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Three-dimensional structure of *Bacillus stearothermophilus* neopullulanase

Hironori HONDOH[1]*, Takashi KURIKI[2] & Yoshiki MATSUURA[1]

[1]*Institute for Protein Research, Osaka University, 3-2 Yamada-oka, Suita, Osaka 565-0871, Japan; tel.: ++ 81 6 6879 8605, fax :++ 81 6 6879 8606, e-mail: hondoh@protein.osaka-u.ac.jp*
[2]*Biochemical Research Laboratory, Ezaki Glico Co. Ltd., 4-6-5 Utajima, Nishiyodogawa-ku, Osaka 555-8502, Japan*

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The three-dimensional structure of *B. stearothermophilus* neopullulanase has been determined by X-ray diffraction at 1.9 Å resolution. The enzyme forms a dimer in the crystal, the monomer being composed of four domains N, A, B, and C. The structure of the active site cleft of a monomer is shallow and wide compared to other α-amylase enzymes. The dimer is formed by monomers folded to make contact with each other via domains N, A and B, making the cleft apparently deeper and narrower. The shape of the cleft in the dimer is slightly different among the enzymes of the subgroup, possibly reflecting the differences in substrate specificities.

Key words: neopullulanase, X-ray structure, *Bacillus stearothermophilus*, $(\beta/\alpha)_8$-barrel, pullulan, cyclomaltodextrinase, α-amylase family.

Introduction

Neopullulanase from *Bacillus stearothermophilus* hydrolyzes not only α-1,4-glucosidic linkages but also α-1,6-glucosidic linkages of several branched oligosaccharides (TAKATA et al., 1992). The reaction of the enzyme on pullulan produces panose, maltose, and glucose, the final ratio being 3:1:1 (IMANAKA & KURIKI, 1989). This enzyme has also been shown to hydrolyze cyclodextrin. It is one of a group of multispecific enzymes that can be classified as cyclomaltodextrinases (PARK et al., 2000). The three-dimensional structures of the enzymes in this group have been determined for maltogenic amylase (KIM et al., 1999) and TVAII (KAMITORI et al., 1999). These enzymes commonly possess a unique N-terminal domain, which is not present in common α-amylases. This paper describes the three- dimensional structure of neopullulanase and compares it to other enzymes in the same group, focusing on the elucidation of the relative specificity of the catalytic reaction.

Experimental

Preparation and purification of neopullulanase
E. coli TG-1 was used as a host for preparation of the neopullulanase from *Bacillus stearothermophilus* TRS 40, as described previously (KURIKI et al., 1996). *E. coli* TG-1 carrying a recombinant plasmid, pUNP129 (structural gene of the neopullulanase from *B. stearothermophilus* TRS40), was

* Corresponding author

incubated overnight in 120 mL of L broth containing 100 μg ampicillin at 30 ℃. Then 1 L of L broth containing 100 μg ampicillin in a 2-L Sakaguchi flask was inoculated with 10 mL of the overnight preculture and shaken at 30 ℃ to mid-log phase (optical density at 660 nm = 0.6). Isopropyl-β-thiogalactopyranoside (final concentration, 1 mM) was added as the inducer of the lac promoter, and cultivation was continued at 30 ℃ for 15 h from the inoculation time. In total 10 L of this cell culture was used for the enzyme purification. The cell culture sample was harvested by centrifugation (8,000 $\times$ g, 5 min) and washed with 20 mM sodium phosphate buffer (pH 6.5; buffer A). The cell pellet was then suspended in buffer A containing 10 mM 2-mercaptoethanol. The cells were disrupted by sonication (19.5 kHz, 4 ℃ for 20 min), and the cell extract was held at 60 ℃ for 10 min to denature the proteins derived from *E. coli*. The precipitate was removed by centrifugation (10,000 $\times$ g, 20 min). Ammonium sulfate (final 70% saturation) was added to the supernatant, and the solution was kept overnight at 4 ℃. After centrifugation (10,000 $\times$ g, 20 min), the precipitate was dissolved in about 100 mL of buffer A containing 10 mM 2-mercaptoethanol and dialyzed against buffer A containing 10 mM 2-mercaptoethanol. The enzyme solution was applied to a column (26 $\times$ 200 mm) of Q-Sepharose (Pharmacia, Uppsala, Sweden) that had been equilibrated with buffer A containing 10 mM 2-mercaptoethanol. Active enzyme was eluted with a 0 to 0.4 M NaCl gradient. Ammonium sulfate was added to the enzyme fraction to a final concentration of 0.6 M, and the mixture was applied to a column (26 $\times$ 100 mm) of Phenyl-Toyopearl 650 M (Tosoh, Tokyo, Japan) that had been equilibrated with buffer A containing 10 mM 2-mercaptoethanol and 0.6 M ammonium sulfate. The active fraction was eluted with a 0.6 to 0 M ammonium sulfate gradient and dialyzed against buffer A containing 10 mM 2-mercaptoethanol. The neopullulanase was finally purified to homogeneity using a Source 15Q (Pharmacia) column (10 $\times$ 100 mm) that had been equilibrated with buffer A containing 10 mM 2-mercaptoethanol. The enzyme was eluted with a 0.15 to 0.5 M NaCl gradient.

