Journal of Enzyme Inhibition, Vol. 16, pp. 313–319
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Kinetics of Inactivation of *Ulva pertusa Kjellm* Alkaline Phosphatase by Ethylenediaminetetraacetic Acid Disodium

DONG YANG[a], JINGYUN WANG[a,*], XIAOJUN PENG[b] and LIJIA AN[a]

[a]*Department of Bioengineering, Dalian University of Technology, No. 158, Zhongshan Road, Dalian 116012, People's Republic of China;*
[b]*State Key Laboratory of Fine Chemicals, People's Republic of China*

(Received 2 March 2001)

***Ulva pertusa Kjellm* alkaline phosphatase (EC 3.3.3.1) is a metalloenzyme, the active site of which contains a tight cluster of two zinc ions and one magnesium ion. The kinetic theory described by Tsou of the substrate reaction during irreversible inhibition of enzyme activity has been employed to study the kinetics of the course of inactivation of the enzyme by EDTA. The kinetics of the substrate reaction at different concentrations of the substrate *p*-nitrophenyl phosphate (PNPP) and inactivator EDTA indicated a complexing mechanism for inactivation by, and substrate competition with, EDTA at the active site. The inactivation kinetics are single phasic, showing that the initial formation of an enzyme–EDTA complex is a relative rapid reaction, following by a slow inactivation step that probably involves a conformational change of the enzyme. The presence of Zn^{2+} apparently stabilizes an active-site conformation required for enzyme activity.**

Keywords: Alkaline phosphatase; Kinetics; Inactivation; Zinc ion

INTRODUCTION

Alkaline phosphatase (EC 3.1.3.1) ALP, which is widely distributed in nature, is characterized by a high pH optima and a broad substrate specificity. It is a zinc-containing metalloprotein which catalyzes the nonspecific hydrolysis of phosphate monoesters.[1] The enzymes from *Escherichia coli* and mammals have been extensively studied,[2] and the X-ray crystal structure of bacterial alkaline phosphatase has been reported to be 2.0 Å resolution in the presence of inorganic phosphate.[3] The active site is a tight cluster of two zinc ions (3.9 Å separation) and one magnesium ion (5 and 7 Å from the two zinc ions). However, alkaline phosphatase from seaweeds has been little studied before, and

*Corresponding author. Fax: +86-411-3685241. E-mail: pxjun@dlut.edu.cn

neither have the kinetics of the course of inactivation. The active site of *Ulva pertusa Kjellm* alkaline phosphatase may also contain two zinc ions and one magnesium ion and the structure of its active site is probably similar to that of bacterial alkaline phosphatase. In the present investigation the complete kinetic course of EDTA inactivation, by monitoring the hydrolysis of *p*-nitrophenyl phosphatase (PNPP) has been studied. The microscopic rate constants for the inactivation reaction were determined. Kinetic analysis of the results suggests a triphasic reaction involving a rapid formation of a reversible enzyme–EDTA complex followed by a relatively slow conformational change at the active site leading to the inactivation of the enzyme before the removal of metal ions.

MATERIALS AND METHODS

The alkaline phosphatase from *Ulva pertusa Kjellm* was prepared by firstly ammonium sulfate fractionation and then the crude preparation was further purified by affinity chromatography and finally filtration through Superdex 75. The final preparation was homogeneous on polyacrylamide gel electrophoresis. The specific activity of the purified enzyme was 48.58 Unit mg^{-1}. PNPP was from Sigma, Superdex 75 was from Amersham Pharmacia and all other reagents were local products of analytical grade.

Enzyme concentration was determined by the Coomassie Brilliant Blue G-250 dyeing method[4] with bovine albumin as standard. Enzyme activity was determined at 37°C by following the increase of absorbance at 405 nm accompanying the hydrolysis of the substrate.

The progress-of-substrate reaction method described by Tsou[5] was employed for the study of the inactivation kinetics of *Ulva pertusa Kjellm* alkaline phosphatase. In the method, 10 μl of 0.274 μM alkaline phosphatase was added to 2.0 ml of reaction mixture containing 3.75 mM substrate in 1 M DEA–HCl (pH 9.8) containing different concentrations of EDTA. The reaction was carried out at a constant temperature of 37°C. Absorption and kinetic measurement were carried out on a Jasco UV-560 spectrophotometer.

