]

$$\text{Free catalase} + H_2O_2 \longrightarrow \text{compound I} + H_2O$$

$$\text{Compound I} + H_2O_2 \longrightarrow \text{Free catalase} + H_2O + O_2 \qquad \text{(route 1)}$$

Over the normal concentration range of H_2O_2 the reaction is assumed to be first order with regard substrate concentration.[8,9] Secondly, inactivation of catalase via conversion of compound **I** to compound **II** by internal electron donors[1,10] occurs:

* Correspondence.

Compound **I** + electron donor → Compound **II** → etc. (route 2)

Compound **II** and its derivatives are catalytically inactive. Thus, during the reaction the active enzyme gradually exits from the catalytic cycle and it is this process which is termed suicide inactivation.[11,12]

The goal of the present study was to determine the kinetic parameters of the suicide inactivation of catalase by H_2O_2 and thereby obtain an overall kinetic equation for the enzymatic decomposition of H_2O_2 by catalase. The obtained semiempirical equations seem to be a simplification of those which have developed by others[11,13] for the special case where apparently first-order processes apply with respect to substrate concentration for catalysis and inactivation, having a partition ratio well above 1.

MATERIALS AND METHODS

Recrystallized bovine liver catalase was obtained from Sigma (C-100), other substances (reagent grade) were obtained from Merck. The buffer utilized throughout the study was sodium phosphate 50 mM, pH 7.0.

Method

The mean velocity of the decomposition reaction of H_2O_2 by catalase was measured on a Shimadzu UV-3100 spectrophotometer in kinetic mode at 240 nm and 27°C. Catalase was dissolved and dialyzed sufficiently against buffer and its concentration estimated by measuring its absorbance at 405 nm, applying the extinction coefficient of 3.24×10^5 l. mol^{-1}.cm^{-1} and using a molecular weight of 240,000 for bovine liver catalase.[14] The molar extinction coefficient of H_2O_2 at 240 nm and pH 7.0 was measured and taken as being 39.6 l. mol^{-1}.cm^{-1} throughout the remaining experiments.

Apparent first-order rate constant of the H_2O_2 decomposition reaction in min^{-1} was used for expression of catalase activity (designated here by α). A concentration of 1.0×10^{-10} M of active catalase offered an initial value of $\alpha = 0.1$ min^{-1} for the reaction at 27°C, pH 7.0.

Incubation-assay

Reaction between catalase and H_2O_2 was initiated by transferring 2×10^{-13} mol of catalase to 1.00 ml of buffer-H_2O_2 solution; the initial rate constant of the reaction was 0.2 min^{-1}. The concentration of H_2O_2 in the reaction mixture within the cuvette (monitored at A_{240} was maintained at the desired value by adding small volumes (less than 15 μl in total) of concentrated H_2O_2 to offset the consumed substrate so that fluctuations in H_2O_2 concentration during incubation were not greater than ± 7%. During each incubation experiment, based on the decrease of the absorbance at 240 nm,[15] catalase was assayed. The assay was repeated 6 times at appropriate intervals. In each assay, the mean velocity of reaction during 20 s was recorded and divided by the concentration of substrate in order to calculate the rate constant of reaction as a direct measure of catalase activity.[15]

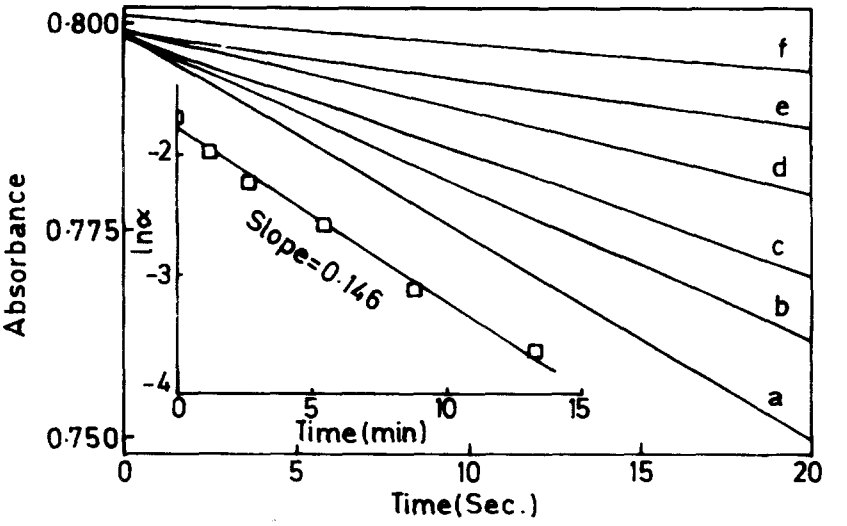

FIGURE 1 An incubation-assay experiment. Catalase (2×10^{-13} mol; 5 μl) was added to 1.00 ml of H_2O_2 solution (20 mM) in phosphate buffer (50 mM), at pH 7.0 and 27°C. The concentration of H_2O_2 was held at 20 ± 1 mM between rate measurements. The traces show changes in A_{240} *vs* time. The incubation time (in min) and mean velocity (Abs. $\times 10^3$/min) were 0 and 148 (a); 1.23 and 110 (b); 2.71 and 86 (c); 5.47 and 60 (d); 8.85 and 35 (e); 13.31 and 20.5 (f), respectively. The slope of the internal plot indicates the k_i^{app} in H_2O_2 (20 mM).

