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Insight into the transition between the open and closed conformations of *Thermus thermophilus* carboxypeptidase

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ABSTRACT

Carboxypeptidase cleaves the C-terminal amino acid residue from proteins and peptides. Here, we report the functional and structural characterizations of carboxypeptidase belonging to the M32 family from the thermophilic bacterium *Thermus thermophilus* HB8 (TthCP). TthCP exhibits a relatively broad specificity for both hydrophilic (neutral and basic) and hydrophobic (aliphatic and aromatic) residues at the C-terminus and shows optimal activity in the temperature range of 75–80 °C and in the pH range of 6.8–7.2. Enzyme activity was significantly enhanced by cobalt or cadmium and was moderately inhibited by Tris at 25 °C. We also determined the crystal structure of TthCP at 2.6 Å resolution. Two dimer types of TthCP are present in the crystal. One type consists of two subunits in different states, open and closed, with a C^{α} RMSD value of 2.2 Å; the other type consists of two subunits in the same open state. This structure enables us to compare the open and closed states of an M32 carboxypeptidase. The TthCP subunit can be divided into two domains, L and S, which are separated by a substrate-binding groove. The L and S domains in the open state are almost identical to those in the closed state, with C^{α} RMSD values of 0.84 and 0.53 Å, respectively, suggesting that the transition between the open and closed states proceeds with a large hinge-bending motion. The superimposition between the closed states of TthCP and BsuCP, another M32 family member, revealed that most putative substrate-binding residues in the grooves are oriented in the same direction.

1. Introduction

Carboxypeptidases, the enzymes that catalyze the release of amino acid residues from the C-termini of peptides and proteins, can be divided into three major classes: metallocarboxypeptidases, serine carboxypeptidases and cysteine carboxypeptidases [1]. Metallocarboxypeptidases are ubiquitous in nature and typically have a single Zn^{2+} bound to the active site. The M32 family is a subclass of metallocarboxypeptidases that was first discovered from the thermophilic bacterium *Thermus aquaticus* YT-1 [2,3]. M32 family carboxypeptidases, which are widely distributed in eukarya, bacteria and archaea, possess the classical HEXXH motif observed in numerous zinc metalloproteases. Biochemical studies have shown that the substrate specificity, metal ion dependency and quaternary structure differ among carboxypeptidases belonging to the M32 family [2,4–11]. For example, the carboxypeptidase from the thermophilic bacterium *Thermus aquaticus* YT-1 (TaqCP) exists as a monomer (56 kDa) in solution and is activated by both Co^{2+} and Zn^{2+} [2,3]. In contrast, the carboxypeptidase from the hyperthermophilic archaeon *Pyrococcus furiosus* (PfuCP) exists as a homodimer (59 kDa x 2) in solution and is activated by Co^{2+} but not Zn^{2+} [9].

Crystal structures of the M32 family carboxypeptidases have been reported for those from *Pyrococcus furiosus* (PfuCP) [12], *Thermus thermophilus* (TthCP) [5], *Bacillus subtilis* (BsuCP) [5], *Fervidobacterium islandicum* (FisCP) [6] and *Trypanosoma cruzi* (TcMCP-1) [13]. Each M32 family carboxypeptidase consists of two domains. The entrance of the substrate-binding groove is located between these two domains. The reported crystal structures of M32

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family carboxypeptidases can be divided into two groups, open and closed conformations, depending on the relative orientation of the two domains and the size of the entrance of the substrate-binding grooves. Those of PfuCP, TthCP, FisCP and TcMCP-1 are in the open conformation, while that of BsuCP is in the closed conformation. Although M32 family carboxypeptidases are believed to transit between these two conformations, the mechanism of the conformational change remains unknown because the structures of these proteins have not been solved in both the open and closed conformations. In the present study, we solved a crystal structure of the carboxypeptidase from *Thermus thermophilus* HB8 (TthCP), which is an extremely thermophilic bacterium and a model organism in structural biology [14–18], at 2.6 Å resolution under conditions that differ from those previously reported [5]. Our crystal contains both the open and closed conformations in an asymmetric unit. Based on these two conformations of TthCP, we propose the conformational transition mechanism of this M32 carboxypeptidase. Moreover, we characterized the enzymatic properties of TthCP by analyzing its substrate preference, temperature and pH optima, as well as metal ion dependency.

2. Materials and methods

2.1. Amino acid specificity of TthCP toward ZAX substrates

The amino acid preference of the C-terminus substrate was determined by quantifying the free amino acids released from a series of ZAX substrates [ZA = N-terminal benzyloxycarbonyl-protected alanine, and X = A, D, E, F, G, H, I, K, L, M, N, P, R, S, V, W, or Y (Bachem Biochemicals)] using the ninhydrin assay [19]. The assay mixture (100 μl) contained 50 mM Tris-HCl (pH 8.5), 0.4 mM $MnCl_2$ and 2 mM ZAX substrate. The reaction was initiated by adding 1 μl of 100 μM TthCP solution, and the mixture was incubated at 80 °C for 10 min, followed by quenching on ice. Subsequently, 750 μl of cadmium-ninhydrin reagent was added and the sample was incubated for an additional 5 min at 80 °C for color development, followed by quenching on ice. The absorbance at 508 nm was read, and the amount of cleaved ZAX substrates was calculated using the standard curve prepared using each amino acid.

