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Unfolding and Inactivation of Abalone (*Haliotis diversicolor*) Alkaline Phosphatase During Denaturation by Guanidine Hydrochloride

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Abstract Abalone, a kind of low poikilothermic invertebrate, is easily exposed to ocean environment stress. Since it is one of the important mariculture animals, the attention paid to the abalone study becomes increasing. Alkaline phosphatase (ALPase, EC 3.1.3.1) is a kind of zinc-contained metalloenzyme, which catalyzes the nonspecific hydrolysis of phosphate monoesters. Unfolding and inactivation of ALPase from abalone (*Haliotis diversicolor*) during denaturation by guanidine hydrochloride (GuHCl) of different concentrations has first been studied. The kinetic theory of the substrate reaction by enzyme was described by Tsou, which was applied to the study on ALPase's kinetic course of inactivation by GuHCl. The result showed that the inactivation of the enzyme by GuHCl was a slow, reversible reaction with fractional remaining activity. The microscopic rate constants were determined. The result, $k_{+0} > k'_{+0}$, showed that the enzyme was protected by the substrate to a certain extent during guanidine denaturation. The changes of conformation of the enzyme in different concentrations of GuHCl have been studied by means of measuring the fluorescence spectra. The results showed that the inactivation occurred before the noticeable conformational changes of the enzyme molecule as a whole can be detected, which suggests that the active site of the enzyme has more flexibility than the whole enzyme molecule. These studies will facilitate the understanding of physiological and biochemical features of the *H. diversicolor* and will also help in the understanding of the abalone immune system.

Keywords *Haliotis diversicolor* · Alkaline phosphatase · Denaturation and inactivation · Guanidine hydrochloride · Kinetics

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Introduction

Abalone is a low poikilothermic invertebrate, which is one of the important mariculture kinds in China. Since the taste of abalone is very palatable and at the same time the shell of abalone can be used as medicine to cure many diseases [1], abalone therefore possesses important economic value and pharmic value, but the mariculture of abalone is not very easy, because it is easily exposed to ocean environment stress and subject to catastrophic infectious disease outbreaks frequently in the mariculture of abalone [2]. So the attention paid to abalone immune and stress responses study is on the increase [3].

Alkaline phosphatase (ALPase, EC 3.1.3.1,), a metalloenzyme, which catalyzes the nonspecific hydrolysis of phosphate monoesters, is an important enzyme in cell phosphate metabolism [4]. The X-ray crystal structure of bacterial ALPase showed that the active site was a pocket containing a tight cluster of two zinc ions and one magnesium ion [4, 5]. In the last decade, ALPase from mammalian and microbes has been extensively investigated because of its important physiological function [6, 7]. Although the enzyme from *Escherichia coli* and human has been extensively studied, little is known about the enzyme from gastropod. A study involving crustaceans showed that ALPase might be involved in calcium secretion [8], while another study on clam suggested that the ALPase activity could be positively related to shell deposition [9]. Another study on response of innate immune factors in *Haliotis diversicolor supertexta* used ALPase as an indicator, and the results showed that ALPase activity changed when the abalone challenged with *Vibrio parahaemolyticus* [10].

Since ALPase is one of the important enzymes in the gastropod and abalone ALPase has not been extensively studied so far, we chose abalone ALPase as our study objective. In previous studies described by author, we purified and illustrated many enzymatic properties of ALPase from *H. diversicolor*, which was composed of two identical subunits with 92 kDa [11]. To explore the relationship between the structure and function of ALPase from *H. diversicolor*, we studied the unfolding and inactivation of this enzyme during denaturation by GuHCl. In our ongoing research, GuHCl was found to be a strong denaturant and inactivator to the enzyme. It can lead to the conformational change of the enzyme molecule and the complete loss of the enzyme activity. It has been reported by many authors that during the denaturation of a number of enzymes under different conditions, inactivation occurred before noticeable conformational change of the enzyme molecule as a whole can be detected [12, 13]. Therefore, Tsou suggested that the enzyme active site was formed by relatively weak molecular interactions and, hence, may be more flexible than the whole enzyme [14]. Unfolding and inactivation of green crab (*Scylla serrata*) alkaline phosphatase [15], prawn (*Penaeus penicillatus*) acid phosphatase [16] and big-vase (*Ampullarium crossean*) glucosidase [17] during denaturation by denaturants have been reported with similar results. Meanwhile, the present paper dealt with a study of the conformational changes during guanidine denaturation monitored by fluorescence properties and with an effort to make a comparison between enzyme inactivation and unfolding. These studies will facilitate the understanding of physiological and biochemical features of the *H. diversicolor* and will also help in the understanding of the abalone immune system.

