〈Brief Note〉

Structural prediction and analysis of the highly reactive alkaline phosphatase from *Shewanella* sp. T3-3

Yuya Shimozawa[1], Hiroshi Aiba[2] and Yoshiaki Nishiya[1,*]

Summary *Shewanella* sp. T3-3 has been reported to produce a highly reactive alkaline phosphatase, called T3-3 alkaline phosphatase (T3-3AP) (Bioscience, Biotechnology, and Biochemistry, 81:1994–2001, 2017). The specific activity of T3-3AP is approximately six-fold higher than that of the other *Shewanella* alkaline phosphatase (SCAP), although the enzymes are homologous and their primary structures are very similar (68% identical amino acids). The tertiary structure of T3-3AP was constructed by homology modeling based on the X-ray crystal structure of SCAP. A comparison of the homology model and the structure of SCAP revealed differences in the shapes of their respective active sites. Moreover, a tyrosine residue near the putative substrate binding site of T3-3AP seemed to be more flexible than the corresponding tyrosine in SCAP. These observations may explain the high reactivity of T3-3AP. Subsequently, the homology model was used to provide insight into how the modification of two lysine residues in T3-3AP likely causes the decrease in residual activity observed after maleimide activation.

Key words: Alkaline phosphatase, Specific activity, Structural model, Substrate docking, *Shewanella*

1. Introduction

Alkaline phosphatase (AP, EC 3.1.3.1) is a nonspecific phosphomonoesterase, that has been commercially used as a labeling enzyme for antibodies in immunoassays[1,2]. In particular, calf intestinal AP (CIAP) is widely used in enzyme-linked immunosorbent assays (ELISA) because of its high specific activity[1,2]. Some commercially available CIAPs have high specific activities of more than 7,000 U/mg[3]. Although bacterial APs of various origins, such as *Escherichia coli*[4], *Bacillus*[5], and *Geobacillus*[6], have much higher thermal stability than CIAP, their specific activities are much lower. However, two APs from the genus

[1]Department of Life Science, Setsunan University, 17-8 Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan.
[2]Healthcare Division, Toyobo Co., Ltd., 2-8 Dojima-Hama, Kita-ku, Osaka 530-8230, Japan.

[*]Corresponding author: Yoshiaki Nishiya, Department of Life Science, Setsunan University, 17-8 Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan.
Tel: +81-72-800-1151
Fax: +81-72-838-6599
E-mail: nishiya@lif.setsunan.ac.jp

Received for Publication: April 21, 2020
Accepted for Publication: May 6, 2020

Shewanella, *Shewanella* sp. SIB1 AP (SIB1AP)[7] and cold-active AP (SCAP)[8], exhibit comparatively high specific activities of 1,900 and 1,500 U/mg, respectively (Table 1).

A highly reactive AP from *Shewanella* sp. T3-3 (T3-3AP)[3] with the specific activity of 8,600 U/mg (Table 1) was previously isolated by our group. T3-3AP has potentially useful biotechnological applications as bacterial recombinant protein expression systems are known to be highly productive, but are generally poor expression systems for eukaryotic proteins, including CIAP. T3-3AP was cloned, overexpressed, and purified from *E. coli*[3]. Recombinant T3-3AP showed comparatively high reactivities with a colorimetric substrate, *p*-nitrophenyl phosphate (*p*NPP), and the luminescent substrates 4-methoxy-4-(3-phosphatephenyl)spiro [1,2-dioxetane-3,2'-adamantane] disodium salt (PPD) and [(4-chlorophenyl)thio](10-methyl-9(10*H*)-acridinylidene)-methanol, 1-(dihydrogenphosphate), disodium salt (APS-5), as compared with CIAP (Table 1). Subsequently, the residual activity of T3-3AP after maleimide activation was improved by introducing amino acid substitutions in two lysine residues. The double mutant enzyme (K161S+K184S) showed much higher residual activity after maleimide activation than the wild type enzyme, and had approximately two-fold increased sensitivity in ELISAs, compared with CIAP[3].

