Biochimica et Biophysica Acta 1844 (2014) 2203–2213

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

# Structural investigation of the cold-adapted acylaminoacyl peptidase from *Sporosarcina psychrophila* by atomistic simulations and biophysical methods

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ARTICLE INFO

*Article history:*
Received 29 April 2014
Received in revised form 19 September 2014
Accepted 23 September 2014
Available online 01 October 2014

*Keywords:*
Psychrophilic enzyme
Acylaminoacyl peptidase
Acylpeptide hydrolase
Molecular dynamics
Three-dimensional structure
beta-Barrel

ABSTRACT

Protein structure and dynamics are crucial for protein function. Thus, the study of conformational properties can be very informative for characterizing new proteins and to rationalize how residue substitutions at specific protein sites affect its dynamics, activity and thermal stability. Here, we investigate the structure and dynamics of the recently isolated cold-adapted acylaminoacyl peptidase from *Sporosarcina psychrophila* (SpAAP) by the integration of simulations, circular dichroism, mass spectrometry and other experimental data. Our study notes traits of cold-adaptation, such as lysine-to-arginine substitutions and a lack of disulphide bridges. Cold-adapted enzymes are generally characterized by a higher number of glycine residues with respect to their warm-adapted counterparts. Conversely, the SpAAP glycine content is lower than that in the warm-adapted variants. Nevertheless, glycine residues are strategically located in proximity to the functional sites in SpAAP, such as the active site and the linker between the two domains.. In particular, G457 reduces the steric hindrance around the nucleophile elbow. Our results suggest a local weakening of the intramolecular interactions in the cold-adapted enzyme. This study offers a basis for the experimental mutagenesis of SpAAP and related enzymes. The approaches employed in this study may also provide a more general framework to characterize new protein structures in the absence of X-ray or NMR data.



## 1. Introduction

The β-propeller is a common architecture based on four-stranded antiparallel and twisted β-sheets that are radially arranged around a central cavity [1]. This fold is associated with a variety of different biological functions, such as regulation of multi-domain proteins [2], structural stabilization, metal-ion binding [3,4], ligand binding [5] and catalysis [6]. Moreover, several proteins containing β-propeller motifs have been reported to be associated with human diseases, such as cancer, neurodegeneration and arthritis [7].

β-propeller domains share common structural features and motifs [8,9] even though their primary sequences are poorly conserved and they are functionally different [10]. The circular toroidal architecture of β-propeller domains has been proposed to be inherently unstable [11]. In this context, the interactions between the first and last blades, which are responsible for ring closure, are likely to protect from misassembly, domain swapping or fibril formation [12]. The ring closure in the β-propeller domains is stabilized by different mechanisms. In particular, some β-propellers feature a molecular *Velcro* mechanism in which the closure is mediated by anti-parallel β-strands formed by residues from the two termini of the β-propeller domain [1]. On the contrary, the ring closures of the *non-Velcro* type are mediated by hydrophobic interactions between continuous β-sheets [12]. Disulfide bonds can also be found between the first and last blades [13,14]. The *non-Velcro* structure allows flexibility in the ring and even a widening of a central cavity. β-propeller domains can employ such a tunnel to coordinate a ligand [3] or to interact with other protein domains [1,7].

The prolyl oligopeptidase (POP) superfamily includes prolyl oligopeptidases, dipeptidyl proteases IV (DPP IV), oligopeptidases B and acylaminoacyl peptidases (or acyl-peptide hydrolases, AAP/APH) and the β-propeller domain in these proteins plays either regulatory or structural roles [15]. In the POP superfamily, the β-propeller domain is located N-terminal with respect to the catalytic domain. It is also characterized by a structural motif, which protrudes from the β-propeller domain and contacts the C-terminal domain. This motif is poorly conserved in sequence and size [16]. In the AAP family, the N-terminal motif contains the helix α1 [17]. The deletion of this helix in the hyperthermophilic AAP from *Aeropyrum pernix K1* (ApAAP, E.C. 3.4.19.1) affects the protein thermal stability and the temperature-dependence of the AAP activity [16] without causing marked changes in the monomeric

