ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 1, pp. 87-91. © Pleiades Publishing, Ltd., 2008.
Published in Russian in Biokhimiya, 2008, Vol. 73, No. 1, pp. 107-112.

An Essential Tryptophan Residue in Alkaline Phosphatase from Pearl Oyster (*Pinctada fucata*)

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Received May 4, 2007
Revision received June 4, 2007

Abstract—Alkaline phosphatases are ubiquitous enzymes found in most species including the pearl oyster, *Pinctada fucata*, where it is presumably involved in nacreous biomineralization processes. In the present study, we have purified alkaline phosphatases from the pearl oyster and modified the tryptophan residues using N-bromosuccinimide (NBS). We show that the resulting inactivation of purified alkaline phosphatase by NBS is dependent on modification of only one of five tryptophan residues in the enzyme. Substrate protection experiments showed that the tryptophan residue was not located at the substrate-binding site but was involved in the catalytic activity.

DOI: 10.1134/S0006297908010136

Key words: alkaline phosphatase, *Pinctada fucata*, chemical modification, kinetics

Alkaline phosphatases (ALP, EC 3.1.3.1) are ubiquitous enzymes found in most species from bacteria to human. They are homodimeric metalloenzymes that catalyze the hydrolysis of a wide variety of phosphomonoesters with release of inorganic phosphate and an alcohol [1]. In the presence of a phosphate acceptor, such as Tris or ethanolamine, alkaline phosphatase also acts as a transphosphorylase [2, 3]. To date, three crystal structures of alkaline phosphatase have been solved—from *E. coli*, shrimp *Pandalus borealis*, and human placenta [4-7]. All three structures are homodimers and share a similar overall structural topology, typically with two zinc ions and one magnesium ion in the active site. The ALP from shrimp *P. borealis* has three magnesium ions [6]. Although the amino acid sequences of ALPs show low similarity, most of the functional residues in the active sites are highly conserved [8-12]. Active site studies have revealed the importance of several amino acid residues in the catalytic action of ALPs. A detailed catalytic mechanism involving three metal ions at the active site of the enzyme has been revealed [13-15].

Although alkaline phosphatase has been extensively investigated, its detailed physiological role has not been fully elucidated. However, the wide distribution of ALP in almost all living beings indicates an involvement in fundamental biochemical processes [16, 17], such as transport of material [18]. Several investigations showed that ALP is necessary for initiating mineralization of bone nodules and matrix vesicle *in vitro*, either by increasing the local concentration of inorganic phosphate or by hydrolyzing inhibitors of mineralization [19]. In marine invertebrates, alkaline phosphatase has been thought to participate in biomineralization [20-23].

In order to explore the possible role of alkaline phosphatase in pearl biomineralization, we previously purified a tissue-nonspecific alkaline phosphatase (PALP) from the pearl oyster, *Pinctada fucata*, the prominent species for saltwater pearl culture, and reported on some of its enzymatic properties [24]. We identified two critical residues at the active site of PALP: an arginine residue involved in substrate binding and a lysine residue involved in catalysis [25]. The present paper reports on another essential amino acid, tryptophan. Chemical modification of PALP with N-bromosuccinimide (NBS) suggests that at least one tryptophan residue is essential for catalysis.

MATERIALS AND METHODS

Animals and chemicals. Adult pearl oysters, *Pinctada fucata*, collected from Beihai Oyster Culture Center,

Abbreviations: ALP) alkaline phosphatase; NBS) N-bromosuccinimide; PALP) *Pinctada fucata* alkaline phosphatase; pNPP) *p*-nitrophenyl phosphate.

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Guangxi Province, China, were transported to the laboratory and kept in a filtered flow-through system with aerated seawater at 20°C prior to enzyme purification. *p*-Nitrophenyl phosphate (pNPP) was purchased from Amresco (USA) and L-histidyldiazobenzylphosphonic acid agarose from Sigma (USA). N-Bromosuccinimide (NBS) and other chemicals sourced locally were of analytical grade.

