KINETICS OF THE THERMAL INACTIVATION OF ALKALINE PHOSPHATASE FROM GREEN CRAB (*SCYLLA SERRATA*)

QING-XI CHEN [a], WEI ZHANG [a], SI-XU YAN [a], TONG ZHANG [b]
and HAI-MENG ZHOU [b,*]

[a] *Department of Biology, Xiamen University, Xiamen 361005, China;*
[b] *Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, China*

The kinetics of thermal inactivation of alkaline phosphatase from green crab (*Scylla Serrata*) has been studied using the kinetic method relating to the substrate reaction during irreversible inhibition of enzyme activity previously described by Tsou.[3] The results show that the thermal inactivation of the enzyme is an irreversible reaction. Comparison of the microscopic rate constants for thermal inactivation of free enzyme and the enzyme-substrate complex shows that the presence of substrate has a certain protective effect against thermal inactivation.

ABBREVIATIONS

ALP – alkaline phosphatase; PNPP – *p*-nitrophenyl phosphate.

INTRODUCTION

Alkaline phosphatase (ALP, EC 3.1.3.1.) is a metalloenzyme which catalyzes the nonspecific hydrolysis of phosphate monoesters.[1] The X-ray crystal structure of bacterial alkaline phosphatase has recently been reported

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* Corresponding author.

at 2.0 Å resolution in the presence of inorganic phosphate.[2] The active site is a pocket containing a tight cluster of 2 zinc ions (3.9 Å separation) and one magnesium ion (5 and 7Å from the two zinc ions). Although the X-ray crystal structure of the alkaline phosphatase from green crab (*Scylla Serrata*) is still unknown, the quantitative analysis of zinc and the complete inactivation of the enzyme after removal of zinc ions show that the active site of this enzyme is similar to that of bacterial alkaline phosphatase. In the present investigation, the kinetic theory relating to the substrate reaction during modification of enzyme activity previously described by Tsou[3] has been applied to a study of the kinetics of the course of thermal inactivatioin of green crab alkaline phosphatase containing multiple metal ions at the active site.

MATERIALS AND METHODS

p-Nitrophenylphosphate (PNPP) was from E. Merck; DEAE-cellulose (DE-32) was from Whatman; Sephadex G-150 and DEAE-Sephadex A-50 were Pharmacia products. All other reagents were local products of analytical grade.

Enzyme Preparation

The alkaline phosphatase was prepared from green crab (*Scylla Serrata*) viscera first according to the method of Yan and Chen[4] to the step of ammonium sulfate fractionation. The crude preparation was further chromatographed by ion-exchange with DEAE-cellulose (DE-32), and then by gel filtration through Sephadex G-150, followed by DEAE-Sephadex A-50. The final preparation was homogeneous on polyacrylamide gel isoelectric focusing and HPLC. The specific activity of the purified enzyme was 3320 μ/mg.
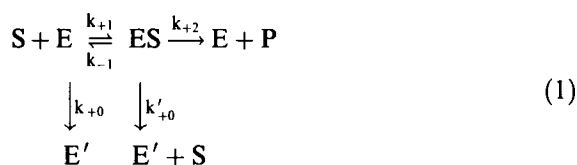
Assay

Protein concentration was determined as described by Lowry.[5] Enzyme activity was determined at 30°C by following the increase of absorbance at 405 nm accompanying the hydrolysis of the substrate (*p*-nitrophenylphosphate) using a molar absorption coefficient of $1.73 \times 10^4 \, M^{-1} \, cm^{-1}$. The reaction system contained *p*-nitrophenyphosphate, 2 mM; $MgCl_2$, 2 mM and $Na_2CO_3/NaHCO_3$ buffer, 0.05 M (pH 10.0). The kinetics of the thermal

inactivation reaction was followed by the substrate reaction during irreversible inhibition of enzyme activity as previously described by Tsou.[3] Kinetic measurements were carried out on a Kontron UV 860 spectrophotometer.

Determination of Microscopic Rate Constants of Thermal Inactivation of the Enzyme

The progress-of-substrate-reaction method previously described by Tsou[3] was used for the study of the thermal inactivation kinetics of ALP. In this method, $5 \mu l$ of $15 \mu M$ ALP was added to $1.0 ml$ of reaction mixture containing $2 mM$ substrate in $0.05 M$ $Na_2CO_3/NaHCO_3$ buffer (pH 10.0) at different temperatures. The course of the thermal inactivation was followed by measuring PNPP hydrolysis spectrophotometrically at $405 nm$. The substrate reaction progress curve was analyzed to obtain the rate constants as detailed below. When the reaction time is sufficiently long, the product concentration, [P], approaches a constant final value $[P]_\infty$, which decreases with elevating temperature. The results showed that the thermal inactivation of ALP was an irreversible reaction without residual activity. This reaction scheme can be written as follows:

$$S + E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P$$
$$\downarrow{k_{+0}} \quad \downarrow{k'_{+0}}$$
$$E' \quad E' + S \tag{1}$$

where S, P, E and E' are the substrate, product, the native and thermal denatured enzymes, respectively. ES is enzyme-substrate complex. The k_{+0} and k'_{+0} are the microscopic rate constants of the thermal inactivation of free enzyme and enzyme-substrate complex, respectively.