RESULTS

Kinetics of Seaweed Alkaline Phosphatase

The kinetic behavior of *Ulva pertusa Kjellm* alkaline phosphatase in the hydrolysis of PNPP has been studied. Under the conditions in the present study, it was found that the hydrolysis of PNPP by *Ulva pertusa Kjellm* alkaline phosphatase followed Michaelis–Menten kinetics, and the results showed that K_m and V_{max} were 0.95 mM and 5.00 μM/min, respectively.

Kinetics of Inactivation of the Enzyme by Removing Metal Ions from the Active Site with EDTA

A conventional method was first employed to study the inactivation of seaweeds alkaline phosphatase during the removal of metal ions by EDTA. Figure 1 shows the course of inactivation of the enzyme at an EDTA concentration of 6.25 mM. The semilogarithmic plot shows a first-order reaction process with a rate constant $k = 1.88 \times 10^{-3}\ s^{-1}$.

Kinetics of the Substrate Reaction in the Presence of Different Concentrations of EDTA

The time course of the hydrolysis of the substrate in the presence of different EDTA concentrations is shown in Fig. 2. The results in Fig. 2 show that, at each concentration of EDTA, the reaction rate decreased with increasing time until a straight line is approached, the slope of which decreases with increasing EDTA concentrations. As discussed by Tsou,[5] the above suggests the slow formation of a reversible inactive enzyme–EDTA complex, comparable with the formation of complexes of trypsin with ovomucoid and soya-bean

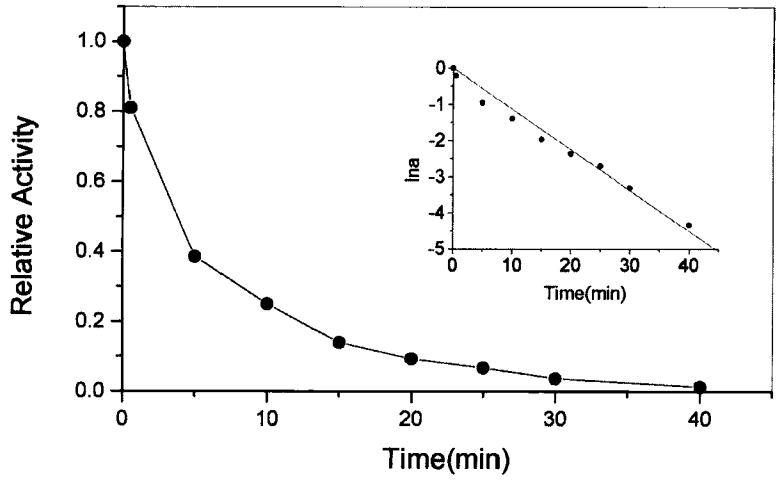


FIGURE 1 Course of inactivation of *Ulva pertusa Kjellm* alkaline phosphatase by EDTA. A mixture of the enzyme (0.137 μM) and EDTA (6.25 mM) in 1 M DEA–HCl buffer (pH 9.8) at 37°C. At different time intervals as indicated, 10 μl portions were taken for activity determination in a 2 ml reaction mixture containing 7.5 mM substrate and EDTA of respective concentration. The inset shows a semilogarithmic plot of the results.