Materials and Methods

Materials

ALPase was prepared from *H. diversicolor* as described previously [11]. The crude preparation was further purified by ion exchange gel DEAE-32, then by filtration

chromatograph through Sephadex G-200, and ion exchange gel DEAE-32. The final enzyme preparation was homogeneous as judged by electrophoresis on polyacrylamide gel in the absence and the presence of SDS. The specific activity of the enzyme is 1,226 units/mg of protein. *p*-Nitrophenyl phosphate (pNPP) was a Sigma product; GuHCl was a Sigma product of ultra grade; DEAE-32 and Sephadex G-200 were Pharmacia products. All other reagents were local products of analytical grade.

Assay

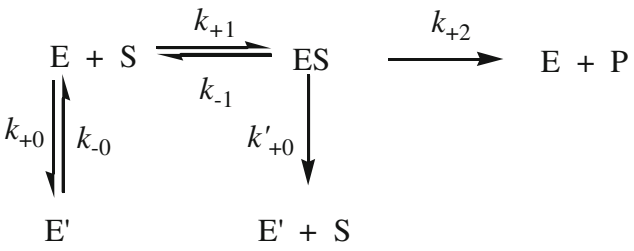
Enzyme concentration was determined as described by Lowry [18]. Enzyme activity was assayed at 37 °C by following the increasing absorbance at 405 nm accompanying the hydrolysis of the substrate (pNPP). Enzyme (10 μl) was added to 2 ml of activity assay system that contained 2 mM pNPP, 0.05 M $NaCO_3$–$NaHCO_3$ buffer, pH 10.1. Absorption measurements were recorded using a Beckman DU-650 spectrophotometer. The enzyme activity was calculated by the increased absorption of the reaction mixture at 405 nm using a molar absorption coefficient 1.73×10^4 M^{-1} cm^{-1} [15].

Enzyme denaturation was carried out at 4 °C in a solution containing different concentration GuHCl in 0.1 M Tris–HCl buffer (pH 7.5) for 24 h. Denatured enzyme solutions were used for activity assay and measurements of fluorescence spectra. Fluorescence spectrum measurements were made with a Hitachi 850 spectrofluorometer. The excitation wavelength was 279 nm.

Inactivation Rate Constants of ALPase in GuHCl Solutions

The progress-of-substrate-reaction method previously described by Tsou [19] was used for the study of the inactivation kinetics of *H. diversicolor* ALPase. In this method, 10 μl of enzyme was added to 2.0 ml of reaction mixture, with 2 mM pNPP in 0.05 M $NaCO_3$–$NaHCO_3$ buffer (pH 10.1) containing different concentrations of GuHCl. The reaction was carried out at a constant temperature of 30 °C. The substrate reaction progress curve was analyzed to obtain the rate constants as detailed below. The time course of the hydrolysis of the substrate in the presence of different GuHCl concentrations showed that the rate decreased with increasing time until a straight line was approached. The results showed that the inactivation of the enzyme was a reversible reaction with fractional residual activity. This can be written as Scheme 1 [15], where S, P, E, and E′ denote substrate, product, and the native and inactivated enzyme, respectively; ES is the native enzyme–substrate complex; and E′S is the inactivated enzyme–substrate complex. k_{+0} and k_{-0} are rate constants for forward and reverse inactivation of the free enzyme, respectively; k'_{+0} is the

Scheme 1 A scheme



inactivation rate constants of enzyme–substrate complex. As is usual for the case [S]»[E_o], the product formation can be written as:

$$[P]_t = \frac{vk_{-0}}{A} \times t + \frac{v}{A^2}(A - k_{-0})\left(1 - e^{-At}\right) \tag{1}$$

where $[P]_t$ is the concentration of the product formed at time t, which is the reaction time; A is the apparent rate constant of inactivation; [S] is the concentration of the substrate; and v is the initial rate of reaction in the absence of denaturant (GuHCl).

$$A = \frac{k_{+0} \cdot K_m + k_{+0}[S]}{K_m + [S]} + k_{-0} \tag{2}$$

$$v = \frac{V_m \times [S]}{K_m + [S]} \tag{3}$$

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

$$[P]_{calc} = \frac{vk_{-0}}{A} \times t + \frac{v}{A^2}(A - k_{-0}) \tag{4}$$

Combining Eqs. 1 and 4 yields:

$$[P]_{calc} - [P]_t = \frac{v}{A^2}(A - k_{-0}) \times e^{-At} \tag{5}$$

$$\ln\left([P]_{calc} - [P]_t\right) = -A \times t + \text{constant}, \tag{6}$$

where $[P]_{calc}$ is the product concentration to be expected from the straight-line portions of the curves as calculated from Eq. 4, and $[P]_t$ is the product concentration actually observed at time t. Plots of $\ln([P]_{calc} - [P]_t)$ versus t give a series of straight lines at different concentrations of denaturant with slopes of $-A$. The apparent rate constant A can be obtained from such graphs. From Eq. 4, a plot of $[P]_{calc}$ against time, t, gives a straight line with a slope of vk_{-0}/A. From the slope of the straight line, k_{-0} can be obtained.