RESULTS AND DISCUSSION

To determine the kinetic parameters of the substrate-induced catalase inactivation process it is necessary to follow the catalase activity for the steady state concentration of H_2O_2. This was possible according to the incubation-assay procedure adopted in this study. Figure 1 shows absorbance *vs* time which indicates the mean velocity of reaction during an incubation assay with 20 mM H_2O_2. The linearity of absorbance *vs* time indicates that no significant decrease in the substrate concentration and enzyme activity occurred during the 20 seconds. Thus, in each assay, the activity, α can be obtained as follows:

$$\alpha = \bar{v}/[H_2O_2] \quad (1)$$

in which $\bar{v}$ is the mean velocity of H_2O_2 decomposition.

As can be seen from Figure 1 (inset), changes in α match first order kinetics when other conditions remain constant. Thus, inactivation of catalase by its suicide substrate is a first order reaction under such conditions. The apparent rate constant of this reaction (k_i^{app}) was determined in different concentrations, from 5.0 to 20 mM, of H_2O_2. The results in Figure 2 indicate the linearity of k_i^{app} *vs* concentration of H_2O_2. Therefore, inactivation of catalase is a first order reaction in relation to the concentration of H_2O_2.

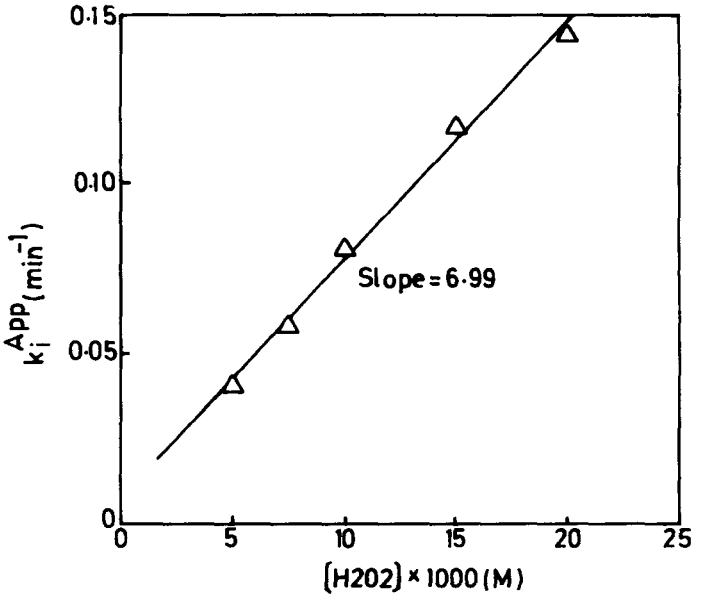

FIGURE 2 Relationship between k_i^{app} and concentration of H_2O_2. The k_i^{app} was measured for different concentrations of H_2O_2 as described in Figure 1. The slope of this plot indicates the k_i of catalase at 27°C.

The dependency of the inactivation rate ($\frac{d\alpha}{dt}$) on substrate concentration, as well as α and k_i, can be formulated as follows:

$$-\frac{d\alpha}{dt} = k_i \alpha s \tag{2}$$

$$-\frac{de}{dt} = k_i e s \tag{3}$$

where k_i is the inactivation rate constant, and s and e are the molar concentrations of H_2O_2 and active catalase respectively.

Recall that less than 10^{-9} M of H_2O_2 (far below the detectable limit) is sufficient to complete inactivation of catalase via route 2. Thus all of the decrease in H_2O_2 concentration in the reactions (may be up to 2×10^{-2} M) is ascribed to the catalytic pathway (route 1).

The decomposition reaction of H_2O_2 by catalase exhibits an unusual kinetic property. Saturation of the enzyme by its substrate is not possible at feasible H_2O_2 concentrations (below 5 M).[15] Reactions which are generally accomplished in H_2O_2 concentrations below 100 mM fall into the first order region of the Michaelis-Menten model. Hence, the rate of decomposition of H_2O_2 by catalase is given by

$$-\frac{ds}{dt} = \alpha s \tag{4}$$

$$-\frac{ds}{dt} = k_c e s \tag{5}$$

where k_c, a measure of catalytic efficiency of enzyme, is the number of H_2O_2 molecules which are decomposed by one molecule of catalase per unit of time and unit concentration of H_2O_2. It is calculated that $k_c = 1.0 \times 10^9$ M^{-1} min^{-1} at 27°C, pH = 7.0. The rate equations for these two concurrent reactions can be combined. Dividing Equation (3) by Equation (5) gives:

$$\frac{de}{ds} = \frac{k_i}{k_c} = P \tag{6}$$

Since $p \ll 1$, in a qualitative sense the reaction must be classified[13,14] as $r \gg 1$ and $s_o \gg e_o$. "r" is the partition ratio.