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http://dx.doi.org/10.1016/j.bbapap.2014.09.018
1570-9639/

and dimeric assemblies [18]. A cold-adapted variant from *Sporosarcina psychrophila* AAP (SpAAP) [19,20] and another thermophilic AAP [21] have been recently isolated and characterized. AAPs not only represent an intriguing model for the study of structure-function-dynamics relationships, but also are of marked pharmacological interest [22–24].

It is currently well-accepted that a detailed description of the protein structure and dynamics is indispensable for the investigation of protein function [25]. In particular, dynamically coupled residues are involved in protein structural stability and activity [26–32] as also shown by mutational studies [30,31,33–36]. NMR data indicate that the motions on the nanosecond (ns) or longer timescales are equally important. Thus, it is expected that the dynamics at the ns-timescale provide information on events that are likely to occur at larger timescale and modulate the most important conformational transitions [37].

In this manuscript, we present a comprehensive study of the psychrophilic AAP (SpAAP) by all-atom molecular dynamics (MD) simulations, methods inspired by graph theory, and experimental investigation. Our data note the structural and dynamical traits of SpAAP. In particular, this psychrophilic enzyme presents lysine-to-arginine substitutions and a lack of disulphide bridges in favour of free cysteines compared with its warm-adapted counterparts. Interestingly, SpAAP also has a lower number of glycines than its warm-adapted counterparts, a result that may sound counterintuitive for a cold-adapted enzyme. Nevertheless, in SpAAP, several glycines are strategically placed in proximity to the active site (G457, G483, G537, G573) or in the linker region between the two domains (as G335), where the helix α3 typical of the hyperthermophilic enzyme is lost in favour of a more disordered structure. In particular, the replacement of a tyrosine (Y444 in ApAAP) by a glycine (G457 in SpAAP) reduces the steric hindrance around the nucleophile elbow. SpAAP is also predicted by *in-silico* saturation mutagenesis to accommodate a large number of substitutions with just a few key residues that are not tolerant to mutations. Residues T8, T15, I18 and T19 in the N-terminal region are predicted to be the most sensitive sites to mutations. These are, thus, suitable candidates for future site-directed mutagenesis to assess the role of helix α1 in protein stability.

## 2. Results and discussion

### 2.1. Structural overview

#### 2.1.1. Three-dimensional (3D) structure of the N-terminal β-propeller and C-terminal hydrolase domains

SpAAP has been classified as a cold-adapted enzyme because still retains 10–15% of its optimum activity at 6 °C [20]. We investigated the thermal stability of the multi-domain SpAAP by intrinsic fluorescence spectroscopy (Fig. 1). This technique suggests a cooperative and apparently two-state unfolding process with a $T_m$ of ~50 °C, which is consistent with the results previously observed by CD spectroscopy [20]. SpAAP thermal unfolding differs from the multiple-step unfolding process observed for the hyperthermophilic counterpart, ApAAP [16]. The two-state unfolding of multi-domain proteins has been already noted in other cold-adapted enzymes [38–40]. Nevertheless, before concluding that it is a trait of enzyme cold adaptation, we should notice that we may still identify unfolding intermediates even when the CD and fluorescence data coincide [41]. It must be observed that multi-domain proteins can often reveal multi-step transitions when studied by other more sensitive methods [42]. Overall, equilibrium data alone are not sufficient to demonstrate the effects of one domain on another [42].

The structural data for SpAAP that have been collected to date remain very limited. Hence, we integrate homology modelling, all-atom explicit solvent MD, circular dichroism and mass spectrometry to provide a picture of the conformational ensemble of SpAAP in solution.