Combining Eqs. 2 and 3 gives:

$$\frac{A}{v} = \frac{K_m}{V_m}(k_{+0} + k_{-0}) \times \frac{1}{[S]} + \frac{k'_{+0} + k_{-0}}{V_m} \tag{7}$$

A plot of A/v versus $1/[S]$ gives a straight line with $K_m \times k_{+0}/V_{max}$ and k'_{+0}/V_{max} as the slope and intercept, respectively. As K_m and V_{max} are known quantities from measurements of the substrate reaction in the absence of GuHCl at different substrate concentrations, the rate constants k_{+0} and k'_{+0} can be obtained from the slope and intercept of the straight line, respectively.

Results

Determination of the Kinetic Parameters of *H. diversicolor* ALPase

The kinetic behavior of *H. diversicolor* ALPase during the hydrolysis of pNPP was studied. Under the conditions employed in the present investigation, the hydrolysis of pNPP by the

enzyme followed Michaelis–Menten kinetics. The kinetic parameters for *H. diversicolor* ALPase obtained from Lineweaver–Burk plot (Fig. 1) showed that K_m was equal to 0.857 mM and V_m was equal to 21.05 μM/min.

Effect of GuHCl on *H. diversicolor* ALPase Activity

The enzyme was incubated with different concentrations of GuHCl in 0.1 M Tris–HCl buffer, pH 7.5 at 4 °C for 24 h. Then, 10 μl of each samples was taken to measure any remaining activity in 2 ml 0.05 M $NaCO_3$–$NaHCO_3$ buffer, pH 10.1 containing 2 mM pNPP as substrate and corresponding concentrations of GuHCl. The effect of GuHCl on the enzyme was concentration dependent (Fig. 2). Increasing GuHCl concentrations caused the enzyme activity to markedly decrease. When the GuHCl concentration reached 3.0 M, the inactivation of the enzyme was irreversible, and the enzyme was completely inactivated.

The Inactivation Mechanism of *H. diversicolor* ALPase During Denaturation in GuHCl Solutions

The inactivation mechanism of the enzyme in GuHCl solution for the hydrolysis of pNPP was studied. Figure 3 showed the relationship of enzyme activity with its concentration in the presence of different concentrations of GuHCl. The plots of the remaining enzyme activity versus the concentrations of enzyme in the presence of different concentrations of GuHCl gave a family of straight lines, which all passed through the origin. Increasing the GuHCl concentration resulted in a decrease in the slope of the line, indicating that the inactivation of GuHCl on the enzyme was a reversible reaction when

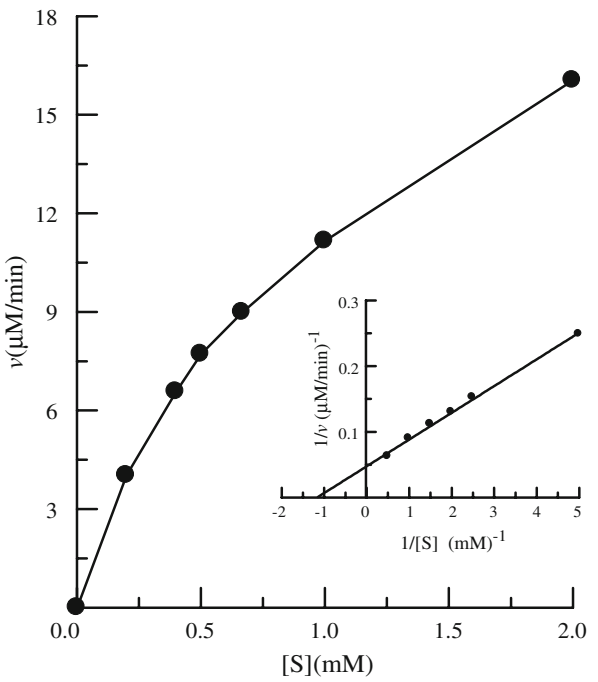


Fig. 1 Lineweaver–Burk plot for the determination of K_m and V_m of *H. diversicolor* ALPase for the hydrolysis of pNPP