Enzyme purification and assay. Alkaline phosphatase was purified as previously described using L-histidyldiazobenzylphosphonic acid agarose affinity chromatography [24, 25]. The final preparations showed homogeneity on polyacrylamide gel electrophoresis in both the absence and presence of SDS. Alkaline phosphatase activity was measured spectrophotometrically utilizing pNPP as substrate [26] in an assay system containing 2 mM pNPP in 10 mM sodium bicarbonate buffer, pH 10.0. The reaction was carried out at 25°C. The released *p*-nitrophenol was determined by measuring the absorbance increase at 405 nm using a molar extinction coefficient of 17.3 $mM^{-1} \cdot cm^{-1}$, determined for the assay conditions. One unit of activity represents the amount of enzyme required to catalyze 1 μmol pNPP per minute under the assay conditions.

Modification of PALP by NBS. PALP (18 nM) was incubated with different concentrations of NBS (25-200 μM) in 50 mM acetate buffer, pH 6.0, for 30 min at 25°C, followed by assay of the residual enzyme activity and recording the absorption spectrum at 280 nm with an Ultraspec 3000 (Pharmacia, Sweden) spectrophotometer with a reference cell containing NBS buffer. A control contained the same amount of enzyme, but no NBS. Before assaying the residual enzyme activity, the reaction mixture was ultra-filtered with a Centricon (Millipore, USA) apparatus to remove the excess NBS and then diluted 10-fold with 20 mM Tris-HCl buffer.

UV absorption and fluorescence spectroscopy. Absorption measurements were performed with an Ultraspec 3000 double-beam spectrophotometer (Amersham Pharmacia Biotech, USA). The UV absorption spectra (240-320 nm) were obtained within 5 min after initiation of the NBS modification. All fluorescence measurements were carried out on a Hitachi F-2500 spectrofluorimeter (Hitachi, Japan). The emission spectra excited at 280 nm were recorded from 300-400 nm at 22°C in a quartz cuvette, light path 1 cm. The final concentration of the enzyme was 18 μM.

Inactivation kinetics of PALP in NBS. PALP was incubated with different concentration NBS in 1 ml 50 mM acetate buffer, pH 6.0. Reactions were stopped and samples were withdrawn at equal intervals within a 30 min period and ultra-filtered to remove unreacted reagents. The residual activities of the modified enzymes were assayed after dilution. Assays were carried out in triplicate. The type of inactivation was determined by plotting the logarithm of residual activity of modified enzyme against the time. The related kinetic constants were calculated by the method of Levy et al. [27]. Linear regression analysis was computed with Prism software.

Stoichiometry of inactivation. The number of modified tryptophan residues per mole PALP induced by different concentrations NBS was determined from the decrease in the absorbance at 280 nm according to the expression proposed by Spande and Witkop [28]. Tsou's plot [29] analysis was used to calculate the number of essential tryptophan residues.

Substrate protection experiments. The enzyme was preincubated at 25°C with different concentrations of substrate (2-glycerol phosphate) or competitive inhibitor (phosphate or tungstate) in 50 mM acetate buffer (pH 6.0) for 5 min. NBS solution was then added. The final concentrations of the enzyme and NBS were 18 nM and 100 μM, respectively. The residual activity of modified enzyme was measured 30 min later, after ultrafiltration and dilution. Control experiments without NBS were run concurrently. The activity of unmodified enzyme was taken as 100%.

Other methods. Protein concentration was measured with BCA protein assay reagent (Pierce, USA) using bovine serum albumin as the standard. The molecular mass of the dimeric enzyme was estimated from gel filtration to be 80 kD.

RESULTS AND DISCUSSION

Inactivation of PALP by NBS. NBS is a commonly used tryptophan-specific modification reagent, which oxidizes the indole residue to an oxindole derivative [30]. Treatment of PALP with increasing concentrations of NBS resulted in a progressive decrease in the activity and absorbance at 280 nm (Fig. 1a). The enzyme was completely inactivated in the presence of 200 μM NBS. The loss of activity could not be restored by dialysis or ultrafiltration, indicating an irreversible reaction.