When the thermal inactivation reaction is irreversible, the product concentration is given[3] by

$$[P]_t = at + b(1 - e^{-At}), \tag{2}$$

where $[P]_t$ is the concentration of the product formed at time t, and t is the reaction time. A is the apparent rate constant.

$$A = (k_{+0}K_m + k'_{+0}[S])/(K_m + [S]) \tag{3}$$

$$[P]_\infty = V_{max}[S]/A(K_m + [S]). \tag{4}$$

Combining equations (3) and (4) yields

$$1/[P]_\infty = (k_{+0}K_m/V_{max})(1/[S]) + k'_{+0}/V_{max}. \qquad (5)$$

Plots of $1/[P]_\infty$ against $1/[S]$ gives a straight line with slope of $k_{+0}K_m/V_{max}$ and intercept k'_{+0}/V_{max}. As K_m is a known quantity, from the ratio of slope to intercept the ratio of the microscopic rates of thermal inactivation of free enzyme to its complex (k_{+0}/k'_{+0}) can be calculated. Therefore, as k_{+0}/k'_{+0} is known, from the apparent rate constant A, the microscopic rate constants, k_{+0} and k'_{+0} can be obtained according to equation (3).

RESULTS

Determination of K_m Values at Different Temperatures

Under the temperature conditions employed in the present study, the hydrolysis of the substrate PNPP catalyzed by ALP follows Michaelis-Menten kinetics. Kinetic parameters for ALP have been determined. The results obtained at 30°C are shown in Figure 1 as Lineweaver-Burk plots.
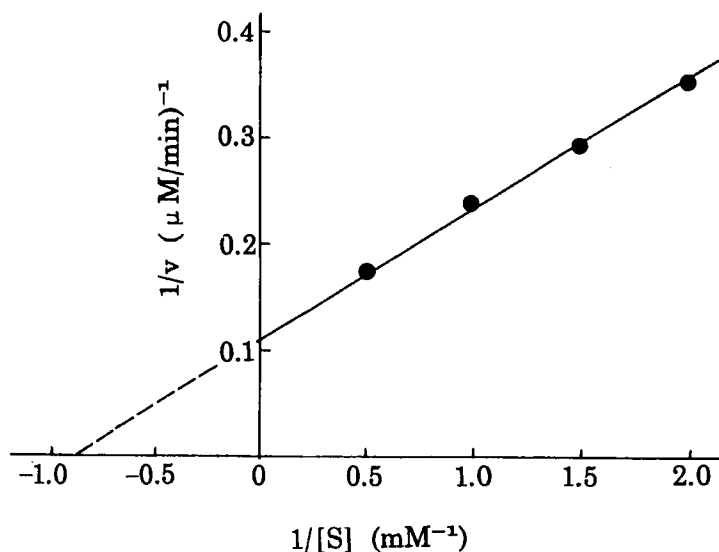

FIGURE 1   Lineweaver-Burk plot for the determination of K_m, for the green crab alkaline phosphatase. Conditions were: 0.05 M $Na_2CO_3/NaHCO_3$ buffer (pH 10.0), 2.0 mM $MgCl_2$ and PNPP at different concentrations at 30°C. Final concentration of the enzyme was 0.09 μM.

The K_m values obtained at different temperatures are very close ($K_m = 1.15$ mM) but V_{max} values are very different from each other.