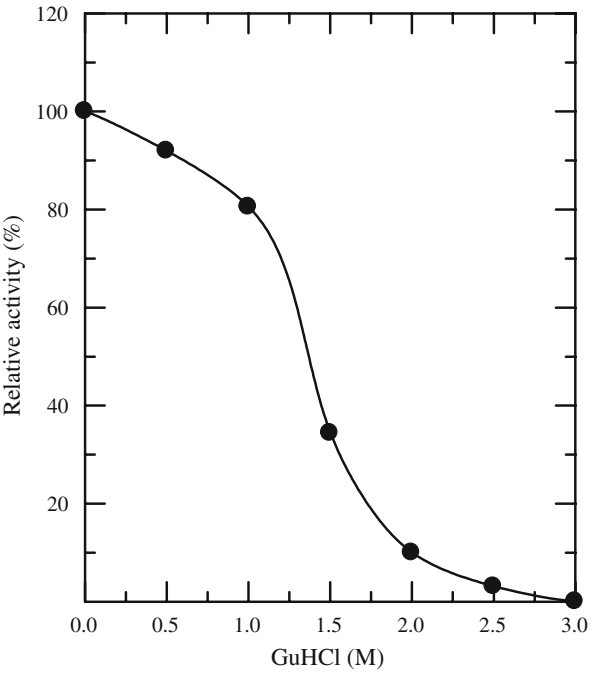


Fig. 2 Inactivation of *H. diversicolor* ALPase in GuHCl solutions of different concentrations. The enzyme was incubated with GuHCl at different concentrations, 0.1 M Tris–HCl buffer, pH 7.5 at 4 °C for 24 h. Then, 10 μl of samples was taken for measurement of remaining activity in 2 ml 0.05 M $NaCO_3$–$NaHCO_3$ buffer, pH 10.1 containing 2 mM pNPP as substrate and corresponding concentrations of GuHCl

the concentration of GuHCl was under 2.0 M. The presence of GuHCl did not bring down the amount of the efficient enzyme, but instead resulted in the inhibition and the decrease in the activity of the enzyme. GuHCl is a reversible inactivator of ALPase for hydrolysis of pNPP.

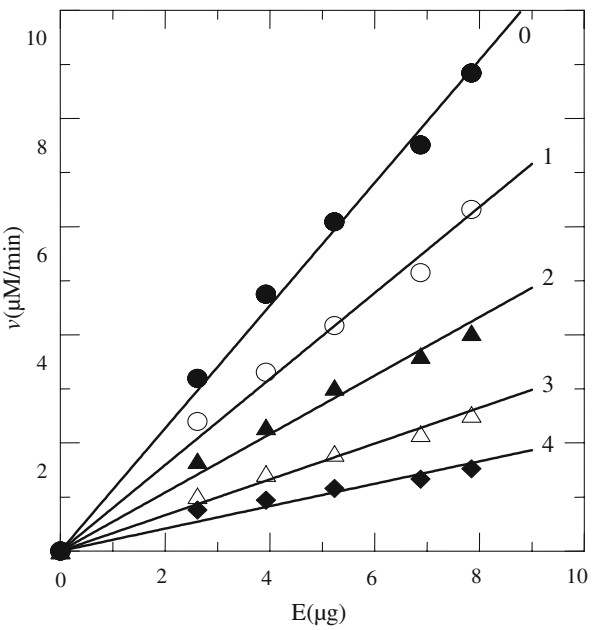


Fig. 3 Effects of ALPase concentration on its activity for the hydrolysis of pNPP at different concentrations of GuHCl. The concentrations of GuHCl for curves *1–4* were 0, 0.5, 1.0, 1.5, and 2 M, respectively

Measurement of Inactivation Rate Constant of *H. diversicolor* ALPase During Denaturation in GuHCl Solutions

The temporal variation of the product concentration during the substrate hydrolysis in the presence of different GuHCl concentrations was shown in Fig. 4. At each concentration of GuHCl, the rate decreased with increasing time until a straight line was approached, the slope of which decreased with increasing GuHCl concentration. The results suggested that denatured enzyme still had partial residual activity (Fig. 4a, curves 1–6). According to Eq. 6, plots of ln ($[P]_{calc}-[P]_t$) versus t give a series of straight lines shown in Fig. 4b. From the slopes of the straight line, the apparent inactivation rate constant A can be obtained.

The kinetic courses of the hydrolysis reaction at different substrate concentrations in the presence of 1.0 M GuHCl were shown in Fig. 5a. In the presence of 1.0 M GuHCl, when the time was sufficiently long, a straight line was approached at each concentration of substrate. Both the initial rate and the slope of the asymptote increased with increasing substrate concentration (Fig. 5a). From Eq. 6, plots of ln ($[P]_{calc}-[P]_t$) versus t give a series of straight lines at different concentrations of substrate, whose slopes are equal to the apparent forward rate constant A (Fig. 5b).