UV absorption and fluorescence spectroscopy. To investigate whether the loss of PALP activity was due to specific modification by NBS, the UV absorption and fluorescence spectra of the modified enzymes were used to monitor the reaction. Figure 1b shows the change in UV spectra of PALP upon reaction with 200 μM NBS. Compared with the spectrum of the unmodified enzyme, a decrease in absorbance at 280 nm and a concomitant increase in absorbance at 250 nm were observed, indicating that the Trp residues were selectively oxidized by NBS [30].

Tryptophan is responsible for the majority of intrinsic fluorescence of proteins, and oxidation by NBS eliminates this fluorescence. The spectral changes of the intrinsic fluorescence of a protein can therefore be used to determine the extent of tryptophan modification. When treated with increasing concentrations of NBS

(from 25 to 200 μM), the enzyme showed a prominent decrease in fluorescence emission intensity at 330 nm (Fig. 2). No red shift appeared in the emission maximum indicating that the loss of enzyme activity was not due to a change in enzyme conformation but was due to a modification of tryptophan residues. In summary, the UV absorption and fluorescence spectra showed that the inactivation of PALP by NBS was due to modification of tryptophan residues.

Kinetics of inactivation of PALP by NBS. After incubating PALP with different concentrations of NBS, the partially modified enzyme was sampled at equal time intervals and assayed for residual activity. A series of straight lines with different slopes were obtained by plotting the logarithm of residual activity of the modified enzyme against the time. This result clearly indicated that inactivation by NBS followed pseudo-first-order kinetics (Fig. 3). Replotting the apparent first-order inhibitory constant (k_{obs}) against NBS concentration yielded a straight line from the slope of which the second-order inactivation constant could be obtained (Fig. 3, insert), $k = 0.001\ \mu M^{-1}\cdot min^{-1}$. Analysis using Levy's method [27] by plotting $\ln k_{obs}$ against the logarithm of NBS concentration yielded a straight line with a slope of 0.806, close to 1, and indicated that at least 1 mole of NBS per mole of active site was required to inactivate the enzyme.

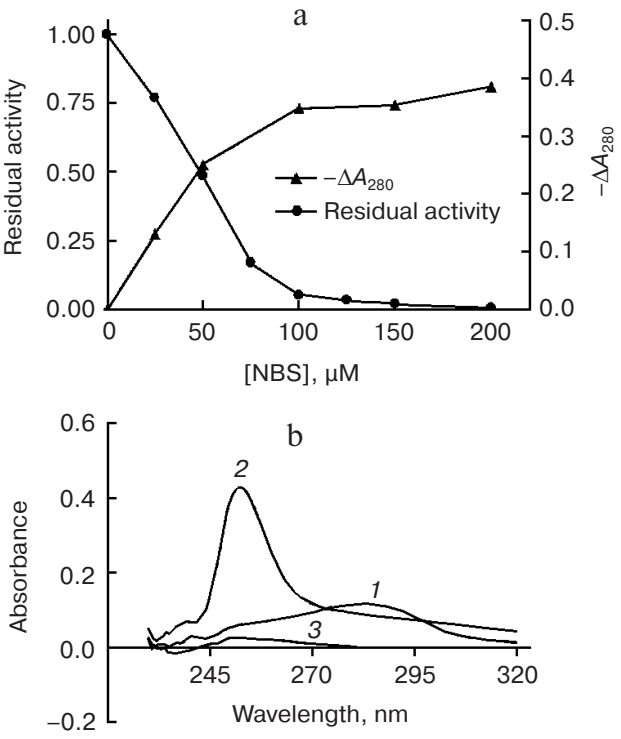

Fig. 1. a) Residual activity and absorbance changes at 280 nm of PALP modified by different concentrations of NBS. For each concentration, three measurements were carried out. b) UV absorption spectra of PALP. The numbers *1*, *2*, and *3* correspond to native PALP, PALP modified by 200 μM NBS, and NBS buffer alone, respectively.