Table 1 Comparison of substrate specificities of CIAP and *Shewanella* APs

Enzyme	Substrate	Specific activity	
		U/mg	Relative unit
CIAP	*p*NPP	≥7,000	
CIAP	PPD		1.6×10^5
CIAP	APS-5		24×10^5
SIB1AP	*p*NPP	1,900	
SCAP	*p*NPP	1,500	
T3-3AP	*p*NPP	8,600	
T3-3AP	PPD		3.5×10^5
T3-3AP	APS-5		33×10^5

Data were cited from Ref. 3.

In this study, a tertiary structure of T3-3AP was predicted using homology modeling, and a structural analysis was performed to explain its high reactivity. The structure provided insight into why modified T3-3AP displayed greater sensitivity in ELISAs.

2. Materials and Methods

MOE (Molecular Operating Environment) software ver. 2019.01 (Chemical Computing Group Inc., Montreal, Canada) was used to align the primary structures of T3-3AP and SCAP, and to build the three-dimensional protein model of T3-3AP using the tertiary structure of SCAP (PDB ID: 3A52a)[9] as a template. The sequence identity between T3-3AP (419 amino acid residues) and SCAP (400 amino acid residues) was approximately 68%, without any gaps, which was deemed to be sufficient for model building. Energy minimization was applied to the model to further refine the structure.

The docking simulation used to model the substrate-enzyme (*p*NPP-SCAP) complex was performed using the induced fit docking protocol in the MOE software to allow for side chain flexibility. The docking site was defined around the active site and contained metal ions.

The *N*-(6-maleimidocaproyloxy)succinimide-enzyme (EMCS-T3-3AP) complex was also built using the MOE software. EMCS is a bifunctional reagent which can modify lysine residues on protein surfaces to allow for maleimide activation[10]. Two molecules of EMCS were covalently bound to the ε-amino groups of two lysine residues (K161 and K184, respectively), and a maleimide-activated model was constructed. Subsequently, the model structure was optimized by energy minimization.

The molecular structures of *p*NPP, PPD, APS-5, and EMCS, were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The MOE and Pymol (https://pymol.org/) software were used for molecular visualization.

3. Results and Discussion

A three-dimensional structural model of

T3-3AP was constructed by homology modeling using a template structure with the highest sequence identity. There were no Ramachandran[11] outliers in the model. The modeled three-dimensional structure used in the present study was therefore of high quality and could potentially enhance understanding of the structure-function relationship in T3-3AP. The structure will also provide a reliable basis for identifying sites for mutagenesis designed to alter the enzyme's activity and stability.

The overall structures of SCAP and T3-3AP superimpose well, with a root mean square deviation (RMSD) for atomic Cα positions of 0.52 Å. A superimposition of the active site regions yielded an RMSD for Cα positions of 0.54 Å. Close-up views of the active site regions of SCAP and T3-3AP are presented in Fig. 1A. The binding site of the *p*NPP substrate on SCAP was predicted by a docking simulation as described in the materials and methods section. *p*NPP and water molecules were also docked into the T3-3AP structure. As expected, both structures are quite similar to each other. However, the active site pocket of T3-3AP was narrower than that of SCAP. Therefore, the strengths of the interactions between the enzymes and the monophosphate groups of the substrates may be influenced by the sizes of the active sites.