Since K_m and V_{max} were quantities known, the values of k_{+0} and k'_{+0} can be obtained from the slope and the intercept of the straight line in Fig. 5c, a plot of A/v versus 1/[S] according to Eq. 7. The above results were shown in Table 1. Similarly, the inactivation rate constants of *H. diversicolor* ALPase at other GuHCl concentrations were also obtained (Table 1).

Fluorescence Emission Spectra of *H. diversicolor* ALPase after Denatured by GuHCl

The fluorescence emission spectra of *H. diversicolor* ALPase denatured in different concentrations of GuHCl were shown in Fig. 6. The emission peak of the native enzyme

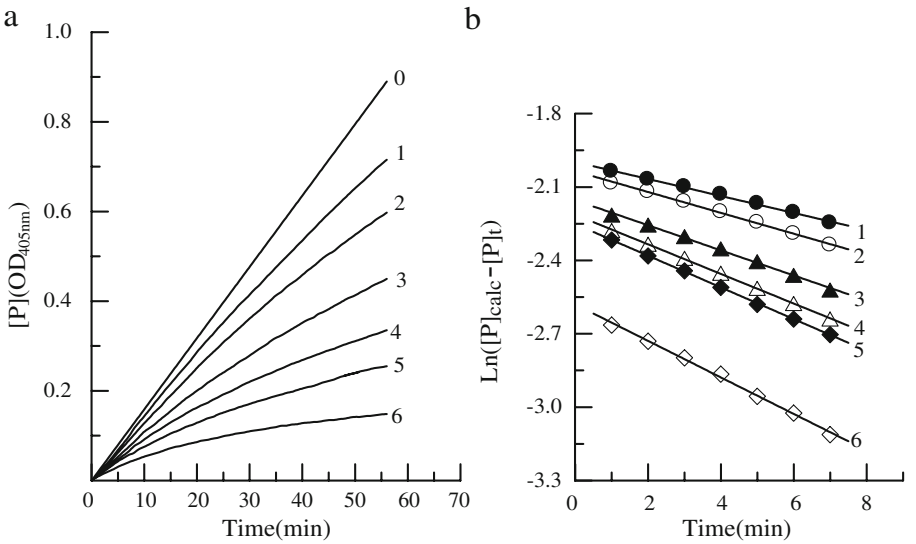


Fig. 4 Course of inactivation of *H. diversicolor* ALPase in GuHCl solution of different concentrations. Final pNPP concentrations were 0.5 mM in 0.05 M $NaCO_3$–$NaHCO_3$ buffer (pH 10.1). Molar concentrations of GuHCl for curves *0–5* were 0, 1.2, 1.4, 1.6, 1.8, and 2 M, respectively. Final enzyme concentration was 0.075 μM

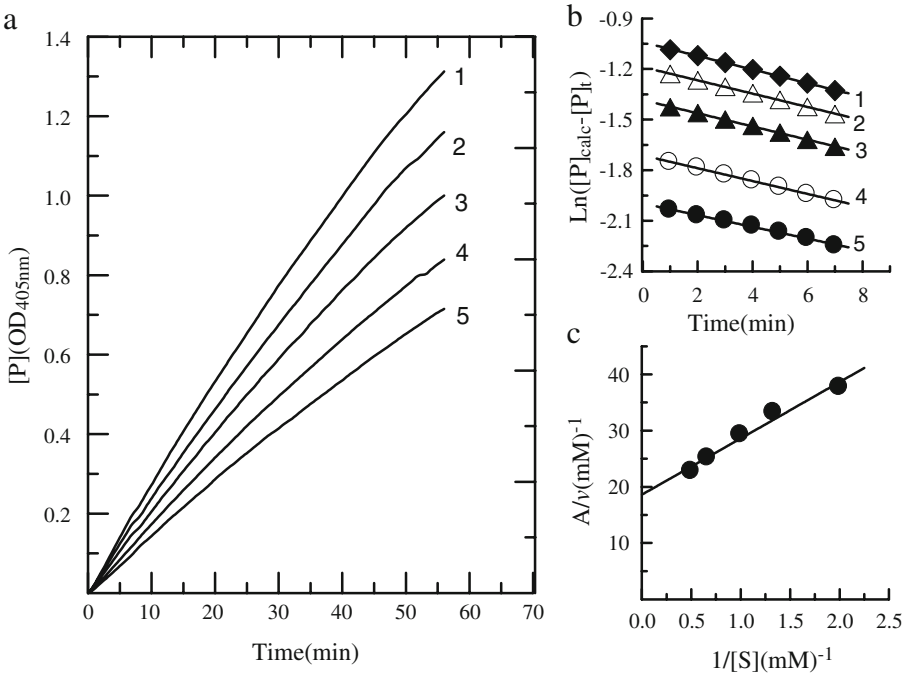


Fig. 5 Determination of inactivation rate constants of *H. diversicolor* ALPase by 1.0 M GuHCl. **a** Substrate reaction course of the enzyme in the presence of 1.0 M GuHCl. Curves *1–5* are progress curves with 2.0, 1.5, 1.0, 0.75, and 0.5 mM of pNPP, respectively. Final enzyme concentration was 0.075 μM. **b** Semilogarithmic plots of ln ($[P]_{calc}-[P]_t$) against time for data given in **a**. The numbers of lines are as for **a**