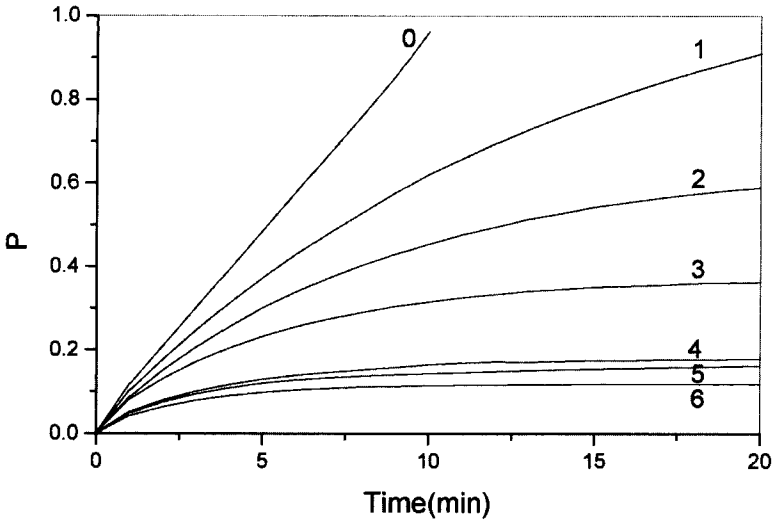
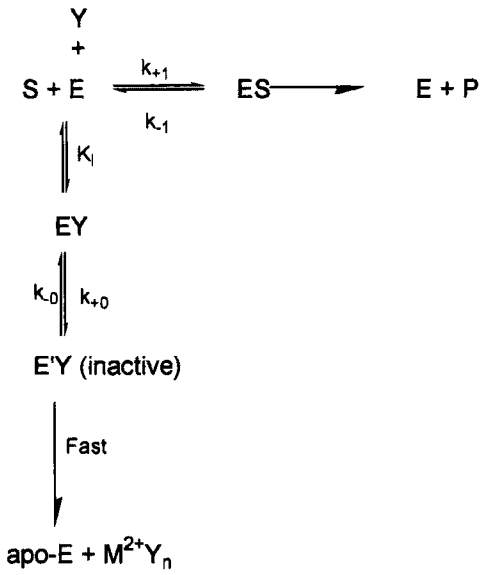


FIGURE 2 Course of the substrate reaction in the presence of different concentration of EDTA. Final conditions were: 1 M DEA–HCl (pH 9.8), 3.75 mM substrate (PNPP), 1.37 nM enzyme at 37°C. Concentration of EDTA for curves 0–6 were 0, 3.125, 6.25, 12.5, 25, 37.5, 50 mM, respectively. The enzyme (10 μl)were added to the reaction mixture (2.0 ml) to start the reaction.

trypsin inhibitor.[6] The reaction mechanism can be written as in Scheme 1, where S, P, Y, E and E′ denote substrate, product, inhibitor (EDTA), the native and conformationally changed enzyme, respectively. ES, EY and E′Y are the respective complexes. k_{+0} and k_{-0} are rate constants for forward and reverse inactivation of the enzyme, respectively. Product formation can be written as

$$[\mathrm{P}]_t = \frac{v}{A[\mathrm{Y}] + B}\left(Bt + \frac{A[\mathrm{Y}]}{A[\mathrm{Y}] + B}(1 - \mathrm{e}^{-(A[\mathrm{Y}]+B)t})\right) \quad (1)$$



SCHEME 1 Reaction mechanism for alkaline phosphatase with EDTA.

and

$$A = \frac{(k_{+0}/K_I)K_m}{K_m(1 + [Y]/K_I) + [S]} \quad (2)$$

where $[P]_t$ is the concentration of the product formed at time t, which is the reaction time, A and B are the apparent rate constants for the forward and reverse reactions between EDTA and the enzyme, respectively. [S] is the concentration of the substrate, and v is the initial rate of reaction in the presence of Y. K_m is the Michaelis constant, K_I is the dissociation constant of the enzyme–EDTA complex.

$$[P]_{calc} = \frac{vBt}{A[Y] + B} + \frac{vA[Y]}{(A[Y] + B)^2} \quad (3)$$

When t is sufficiently large, the curves become straight lines and the product concentration is written as $[P]_{calc}$ (Eq. (3)).

The slope and x-axis intercept are

$$\text{Slope} = vB/(A[Y] + B) \quad (4)$$

$$\text{Intercept} = -A[Y]/B(A[Y] + B) \quad (5)$$

Combining Eqs. (1) and (3) we get

$$\ln([P]_{calc} - [P]_t) = \ln\frac{vA[Y]}{A[Y] + B} - (A[Y] + B)t \quad (6)$$

where $[P]_{calc}$ and $[P]_t$ are the product concentrations to be expected from the straight-line portions of the curves as can be calculated from Eq. (3) and the value actually observed at time, respectively. Plots of $\ln([P]_{calc} - [P]_t)$ against t gives a series of straight lines at different concentrations of EDTA with slopes of $-(A[Y] + B)$. The apparent forward rate constant A and reverse rate constant B can be obtained.