Kinetic Course of the Substrate Reaction at Different Temperatures

The time courses by the hydrolysis of the substrate by green crab alkaline phosphatase at different temperatures are shown in Figure 2. It can be
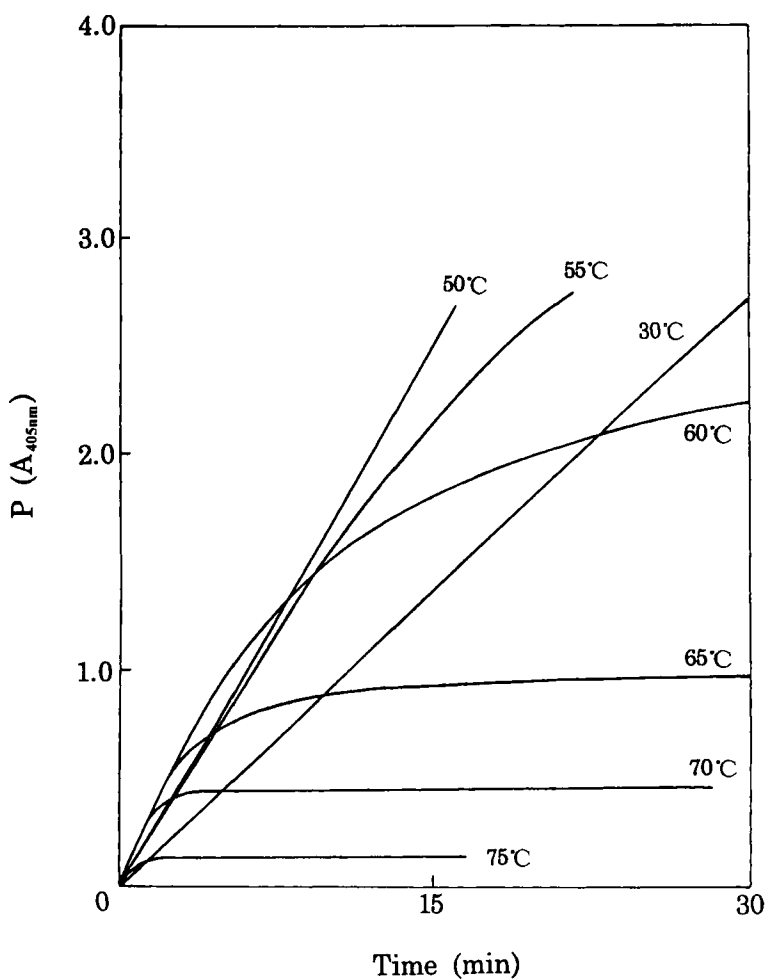

FIGURE 2 Courses for the substrate reaction of green crab alkaline phosphatase during thermal inactivation at different temperatures. Conditions were as for Figure 1 except that the concentration of PNPP was 2.0 mM. Final concentration of the enzyme was 0.09 μM. The reaction temperatures were as indicated.

seen that at a temperature upto 50°C, the progress curves remained linear showing no decrease in activity in 10 min and at 55°C there was some slight inactivation as shown by the deviation of the progress curve from linearity. At higher temperatures than 60°C, the reaction rate decreased rapidly and inactivation was complete in 15 min. When reaction time is sufficiently long, the concentration of the product, [P], approaches a constant final value $[P]_\infty$, which decreases with the elevation of temperature. The results show that the thermal inactivation of ALP is an irreversible reaction.

The semilogarithmic plots (Figure 3) for the product release show that thermal inactivation at different temperatures follows the course of a monophase first-order reactions. The values for the apparent reaction rate constant A can be obtained from the slopes and are summarized in Table I.
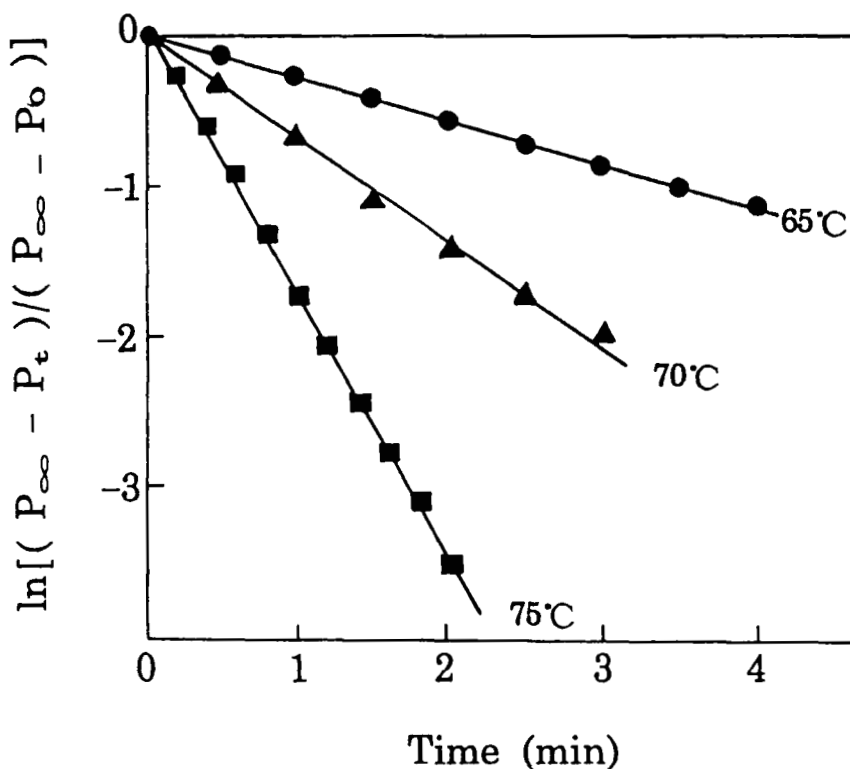

FIGURE 3 Semilogarithmic plots of the product release at different temperatures. Data were taken from corresponding curves in Figure 2. From the slopes of the straight lines the apparent rate constants of the thermal inactivation at different temperatures were obtained.