Equation (6) in its integrated form can be written as;

$$e_o - e = P(s_o - s) \tag{7}$$

Rearrangement of Equation (7) gives

$$\alpha_o - \alpha = k_i(s_o - s) \tag{8}$$

The subscript 'o' indicates the initial value of each parameter and e and α are remaining active catalase concentration and its activity, respectively, when the substrate concentration reaches "s". Equations (7 and 8) show that the ratio of reacted substrate to inactivated enzyme remains constant during the reaction; P is the number of moles of catalase which are inactivated concurrent with the decomposition of one mole of H_2O_2 and inversely proportional to the "r".

Extraction of "e" from Equation (7) and insertion into Equation (5) gives:

$$\frac{-ds}{s[e_o - P(s_o - s)]} = k_c dt \tag{9}$$

Integration of Equation (9) between time 0 and t results in;

$$\ln \frac{e_o s_t}{s_o[e_o - P(s_o - s_t)]} = k_c(Ps_o - e_o)t \tag{10}$$

in which s_t is the concentration of H_2O_2 at time t.

Equation (10) can be written as a practically useful formula:

$$\ln \frac{\alpha_o s_t}{s_o[\alpha_o - k_i(s_o - s_t)]} = (k_i s_o - \alpha_o)t \tag{11}$$

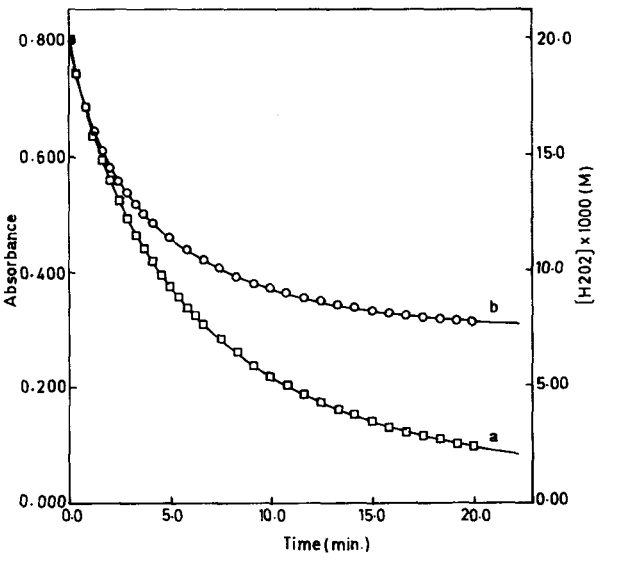

FIGURE 3 Progress curve of decomposition of H_2O_2 in the presence of catalase. The concentration of H_2O_2 was monitored at A_{240} after addition of catalase at 27° (□) and 37°C (○) to H_2O_2 solution (20 mM). The amount of added enzyme was adjusted so that the initial rate constants at both temperatures were close (0.194 and 0.187 min^{-1} for 27° and 37°C, respectively) for better comparison. Calculated k_i's are represented in Table 1. Traces are computer drawn and correspond to plots of Equation (11) at (a) 27°C and (b) 37°C using the corresponding determined parameters.

which shows the overall relationship between the concentration of substrate and the time of reaction and which also describes the progress curve.

Equation (11) was evaluated by monitoring the concentration of H_2O_2 over the long term in the presence of catalase of known initial activity. Good agreement between the experimental data and the proposed equation with exact parameters is shown in Figure 3.

Since k_i is assumed to be constant in Equation (10), each set of data utilized in the progress curve must give the same value for k_i. To examine this condition the progress curve was divided into separate segments. The data set within each segment fitted to the equation to calculate k_i. The results were consistent with each other as can be seen from Table 1.

Equation (8) shows that at the end point of the reaction, depending on, s_o, α_o and k_i, either some substrate or active enzyme may remain, unreacted.

The non-zero value of either one at infinite time is given by

$$s_\infty = s_o - (\alpha_o / k_i) \tag{12}$$

or

$$\alpha_\infty = \alpha_o - k_i s_o \tag{13}$$

TABLE 1
Calculated k_i for bovine liver catalase by fitting the data* to Equation (10).

reaction time (min)	k_i, 27°C	k_i, 37°C
0–4	6.82	13.87
4–8	6.83	14.58
8–12	6.83	14.67
12–16	6.80	14.65
16–20	6.81	14.64
0–20	6.82	14.65

* Same data was used for Figure 3.