Crystallization

Prior to crystallization, the protein solution was checked for suitability. Dynamic light scattering was measured by using Dyna-Pro 810TC (Protein Solution Inc.), with 10mg/mL protein concentration in 5 mM phosphate buffer (pH 6.5) containing 10 mM 2-mercaptoethanol at 20 ℃. The result showed a monomodal profile centered at 4.8 nm radius and 131kDa molecular weight. Secondly an ultracentrifugation experiment was carried out by using Optima XL-A (Beckman Inc.) with a solution of 0.58 mg/mL protein in 20 mM sodium phosphate (pH 6.5) containing 100 mM NaCl at 4 ℃. The result was consistent with that from light scattering showing a molecular weight of 138kDa. These results indicated that neopullulanase exists as a dimer in these solution conditions.

The crystallization for diffraction grade crystals was carried out by hanging drop vapor diffusion with a protein concentration of 10mg/mL and 18% PEG6000 as precipitant in 100mM sodium cacodylate buffer solution at pH 6.5. The crystal grew to a typical size of 0.3 mm in two weeks. The space group and cell dimensions are monoclinic $P2_1$, a = 68.7 Å, b = 72.8 Å, c = 123.6 Å, $\beta = 90.8^\circ$ with one dimer in the asymmetric unit.

Intensity data

The intensity data were initially collected by using R-AXIS IV with a copper rotating anode X-ray generator to a resolution of 2.4 Å. This has been used for structure solution by the molecular replacement method. The intensity data for the refinement of structure were collected at the synchrotron facility SPring8 BL40B2 equipped with a CCD area detector system (ADSC Co.) to a resolution of 1.9 Å. Both experiments were performed at cryo-conditions. The indexing and integration of intensity data were processed by using program *DPS/MOSFLM* (Rossmann et al., 1999).

Structure analysis and refinement

The initial structure model was obtained by the molecular replacement (program *AMoRe/*CCP4 (NAVAZA, 1994)) method using the structure of TVAII (PDB id-code 1BVZ) as search model. The refinement was carried by using the program *CNS* (Brünger et al., 1998) and a graphics program *O* (Jones et al., 1991) at 1.9 Å resolution. The final structure model consisted of 9778 (4889 $\times$ 2) protein atoms and 1632 bound water molecules giving R and $R_{\rm free}$ values of 16.4 and 20.3%, respectively. Statistics of intensity data collection and structure refinement are listed in Table 1.