Kinetics of the Reaction at Different Substrate Concentrations During Thermal Inactivation

The kinetic course for the substrate reaction in the presence of different PNPP concentrations during thermal inactivation at 75°C are shown in Figure 4(a). The plot of $1/[P]_\infty$ against $1/[S]$ gives a straight line with slope $k_{+0}K_m/V_{max}$ and intercept k'_{+0}/V_{max} (Figure 4(b)). According to equation (3), from the slope and intercept the microscopic rate constants for the thermal inactivation, k_{+0} and k'_{+0} can be obtained (Table I).

TABLE I Rate constants for thermal inactivation green crab alkaline phosphatase at different temperatures

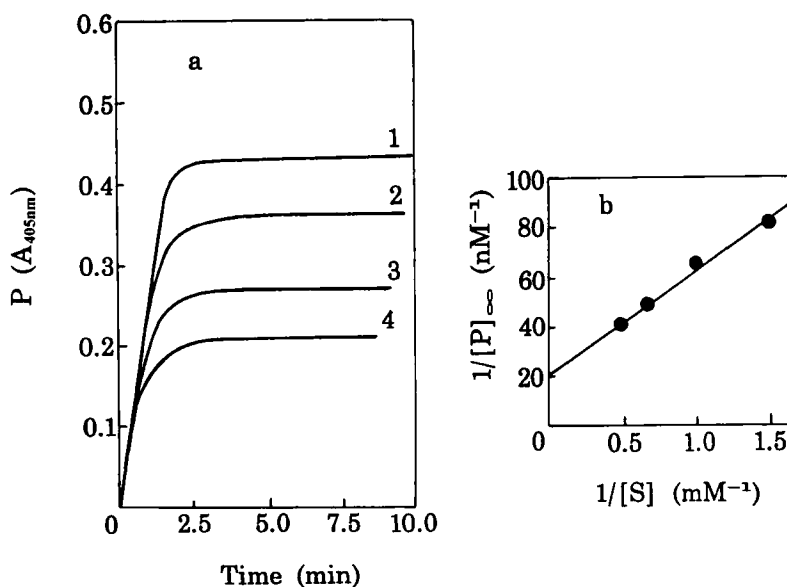| Temperature (°C)	k_{+0}/k'_{+0}	Rate constant of inactivation $(\times 10^3 \, s^{-1})$		
		$k_{app}(A)$	k_{+0}	k'_{+0}
70	1.28	11.67	12.98	10.15
75	1.78	19.17	24.05	13.50

FIGURE 4 Courses for the substrate reaction during thermal inactivation at 75°C. (a) Experimental conditions were as for Figure 1 except the presence of substrate at different concentrations. For curves 2–4, the substrate concentrations were 2.0, 1.5, 1.0 and 0.67 mM, respectively. (b) The plot of $1/[P]_\infty$ against $1/[S]$.

DISCUSSION

It is well known that the presence of metal ions helps to keep the conformation of the active site of an enzyme in a strained state.[6] For a metalloenzyme after removal of metal ions, the ordered structure of the active site is lost markedly,[7] leading to complete inactivation of the enzyme.[8] Although it is generally recognized that a metalloenzyme has a higher stability against thermal inactivation, the kinetics of the substrate reaction during thermal inactivation of metalloenzyme have been little explored. In the present investigation, the kinetic theory relating to the substrate reaction during irreversible modification of enzyme activity previously described by Tsou[3] has been applied to a study of the kinetics of thermal inactivation of ALP. Kinetics studies showed that at higher temperatures than 60°C, enzyme activity was completely lost in 15 min, and when the reaction period was sufficiently long, the concentration of the product, [P], approached a constant final value $[P]_\infty$, which decreased with elevation of the temperature. The results show that the thermal inactivation of ALP is an irreversible reaction without a residual activity. Although substrate protection against chemical modification of the essential groups at the active sites of enzymes has been previously reported,[9,10] the protection by the substrate against the thermal inactivation is of interest. Similar results were observed during inactivation of aminoacylase[11] and papain[12] by guanidinium chloride. It seems that the substrate binds at the active site by coordination with Zn^{2+} and replacing a water molecule. In addition, the substrate may also bind with other binding groups at the active site, resulting in an increase in the conformational stability of the active site.

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