The starting structures selected for the different MD simulations are reported in Fig. 2, along with the template structure used for homology

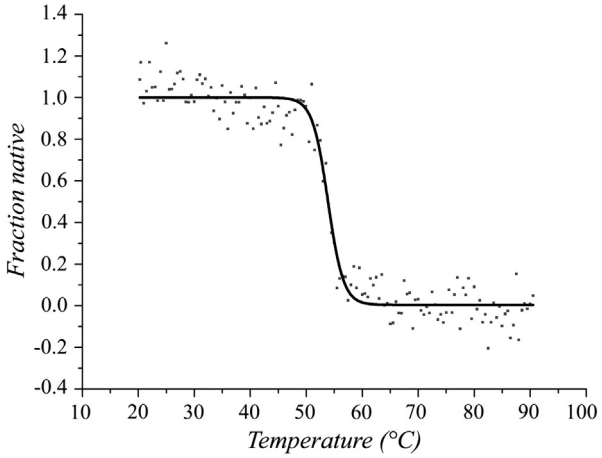


**Fig. 1.** Thermal stability of SpAAP. The thermal unfolding of SpAAP was monitored by intrinsic fluorescence spectroscopy with excitation and emission wavelengths of 280 nm and 330/350 nm, respectively, and fitted to a two-state model. The protein samples were ~ 7 μM in 10 mM sodium phosphate buffer at pH 7.5.

modelling (details in the Method Section). The N-terminal domain of SpAAP (residues 15-333) is a β-propeller with seven blades. Each blade consists of a three- or four-stranded antiparallel β-sheet, with some β-strands shorter than those in the template structure. In the MD-derived structures, blades 1 (residues 20–60), 2 (residues 66–109), 4 (residues 160–186), 6 (residues 254–266) and 7 (residues 295–326) are three-stranded antiparallel β-sheets, whereas blades 3 (residues 112–148) and 5 (residues 204–240) are four-stranded antiparallel β-sheets. SpAAP exhibits a *non-Velcro* mechanism for ring closure that is mainly based on hydrophobic interactions. Indeed, the β-propeller termini fold into separate, adjacent blades (Fig. 1S). This structure is typical of AAP-like proteins and other POP members [1]. On the contrary, several other β-propeller proteins feature a *Velcro* structure [1] in which the first and last β-strands contribute to the same blade (Fig. 1S).

Helix α3 connects the C-terminal part of the β-propeller domain to the C-terminal domain in ApAAP and other warm-adapted AAPs (PDB entries 3AZO [43] and 4HXF [44]). This helix is lost in SpAAP and replaced by a more disordered linker. The C-terminal domain (residue 334–596) has a canonical α/β hydrolase fold with a central eight-stranded, mixed β-sheet flanked by α-helices on both sides. A short α-helix (helix α1, residues 5–14) in the N-terminal region protrudes from the β-propeller domain and connects it to the hydrolase domain.

#### 2.1.2. Secondary structure content by far-UV CD

MD simulations were performed starting from the models and the results were compared with Circular Dichroism (CD) data. At 25 °C, the far-UV CD spectrum (Fig. 3A) of the recombinant SpAAP in solution has a high percentage of well defined α- and β-structures, as expected for a protein belonging to the POP superfamily. We recorded three CD spectra for independent protein preparations and performed deconvolution using the *CDPro* software [45] (Fig. 3). CD spectra deconvolution can be affected by wide variability depending on the reference set used. Thus, we employed three different data sets (1, 3 and 4) that were overall coherent in the calculated secondary structure content. The CD deconvolution data were used to filter out the most unlikely SpAAP structures sampled in the MD simulations. Thus, the MD replicates with average contents in α-helices and β-structures that deviated from experimentally derived data were excluded from the following analyses (Fig. 3B). Replicates 3 and 4 feature a lower average content of α-helices (50% and 30% for replicate 3 and 4, respectively) and β-structures (20%) than the experimental data and were therefore discarded. Replicate 7 was also discarded because most