Results

Overall structure

A dimer of the neopullulanase molecule is contained in the asymmetric unit of the crystal. Each monomer of the dimer has the same polypeptide fold composed of four domains: N (residues 1–

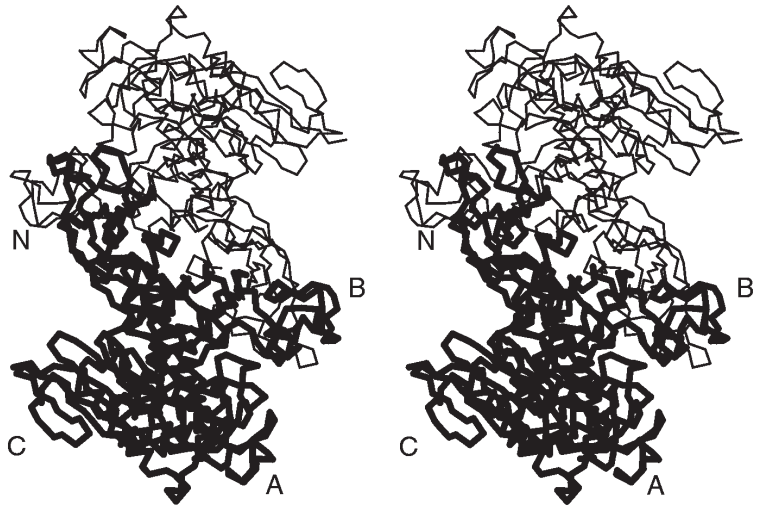

Fig. 1. A stereoscopic view of a dimer with main chain tracing. The two monomers are distinguished by thin and thick lines. Domains are labeled with letters N, A, B and C. [All the figures in this paper except figure 3 were generated using *MOLSCRIPT* (Kraulis, 1991).]

Table 1. Statistics of intensity data collection and structure refinement.

Data collection statistics	
Resolution range (Å)	35–1.9
Observed reflections[a]	321,350
Independent reflections	90,890
Completeness (%)	94.5
R_{merge}	0.058
Refinement statistics	
Resolution range (Å)	10–1.9
R-factor/$R^{\mathrm{b}}_{\mathrm{free}}$	0.164/0.203
r.m.s. deviation bond lengths (Å)/ angles (deg.)	0.005/1.3
No. of amino acids	1176 (588×2)
No. of solvent atoms	1632

[a]For reflections with $I > 3\,\sigma(I)$. [b]R_{free} was calculated with 5% of the data.

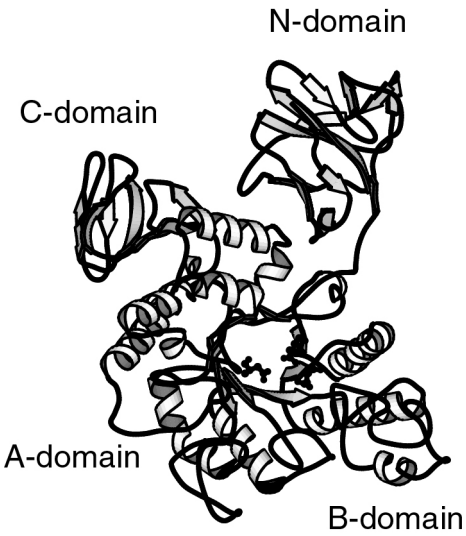

Fig. 2. A ribbon drawing of a monomer with catalytic residues and domain labels.

123), A (124–246, 300–506), B (247–299), and C (507–588). Domain N consists of an antiparallel β-sheet. Domain A contains a $(\beta/\alpha)_8$ -barrel structure, and B one α-helix and other non-typical secondary structures. Domain C also consists of an antiparallel eight-stranded β-sheet.

The dimer is formed by folding of the monomers to give a pseudo two-fold symmetric contact with each other at the surfaces of domains N, A and B as shown in Figure 1. The contact is composed of 44 hydrogen bonds between amino acid residues and other interactions such as van der Waals interactions.

The polypeptide folding of a monomer is shown in Figure 2 as a ribbon representation of the main chain trace. The topological arrangement of the secondary structures is shown in Figure 3 with the residue numbers of the amino acids making up the secondary structures. In the $(\beta/\alpha)_8$-barrel components, an α-helix Aα5 is only a half-turn helix, and Aβ7 is formed by a strand of irregular β-type conformers.