was at 335.4 nm; which might contain contributions from both Trp residues and Tyr residues. The emission peak at 335.4 nm decreased in intensity and red-shifted with the increasing concentrations of GuHCl. As increasing concentration of denaturant to 2.0 M, a small shoulder fluorescence emission peak appeared at about 315 nm. With increasing concentration of GuHCl, marked shoulders were observed. The appearance of the 315 nm peak indicated the disruption of the energy transfer between Tyr and Trp

Table 1 Microscopic rate constants of the inactivation of *H. diversicolor* ALPase in GuHCl solutions.

GuHCl (M)	Rate constants ($\times 10^3$ s^{-1})			Residual activity (%)
	k_{+0}	k_{-0}	k'_{+0}	
0				100
1.0	0.029	0.023	0.007	79
1.2	0.039	0.023	0.008	62
1.4	0.047	0.019	0.009	43
1.6	0.057	0.015	0.010	31
1.8	0.058	0.011	0.012	18
2.0	0.070	0.006	0.013	11

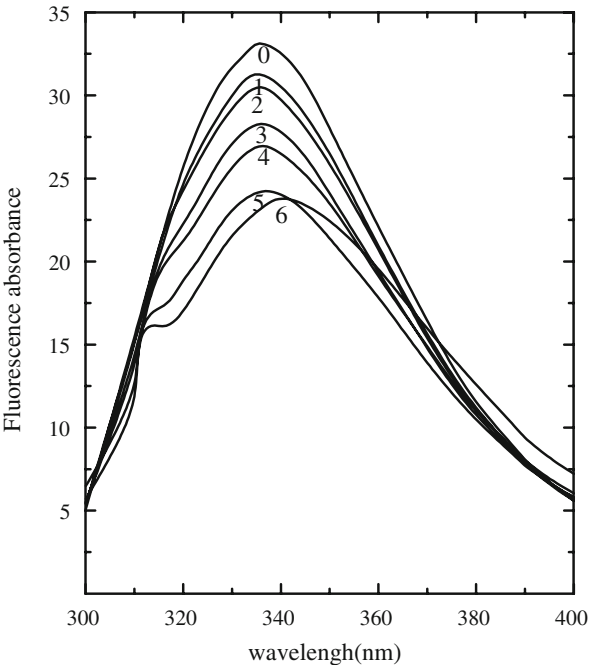


Fig. 6 Fluorescence emission spectra of *H. diversicolor* ALPase denatured in GuHCl solutions of different concentrations. The enzyme was dissolved in 0.05 M $NaCO_3$–$NaHCO_3$ buffer at pH 10.1 containing GuHCl at the desired concentrations. The solutions were allowed to stand at 4 °C for 24 h before fluorescence measurement. The excitation wavelength was 279 nm. Concentrations of GuHCl for curves *0–6* were 0, 0.5, 1, 1.5, 2, 2.5, and 3 M, respectively

residues as a result of the unfolding of the peptide chain. Further increase of GuHCl concentration to 3.0 M produced slight decrease of the fluorescence emission intensity, and the red shift of the emission maximum continued to reach a final value of 339.4 nm.

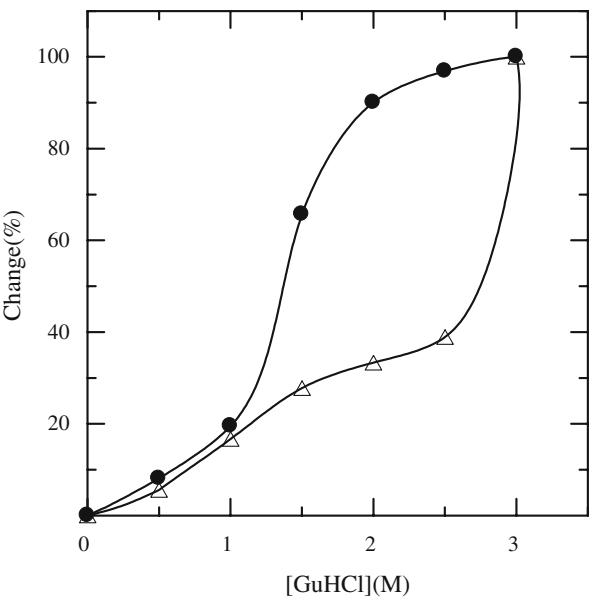


Fig. 7 Comparison of inactivation and unfolding of *H. diversicolor* ALPase during denaturation by GuHCl. Experimental conditions of inactivation were as for Fig. 2. Unfolding data were taken from Fig. 6. Extent of inactivation (*circle*); red shift of fluorescence emission maximum (*triangle*)