The value of B directly gives the microscopic rate constant, $k_{-0} = 0.404 \times 10^{-3}\ s^{-1}$, for the reverse reaction and the value of k_{+0} can be obtained by suitable plots of $1/A$ against [Y] as detailed below.

It is well known that plots of $1/A$ against [Y] can differentiate complexing types of inactivation according to Tsou's method. Figure 3 shows the plot of $1/A$ against [Y], indicating that EDTA reaction with *Ulva pertusa Kjellm* alkaline phosphatase is of a complexing type. The complexing step is fast relative to the subsequent inactivation reaction.

From Eq. (2), the following equation can be derived:

$$\frac{1}{A} = \frac{K_I}{k_{+0}}\left(1 + \frac{[S]}{K_m}\right) + \frac{1}{k_{+0}}[Y] \quad (7)$$

A plot of $1/A$ against [Y] gives a straight line (Fig. 3), as K_m is a known quantity, and from the slope of the straight line the microscopic rate constant can be obtained $k_{+0} = 6.38 \times 10^{-3}\ s^{-1}$.

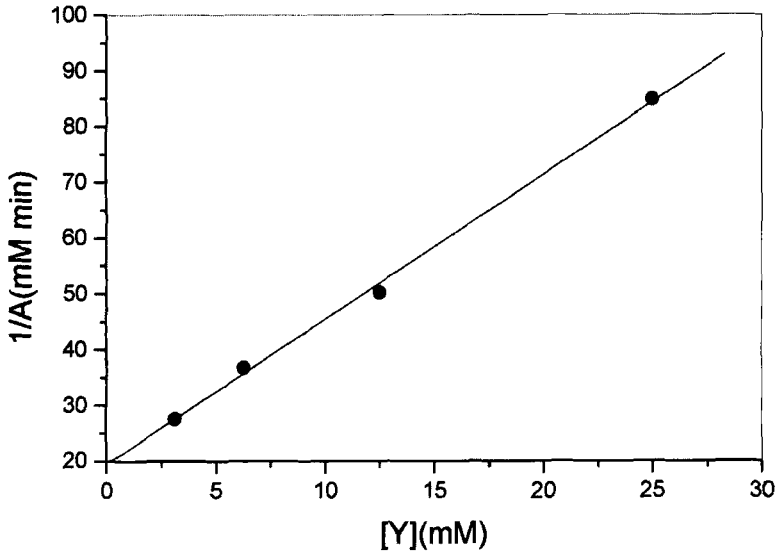


FIGURE 3 Plot of $1/A$ against EDTA concentration, The values of the apparent rate constant A were calculated from Fig. 2 through semilogarithmic plots of $\ln([P]_{calc} - [P]_t)$ against time. For details see the text.

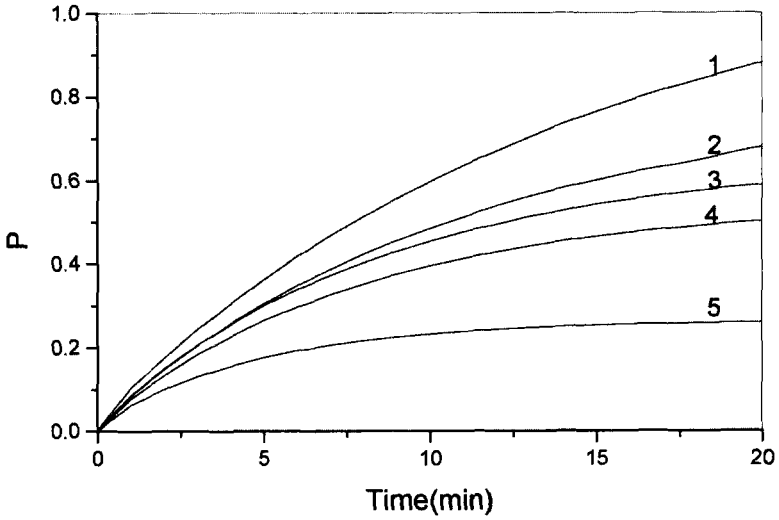


FIGURE 4 Course of the reaction at different substrate concentrations in the presence of EDTA. Final concentrations were 6.25 mM EDTA and 1.37 nM enzyme. Concentration of the substrate for curves 1–5 were 6.0, 4.5, 3.75, 3.0, 1.5 mM, respectively. The enzyme was added to the reaction mixture to start the reaction.