Active site

The active site is located at the C-terminal side of the central β-sheet of the $(\beta/\alpha)_8$-barrel in domain A, at the bottom of the cleft formed between domains A and B. The shape of the cleft of the monomer enzyme is wider and shallower than in other α-1,4 specific enzymes. This suggests the ability of adapting to the substrate pullulan which has α-1,6-glucosidic linkages, although the dimer formation makes the cleft apparently narrower and deeper. The structure of the active site is shown in Figure 4. The active site cleft in the dimer is formed by domains A and B of one monomer, and domain N of another.

The catalytic residues Asp328, Glu357 and Asp424 have a spatial arrangement similar to that of equivalent residues in other α-amylase enzymes. The bound water molecule which is universally found in α-amylase family enzymes also exists in this enzyme. Important amino-acid residues in the

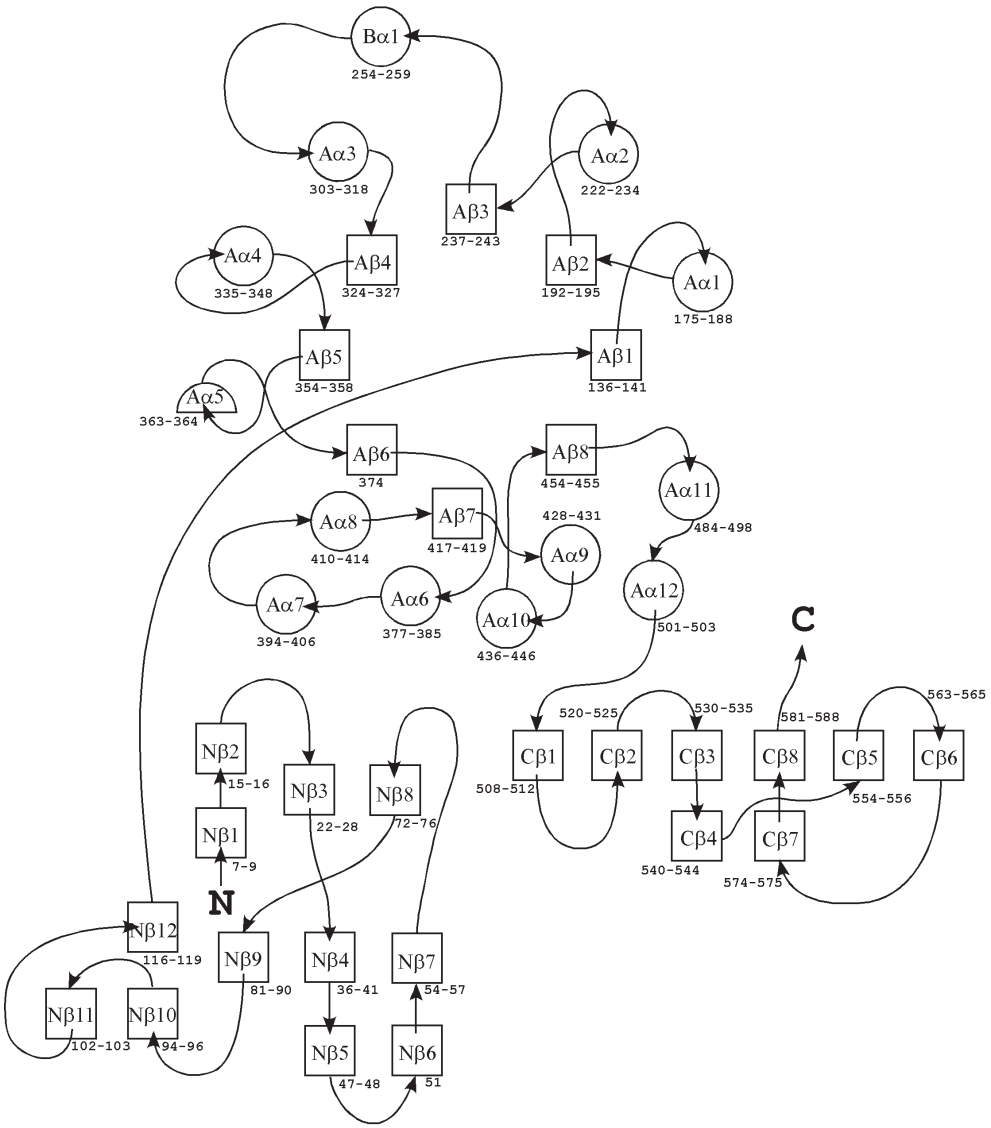

Fig. 3. A topological diagram showing secondary structure arrangement. Residue numbers composing secondary structure units are indicated.

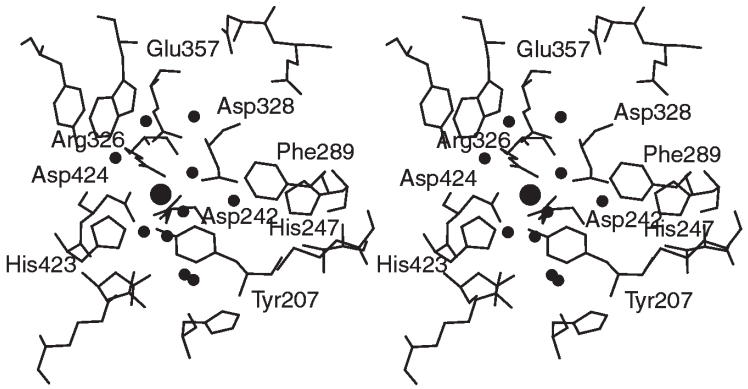

Fig. 4. A stereoscopic view of the catalytic site. Water molecules are marked by •, with a conserved water molecule (large •) located near the center of the catalytic residues, Asp328, Glu357, and Asp424.

four conserved regions of the α-amylase family (Kuriki & Imanaka, 1999) are also found in the active site, with conserved spatial arrangements (Katsuya et al., 1998; Matsuura, 2002).

Discussion

The formation of the dimer structure in this subgroup of enzymes may be essential for the substrate specificity. The structures in the crystals showed that the catalytic cleft formed by the dimerization (Fig. 1) is narrower in neopullulanase and *Thermus* maltogenic amylase (Kim et al., 1999) than in TVAII (Kamitori et al., 1999). This difference may be responsible for the difference in substrate specificities (Park et al., 2000). In the dimer structure, the