Both SCAP and T3-3AP are homodimers. SCAP has a tyrosine residue (Y283) in one subunit that is located near the putative substrate binding site of the other subunit (Fig. 1A). As shown in Fig. 1B, Y297 of T3-3AP, which is found in the same

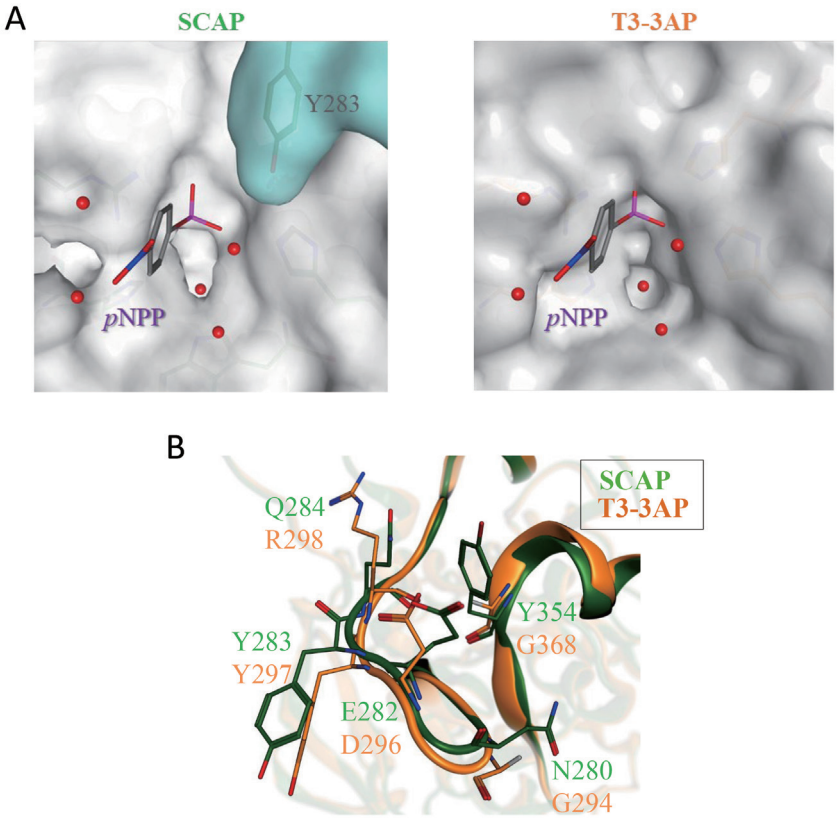

Fig. 1 Comparison of the SCAP structure and T3-3AP homology model. (A) Close-up views of the active site in the *p*NPP-fitted models of SCAP and T3-3AP. The enzymes are shown in stick and surface representations while the substrate, *p*NPP, is shown in a stick representation. Water molecules are represented by red balls. (B) Superimposition of T3-3AP on SCAP. Secondary structures and non-identical amino acid residues are shown in ribbon and stick representations.

corresponding location as Y283 of SCAP, is potentially more flexible because it is surrounded by comparatively smaller amino acid residues (Fig. 1B). The side chain of Y297 is likely to be relatively accessible to substrates compared with the corresponding residue in SCAP.

Maleimide activation is performed to create enzyme-labeled antibodies that are used in immunoassays, and has been shown to decrease catalytic activity in T3-3AP[3]. However, T3-3AP catalytic activity was previously shown to be drastically improved by the introduction of the K161S+K184S double mutation[3]. To provide insight into the reason for the decreased activity and the effects of the mutations, a structural model of the EMCS-T3-3AP complex covalently bound to two molecules of EMCS via K161 and K184 was constructed. As shown in Fig. 2, the EMCS-T3-3AP complex obviously has a narrowed entrance in the active site pocket compared to unmodified T3-3AP. Therefore, the EMCS modification may result in a decrease in the turnover of the phosphatase reaction.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgements

This work was supported by JSPS KAKENHI; Grant Number 18K06616. We would like to thank Editage (www.editage.jp) for English language editing.