2.2. Temperature optimization

The optimal temperature of TthCP was determined by measuring the activity at various temperatures from 20 to 100 °C. The assay solution (20 μl) contained 50 mM HEPES-NaOH (pH 7.6), 0.4 mM $MnCl_2$ and 2 mM ZAG substrate. The reaction was initiated by adding 2 μl of 10 μM TthCP solution to the assay solution, and the mixture was incubated at an appropriate temperature for 10 min, followed by quenching on ice. Subsequently, 140 μl of cadmium-ninhydrin reagent was added, and the sample was incubated for an additional 5 min at 80 °C for color development, followed by quenching on ice.

2.3. pH optimization

The optimal pH of TthCP was determined by measuring the activity at various pH values using the following buffers: MES, 5.2–6.8; MOPS, 6.4–7.2; HEPES, 6.8–8.0; Tris, 7.6–8.8; borate, 8.0–9.2; carbonate, 8.4–9.2; and CHES, 9.0–10.0. The assay solution (20 μl) contained 20 mM of the appropriate buffer, 0.4 mM $MnCl_2$ and 2 mM ZAG substrate. The reaction was initiated by adding 2 μl of 10 μM TthCP solution and the mixture was incubated at 25 °C for 10 min, followed by quenching on ice. Subsequently, 140 μl of cadmium-ninhydrin reagent was added, and the sample was incubated for an additional 5 min at 80 °C for color development, followed by quenching on ice.

2.4. Metal ion dependency

The divalent ion dependence of TthCP activity was examined by performing the enzymatic assay under the presence of the following divalent metal chlorides: $CaCl_2$, $CdCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $NiCl_2$ and $ZnCl_2$. TthCP was de-metallized by dialyzing against 50 mM HEPES-NaOH (pH 7.6), 150 mM NaCl, 1 mM DTT and 5 mM EDTA for 24 h at room temperature, followed by dialysis against 50 mM HEPES-NaOH (pH 7.6), 150 mM NaCl and 1 mM DTT for 24 h at room temperature to remove EDTA. The assay solution (20 μl) contained 50 mM HEPES-NaOH (pH 7.6), 0.4 mM of the appropriate divalent metal chloride and 2 mM ZAG substrate. The reactions were initiated by adding 2 μl of 10 μM de-metallized TthCP solutions and the mixtures were incubated at 80 °C for 10 min, followed by quenching on ice. Subsequently, 140 μl of cadmium-ninhydrin reagent was added, and the sample was incubated for an additional 5 min at 80 °C for color development, followed by quenching on ice.

2.5. Structure determination and refinement

Purification, crystallization and data collection were reported previously [20]. The initial structure was obtained by molecular replacement with the program MOLREP [21] using the crystal structure of PfuCP (PDB ID: 1KA2) as the search model. The crystal packing did not show any bad contacts between molecules. The model was subjected to cycles of simulated annealing refinement using the non-crystallographic symmetry with CNS [22] to avoid model bias, along with a manual model building with the software Coot [23]. Refinements were performed with Phenix.refine [24]. The stereochemical quality of the final model was assessed using the Ramachandran plot obtained with MolProbity [25].

3. Results and discussion

3.1. Biochemical characterization of TthCP

The substrate specificity of TthCP was determined at 80 °C using ZAX substrates. As shown in Fig. 1a, the order of substrate preference was as follows: W > G > N > A > K > H > F > M > S > R > V > Y > E > L > I. TthCP had a relatively broad specificity for neutral, aliphatic, basic, polar and aromatic C-terminal residues but not for aspartic acid or proline residues.

The effects of pH, temperature and metal ion dependency on TthCP activity were also investigated. Weak activity was observed at 20–30 °C and a linear temperature dependence of activity was observed at 50–75 °C (Fig. 1b). The optimum temperature was 75–80 °C and the activity fell linearly at 85–100 °C (Fig. 1b). The activity at 100 °C was as high as that at 30 °C. The optimum temperature of TthCP (75–80 °C) was comparable to those of TNA1_CP (70–80 °C) [4] and TaqCP (80 °C) [2] and lower than that of PfuCP (90–100 °C) [9]. The linear temperature dependence of TthCP activity in the temperature ranges of 50–75 °C and 85–100 °C suggest that the enzyme does not undergo drastic conformational changes as the temperature is increased.

The pH dependence of TthCP activity was measured at 25 °C. TthCP was highly active in a neutral pH range of 6.0–7.6, and the activity decreased gradually on either side of the optimum pH 6.4–7.2 (Fig. 1c). Interestingly, TthCP exhibited relatively lower activity in Tris-HCl buffer compared to its activity in HEPES and borate buffers. These data clearly demonstrate the moderate inhibitory effect of Tris on TthCP activity. This is the first report on