N-domain of the second monomer seems to replace the structure of domain B in a common α-amylase to form an appropriate structure for the cleft (Fig. 1). The substrate specificities of these enzymes are only known for dimer enzymes, and the catalytic activity of an isolated monomer has not been reported so far.

The structure of the region surrounding the active site of the monomer of this enzyme does not form a cleft as seen in common α-amylases, but is quite shallow and open to the outside. By superimposing the structure of Taka-amylase on this enzyme, a long loop around residue 160 in TAA overhangs the subsites of the active site binding the reducing aglycon end of the substrate in neopullulanase. The superposition of the dimer of neopullulanase and TAA showed that part of the N-domain of the second monomer of the neopullulanase dimer overlaps the B domain of superimposed TAA, compensating for the structure of part of the active site cleft. This suggests that dimer formation may be necessary to bind some specific types of substrate.

The potential subsite region in the monomer of neopullulanase does not seem to be extended. By analogy with complexed α-amylases, only the subsites -1 and -2 seem to exist in the active site of this enzyme. This is consistent with recognition of the maltoside moiety, that is the common structural portion of the multiple substrates of this enzyme. On the other hand in the dimer structure, the subsites, especially in the region binding the aglycon of the substrate, might be reinforced by the structure of the N-domain of the second monomer. Actually the loops of the 47–51th and 106–113th residues of the second N-domain overhang the active site. These regions may provide subsites suitable for binding certain types of substrate.

The interrelation between substrate specificities of this subgroup of enzymes and the state of oligomerization will be a focus of attention, and awaits further investigation.

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