Comparison of Inactivation and Unfolding of *H. diversicolor* ALPase During Denaturation in GuHCl Solutions

The extent of the red shift of the fluorescence emission maxima of *H. diversicolor* ALPase during denaturation in GuHCl symbolizes the unfolding of the enzyme. The extent of inactivation of the enzyme was compared with the extent of the unfolding of the enzyme. The results, shown in Fig. 7, showed that much lower concentrations of GuHCl were required to bring about inactivation than were required to produce significant conformational changes of the enzyme molecules.

Discussion

It is well known that the activity of enzymes is strongly dependent on their conformational integrity. Tsou [19, 20] suggested that enzyme active sites were usually situated in a limited region of the enzyme molecule, which was more flexible than the enzyme molecule as a whole. Some authors have studied the relationship between the inactivation and unfolding of the enzymes containing metal ions as prosthetic groups [21–23]. The metal ions as prosthetic groups are usually situated in the enzyme active site and are helpful for keeping the conformation of the active site in a strained state [24]. The present paper compared the inactivation with conformational changes of the enzyme during denaturation by GuHCl. The results clearly indicated that the conformational integrity was important for enzyme activity. In addition, the results in Table 1 showed that for a given denaturant concentration, the inactivation rate constants were much faster than the unfolding rate constants for the enzyme. This may be due to the substrate protecting of the enzyme during inactivation by GuHCl. Similar results were obtained during inactivation of aminoacylase by guanidine hydrochloride [21] and urea [22], as well as during inactivation of papain [25]. Those authors suggested that the substrate should be situated and bound at the active site of the enzyme and that the binding of the substrate and the enzyme should increase the conformational stability of the active site. However, even though the substrate protects the enzyme activity, the inactivation rate constants are much faster than the unfolding rate constants of the enzyme. Therefore, the above results suggested that the active sites of *H. diversicolor* ALPase should be situated in a limited region that has more conformational flexibility than the enzyme molecule as a whole.

Nevertheless, in the present study, we also compared the inactivation and unfolding of *H. diversicolor* ALPase during denaturation in GuHCl solutions. The result showed that much lower concentrations of GuHCl were required to bring about inactivation than were required to produce significant conformational changes of the enzyme molecules, which also supports Tsou's theory that enzyme active sites were usually situated in a limited region of the enzyme molecule and were more flexible than the enzyme molecule as a whole.

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References

1. Yan, J. P., & Liu, S. L. (1998). Status of culturing abalone (*Haliotis Discus Hannai*) and its problems and countermeasures for development in China. *Marine Fisheries Research*, *19*, 91–96.