References

1. Yolken RH, Kim HW, Clem T, Wyatt RG, Kalica AR, Chanock RM, and Kapikian AZ: Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. Lancet, 310: 263-267, 1977.
2. Cejka J: Enzyme immunoassay for Factor VIII-related antigen. Clin Chem, 28: 1356-1358, 1982.
3. Aiba H, Nishiya Y, Ojima Y, and Azuma M: Overexpression, characterization, and modification of highly active alkaline phosphatase from a *Shewanella* genus bacterium. Biosci Biotechnol Biochem, 81: 1994-2001, 2017.
4. Kim EE and Wyckoff HW: Reaction mechanism of alkaline phosphatase based on crystal structures. J Mol Biol, 218: 449-464, 1991.
5. Sugawara T, Konno Y, Ohta H, Ito K, Kaneko J, Kamio Y, and Izaki K: Purification and properties of two membrane alkaline phosphatases from *Bacillus subtilis* 168. J Bacteriol, 173: 1824-1826, 1991.
6. Zhang Y, Ji C, Zhang X, Yang Z, Peng J, Qiu R, Xie

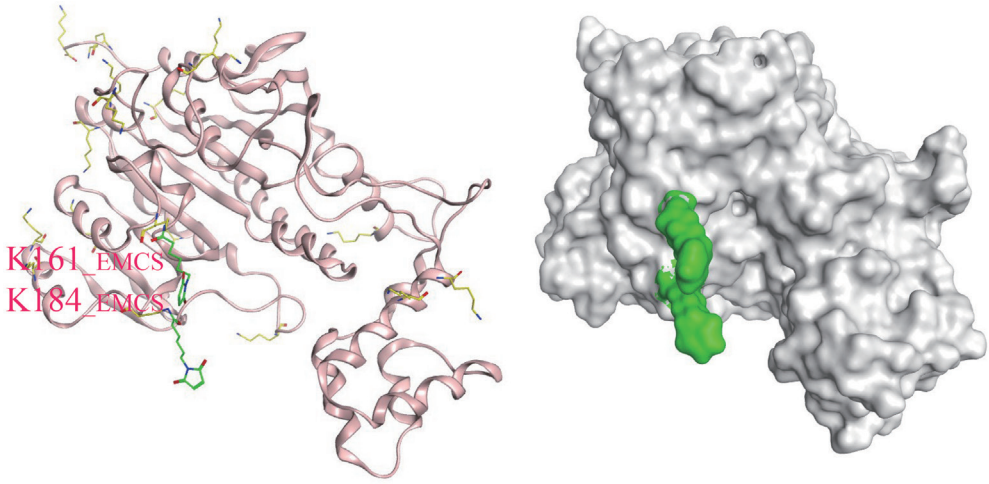

Fig. 2 Structural model of T3-3AP in complex with two EMCS molecules. The overall structure is shown in a ribbon representation (left) and surface representation (right). EMCS is colored in green.

Y, and Mao Y: A moderately thermostable alkaline phosphatase from *Geobacillus thermodenitrificans* T2: cloning, expression and biochemical characterization. Appl Biochem Biotechnol, 151: 81-92, 2008.

7. Suzuki Y, Mizutani Y, Tsuji T, Ohtani N, Takano K, Haruki M, Morikawa M, and Kanaya S: Gene cloning, overproduction, and characterization of thermolabile alkaline phosphatase from a psychrotrophic bacterium. Biosci Biotechnol Biochem, 69: 364-373, 2005.
8. Murakawa T, Yamagata H, Tsuruta H, and Aizono Y: Cloning of cold-active alkaline phosphatase gene of a psychrophile, *Shewanella* sp., and expression of the recombinant enzyme. Biosci Biotechnol Biochem, 66: 754-761, 2002.
9. Tsuruta H, Mikami B, Higashi T, and Aizono Y: Crystal structure of cold-active alkaline phosphatase from the psychrophile *Shewanella* sp. Biosci Biotechnol Biochem, 74: 69-74, 2010.
10. Desai S, Stanaitis M, and Ganapathy R: Polymerized conjugates for biological applications. US Patent 8,007,998, 2011.
11. Gerard JK and Jones TA: Phi/Psi-chology: Ramachandran revisited. Structure, 4: 1395-1400, 1996.