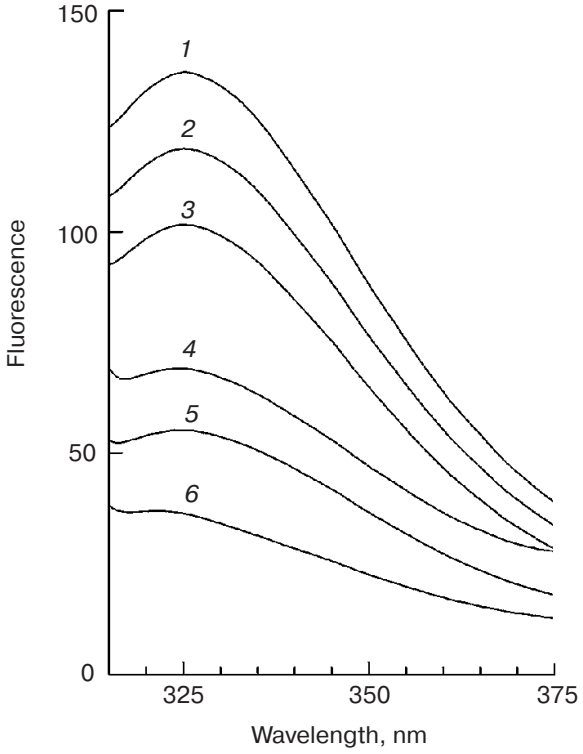

Fig. 2. Changes in intrinsic fluorescence emission spectra of PALP by different concentrations of NBS. Lines *1-6* correspond to NBS concentrations of 0, 25, 50, 100, 150, and 200 μM, respectively.

Stoichiometry of inactivation by NBS. The number of modified tryptophan residues per mole PALP induced by different concentrations NBS could be calculated from the decrease in the absorbance at 280 nm according to the expression proposed by Spande and Witkop [28]. This showed that PALP could be completely inactivated when five tryptophan residues in the enzyme were modified. In another words, there are five reactive tryptophan residues in each molecule of the enzyme.

Tsou's plot [29] analysis was further used to calculate the number of essential tryptophan residues for inactivation. If we assume that the reactivities of all n reactive sites including essential one(s) toward the residue-specific reagents are approximately equal and modification of any essential residues would inactivate the enzyme, the relationship between the fraction of residual activity of the enzyme (a) against the fraction of residual tryptophan residues (x) will be $a^{1/i} = x$, where $x = (n - m)/n$, m is the number of modified residues, and i is the tryout value for the number of essential residues. Figure 4 shows that only with $i = 1$ can a straight line be obtained when plotting $a^{1/i}$ against x, which indicates that only one of the five tryptophan residues is critical to the activity of PALP. Similar results have been reported for alka-