2. Zhang, Z., Wang, J., Su, Y., Yah, Q., Chi, X., Zhou, H., & Zhou, Y. (2001). Pathogeny and bistopathology of the epidemic disease in *Haliotis diversicolor supertexta*. *Journal of Xiamen University*, *40*, 949–956, Natural Science.
3. Celia, H., Rob, D., Ron, S., Judith, H., & Kirsten, B. (2007). Stress and immune responses in abalone: Limitations in current knowledge and investigative methods based on other models. *Fish & Shellfish Immunology*, *22*, 363–379. doi:10.1016/j.fsi.2006.06.009.
4. Coleman, J. E. (1992). Structure and mechanism of alkaline phosphatase. *Annual Review of Biophysics and Biomolecular Structure*, *21*, 441–483. doi:10.1146/annurev.bb.21.060192.002301.
5. Jones, S. R., Kindman, L. A., & Knowles, J. R. (1978). Stereochemistry of phosphoryl group transfer using a chiral [16O, 17O, 18O] stereochemical course of alkaline phosphatase. *Nature*, *275*, 564–565. doi:10.1038/275564a0.
6. Halford, S. E., & Schlesinger, M. J. (1974). Mutationally altered rate constants in the mechanism of alkaline phosphatase. *The Biochemical Journal*, *141*, 845–852.
7. Hamilton, T. A., Gornicki, S. Z., & Sussman, H. H. (1979). Alkaline phosphates from human milk. Comparison with isoenzymes from placenta and liver. *The Biochemical Journal*, *177*, 197–201.
8. Meyran, J. C., & Graf, F. (1986). Ultrahistochemical localization of Na^+–K^+ ATPase, Ca^{2+}–ATPase and alkaline phosphatase activity in a calcium-transporting epithelium of a crustacean during moulting. *Histochemistry*, *85*, 313–320. doi:10.1007/BF00493483.
9. Blasco, J., Puppo, J., & Sarasquete, C. (1993). Acid and alkaline phosphatase activities in the clam Ruditapes philippinarum. *Marine Biology (Berlin)*, *115*, 113–118. doi:10.1007/BF00349392.
10. Wang, S. H., Wang, Y. L., Zhang, Z. X., Ralph, J., Weng, Z. H., Zou, Z. H., et al. (2004). Response of innate immune factors in abalone *Haliotis diversicolor supertexta* to pathogenic or nonpathogenic infection. *Journal of Shellfish Research*, *23*, 1173–1177.
11. Liao, J. H., Chen, Q., Lin, L. R., Hong, Y. F., & Chen, Q. X. (2005). Purification and Characterization of Alkaline Phosphatase from Haliotis diversicolor. *Journal of Xiamen University*, *44*, 272–275, Natural Science.
12. Zhou, H. M., Zhang, X. H., Yin, Y., & Tsou, C. L. (1993). Conformational changes at the active site of creatine kinase at low concentrations of guanidinium chloride. *The Biochemical Journal*, *291*, 103–107.
13. Ma, Y. Z., & Tsou, C. L. (1991). Comparison of the activity and conformation changes of lactate dehydrogenase H4 during denaturation by guanidinium chloride. *The Biochemical Journal*, *277*, 207–210.
14. Tsou, C. L. (1993). Conformational flexibility of enzyme active sites. *Science*, *262*, 380–381. doi:10.1126/science.8211158.
15. Chen, Q. X., Zhang, W., Zheng, W. Z., Zhang, Z., Yan, S. X., Zhang, T., et al. (1996). Comparison of inactivation and unfolding of green crab (*Scylla serrata*) alkaline phosphatase during denaturation by guanidinium chloride. *Journal of Protein Chemistry*, *15*, 359–365. doi:10.1007/BF01886862.
16. Chen, S. L., Chen, Q. X., Yang, P. Z., Qiu, W. J., Wang, L. T., & Zhou, H. M. (1997). Unfolding and inactivation of *Penaeus penicillatus* acid phosphatase during denaturation by guaniding hydrochloride. *Biochemistry and Molecular Biology International*, *42*, 517–526.
17. Chen, Q. X., Zhang, Z., Huang, H., Zhao, F. K., & Xu, G. J. (2003). Unfolding and inactivation of *Ampullarium crossean*, β-glucosidase during denaturation by guanidine hydrochloride. *The International Journal of Biochemistry & Cell Biology*, *35*, 1227–1233. doi:10.1016/S1357-2725(02)00266-2.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Ranadall, R. J. (1951). Protein measurement with the Folin–phenol reagent. *The Journal of Biological Chemistry*, *193*, 265–275.
19. Tsou, C. L. (1988). Kinetics of substrate reaction during irreversible modification of enzyme activity. *Advances in Enzymology and Related Areas of Molecular Biology*, *61*, 381–436. doi:10.1002/9780470123072.ch7.
20. Tsou, C. L. (1986). Location of the active sites of some enzymes in limited and flexible molecular regions. *Trends in Biochemical Sciences*, *11*, 427–429. doi:10.1016/0968-0004(86)90178-7.
21. Wang, H. R., Zhang, T., & Zhou, H. M. (1995). Comparison of inactivation and conformational changes of aminoacylase during guanidinium chloride denaturation. *Biochimica et Biophysica Acta*, *1248*, 97–106.
22. Wang, H. R., Wang, X. C., Zhang, T., & Zhou, H. M. (1995). Comparison between conformational change and inactivation rates of aminoacylase during denaturation in urea solutions. *Science in China. Series B, Chemistry, Life Sciences & Earth Sciences*, *38*, 328–335.
23. He, B., Zhang, Y., Zhang, T., Wang, H. R., & Zhou, H. M. (1995). Inactivation and unfolding of aminoacylase during denaturation in sodium dodecyl sulfate solutions. *Journal of Protein Chemistry*, *14*, 349–357. doi:10.1007/BF01886792.
24. Vallee, B. L., & Williams, R. J. P. (1968). Metalloenzymes: The entatic nature of their active sites. *Proceedings of the National Academy of Sciences of the United States of America*, *59*, 498–505. doi:10.1073/pnas.59.2.498.
25. Xiao, J., Liang, S. L., & Tsou, C. L. (1993). Inactivation before significant conformational change during denaturation of papain by guanidine hydrochloride. *Biochimica et Biophysica Acta*, *1164*, 54–60.