Integrity of the Equations (12) and (13) was investigated by measurement of remaining H_2O_2 and catalase activity after sufficient incubation of different initial activities of catalase with constant initial concentration of H_2O_2. Figure 4 shows a linearity between residual $[H_2O_2]$ and initial catalase activities. On the other hand, when the remaining activities of catalase is significant, there is also a linearity between the later and initial activities of enzyme with the slope of 1.00.

The equations which have introduced here can be used for determination of kinetic parameters of reaction as follows:

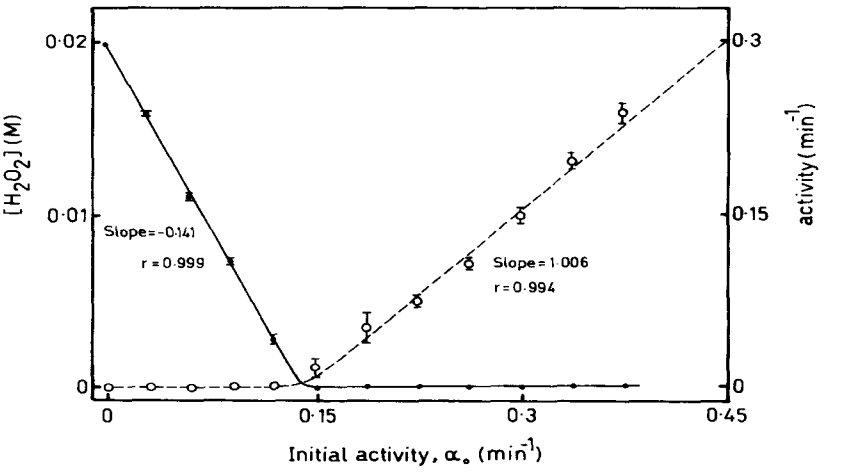

FIGURE 4 Residual $[H_2O_2]$ and remainder activity *vs* α_o in the constant s_o. Different initial activities of catalase were reacted with H_2O_2 (0.020) at 27°C, pH = 7.0. Remainder substrates (●) were measured at 240 nm after the practical end-point of the reactions. Remainder enzyme activities (○) were measured when $[H_2O_2]$ reached nonsignificant values by further addition of H_2O_2 (20 mM) and measurement of activity, immediately. Slope of diagonal segment of the solid line and the intercept of the solid and dashed lines on abscissa indicate the $-\frac{1}{k_i}$ and $k_i s_o$, values respectively.

TABLE 2
k_i and r values for bovine liver catalase at 27°C, pH = 7.0 (± SD).

method of measurement	using Equation (11)	using Equation (12)
k_i (M^{-1} min^{-1})	6.86 (± 0.19)	7.1 (± 0.28)
partition ratio, r	1.82×10^7 ($\pm 5.0 \times 10^5$)	1.76×10^7 ($\pm 6.9 \times 10^5$)

Determination of k_i and r. The partition ratio is the most important determinant in reaction between an enzyme and its suicide substrate. In the case of the enzymatic reaction represented here it can be calculated from the k_i value. r is the number of catalytic events (turnovers) per inactivation event.[12] Inactivation of one molecule of catalase associates with four (the number of active sites per molecule) inactivation events and during each catalytic event decomposition of two molecules of H_2O_2 occurs, thus

$$r = \frac{1}{8P} = \frac{k_c}{8} \times \frac{1}{k_i} \tag{14}$$

The progress curve data may be fitted to equation (11) by non-linear regression for determination of k_i and calculation of r.

Table 2 shows the results of such solutions employing EUREKA software, a common computer program.

The k_i value can also be determined using Equations (12) or (13) by measurement of s_o, α_o, and s_∞ or α_∞. The results, based on s_∞ measurements are also shown in Table 2. The k_i value can be determined from the slope of the solid line or the intercept of the solid or dashed lines on the abscissa in Figure 4.

Accurate determination of initial activity. Estimation of the catalase activity is an inexact process[9,10] due to loss of some activity after exposure of enzyme to H_2O_2 and among it during assay. Equation (11) can be used, not only for determination of k_i, but also for a refined value of activity at the starting time of measurement, α_o. Further, extrapolation of activity to the time before mixing is obtained from Equation (15):

$$\alpha_i = \alpha_o + k_i(s_i - s_o) \tag{15}$$

in which a_i and s_i are the intact values of the corresponding parameters.

The experimental-based equations represented in this paper are applicable to determination of some important kinetic constants and parameters of the enzyme catalase, as discussed above. It may be extended to the reaction between an enzyme and its suicide substrate when the substrate concentration used is well below the K_m of the enzyme.

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