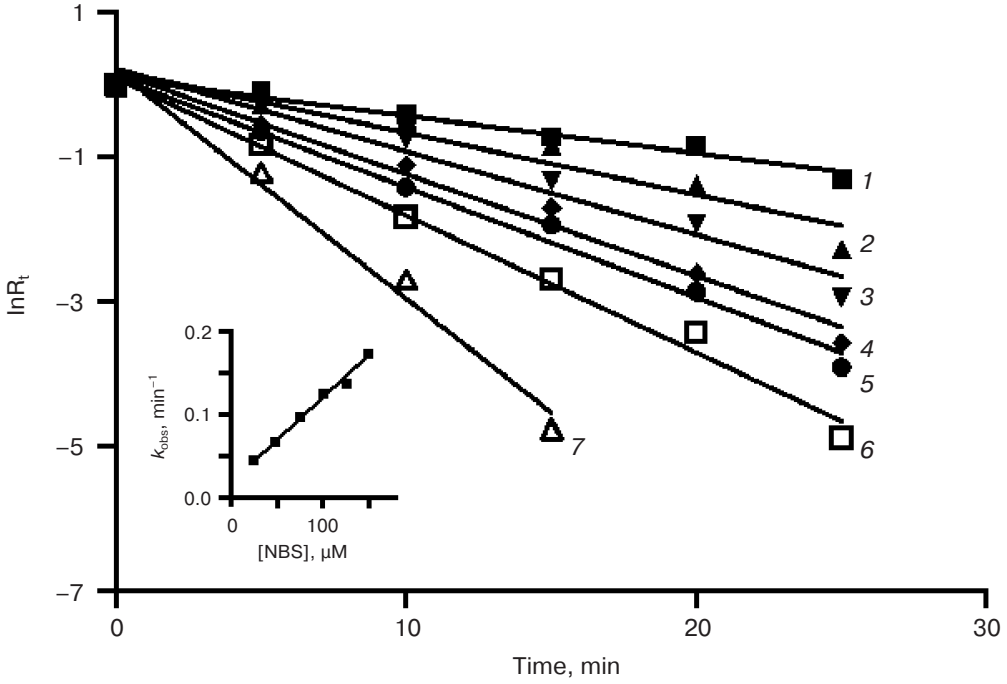

Fig. 3. Kinetics of inactivation of PALP by NBS. The apparent rate constants for inactivation by different concentrations of NBS (lines *1-7* correspond to 25, 50, 75, 100, 125, 150, and 200 μM NBS, respectively) obtained from the slopes of lines by plotting the logarithm of residual activity of modified enzyme against the time. For each concentration, three measurements were carried out. The second-order rate constant for inactivation was determined from the slope of line yielded by plotting k_{obs} against the various concentrations of NBS (insert).

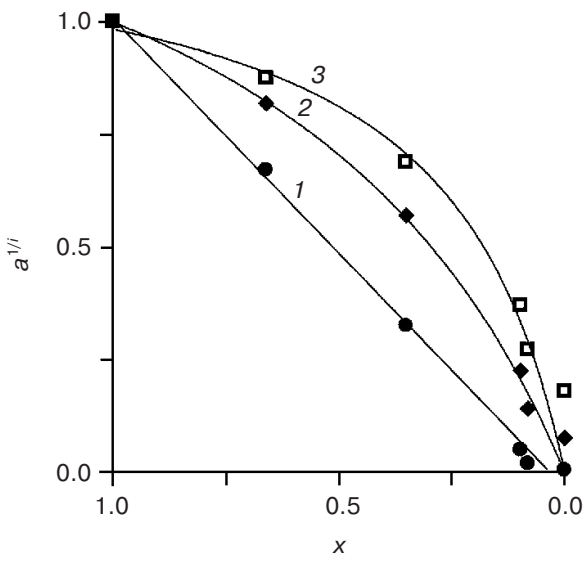

Fig. 4. Correlation between the fraction of residual Trp residues and the fraction of remaining activity of PALP modified by NBS. The data are presented as a Tsou's plot for $i = 1$, 2, and 3 (curves *1-3*, respectively).

line phosphatase from the green crab (*Syclla serrata*) [31].

Substrate protection experiments. Protection experiments, against inactivation by NBS, were performed by pre-incubating the enzyme with 2-glycerol phosphate, the substrate of ALP, or with competitive inhibitors such as phosphate or tungstate. It has been shown that the activity retained by the NBS modified enzyme is about 25, 28, and 29%, respectively, close to that (22%) of enzyme modified by NBS alone. This means that the NBS-mediated inactivation of PALP cannot be appreciably prevented in the presence of substrates or competitive inhibitors, and hence the essential tryptophan residue is not located at the substrate-binding site of PALP. Nevertheless, the activity loss upon NBS modification clearly indicates the involvement of tryptophan in the catalytic activity. Tryptophan may help in maintaining the active center conformation that favors the most efficient catalysis and/or coordination of metal cofactors.

However, according to the sequence analysis of ALPs from different species, tryptophan residue is absent from the active sites of all the known ALPs from eukaryotes including arthropods and vertebrates [8-12, 32, 33]. This clearly indicates the uniqueness of this tryptophan in PALP.

The NBS-mediated inactivation of PALP derives from the reaction of NBS with the indole groups of tryptophan residues. Analysis by Tsou's plot shows that one tryptophan residue among five is essential for the NBS-mediated inactivation of PALP. Protection experiments showed that the essential tryptophan residue was not

directly involved in substrate binding but was involved in the catalytic activity of PALP.

We thank Dr. Bruce Livett, University of Melbourne, Australia, for comments on the manuscript.

This work is supported by Grant 2006AA09Z441 from the National High Technology Research and Development of China and National Science Foundation of China (30530600, 30221003, J0630647).

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