Kinetics of the Reaction of Seaweed Alkaline Phosphatase at Different Substrate Concentrations in the Presence of EDTA

Figure 4 shows the course of the substrate reaction at different PNPP concentrations in the presence of EDTA. It can be seen that when time is sufficiently large both the initial rate and the slope of the asymptote increase with the increase in substrate concentration. Similarly plots of $\ln([P]_{calc} - [P]_t)$ against t give a series of straight lines at different concentrations of the substrate with slope of $-(A[Y] + B)$. The apparent forward rate constant A and B can be

obtained, the value of B directly gives the microscopic rate constant, $k_{-0} = 0.406 \times 10^{-3}\,s^{-1}$, for the reverse reaction, which is almost the same as that obtained previously. Figure 5 shows that the apparent forward rate constant A obtained is dependent on substrate concentration and a plot of $1/A$ against [S] gives a straight line with a positive intercept at the y-axis, indicating competition between EDTA and the substrate, as discussed previously by Tsou.[5] The dissociation constant $K_I = 2.55$ mM, can be obtained from the slope of the straight line.

DISCUSSION

The action of EDTA on zinc enzymes has been extensively studied, and it is generally accepted that EDTA inactivates by chelating with or by removal of Zn^{2+} and that the activity can be restored by the addition of Zn^{2+} or other metal ions.[7–9] Kinetic studies now show that the dissociation is preceded by a relatively rapid step of reversible binding at the active site, presumably by complexing with Zn^{2+}, followed by a slow inactivation step. The second slow step probably involves changes from a strained, entactic and active state to a conformationally more stable and inactive state. In the third step, zinc ions are rapidly removed from the active site of the enzyme to form a complex of Zn^{2+}–EDTA. The second step is rate-limiting, so the rate of this step represents the inactivation reaction rate. The semilogarithmic plots for the inactivation reaction obtained in the present study are monophasic, indicating that the above reaction mechanism is suitable.

Although substrate protection against EDTA inactivation has been previously reported for other zinc enzymes, the competition between EDTA and the substrate is of interest. It seems that the substrate binds at the active site by co-ordinating with zinc ion and replacing a water molecule, as suggested by Henseling and Röhm[10] and that EDTA competes with the substrate in co-ordinating with zinc ion. It is possible that EDTA binding, like that of the substrate, results in a change releasing the strained conformational state which is required for catalysis by the enzyme.

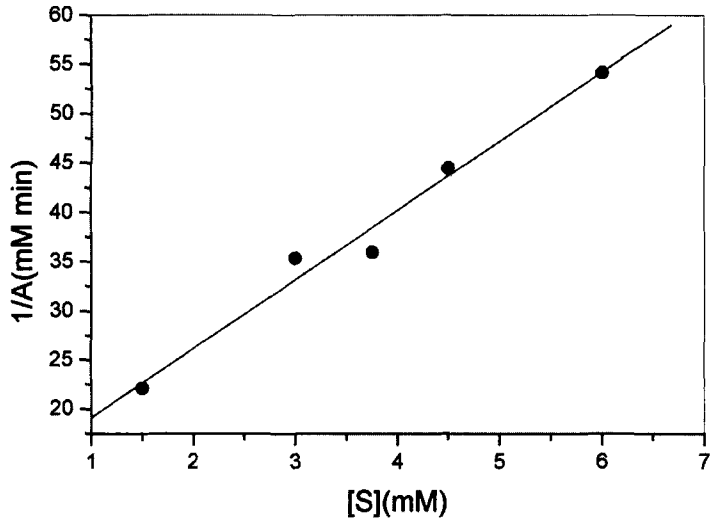


FIGURE 5 Plot of $1/A$ against substrate concentration. The value of the apparent rate constant A was calculated from data in Fig. 4 through semilogarithmic plots of $\ln([P]_{calc} - [P]_t)$ against time. For details see the text.

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