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Dynamic properties of extremophilic subtilisin-like serine-proteases

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ABSTRACT

The investigation of the structural determinants of enzymatic temperature adaptation is a crucial prerequisite both in terms of fundamental research and industrial applications to develop new biocatalysts active at different temperature ranges. In several cases, the differences related to cold- or warm-adaptation are related to subtle structural and aminoacidic differences at the molecular level, often hard to detect. In this context, we present a comparative study of psychrophilic, mesophilic and thermophilic subtilisin-like serine proteases by all-atom molecular dynamics (MD) simulations in explicit solvent using a multiple-*replica* approach. Our results strongly enforce the current view on localized flexibility in crucial functional regions for cold-adapted serine proteases and point out a different optimization and usage of salt–bridge interactions and networks in cold- and warm-adapted enzymes. The analyses allow to identify a subset of structural and dynamic features strictly associated to cold adaptation and which change from cold- to heat-active subtilisins. In particular, the thermophilic subtilisin presents a high affinity calcium binding site which is not structurally conserved in the mesophilic and psychrophilic counterparts, which, as it turns out from the MD analyses, at the same position show a stable salt bridge network and no stabilizing intra-molecular interactions, respectively. These aspects, along with differential flexibility in regions close to the active site or substrate binding pocket, can be an indication of evolution at this protein site toward a lower stability moving from high to low temperature conditions.

1. Introduction

Several organisms and micro-organisms have been considered, from a anthropocentric point of view, to successfully thrive in extreme environmental conditions thanks to a diverse array of adaptative strategies spanning from structural to physiologic adjustments (Rothschild and Mancinelli, 2001). Temperature is one of the most important environmental factors for life and the clarification of adaptation mechanisms at different temperature conditions are of crucial relevance both for fundamental research (D'Amico et al., 2006, 2002; Muga and Moro, 2008; Somero, 2004; Trivedi et al., 2006) and industrial applications aimed at developing new biocatalysts, active in different temperature ranges (Daniel et al., 2008; Schiraldi and Rosa, 2002).

On the two opposite sides of the scale of temperature adaptation, psychrophilic and hyperthermophilic/thermophilic organisms have to cope with extremely low or high temperature habitats. Among the several adaptative strategies, they have evolved their enzymatic repertory. It has been suggested that the optimization of enzyme function at a given temperature requires a proper balance between a sufficient structural rigidity for the maintenance of the enzyme three-dimensional (3D) architecture and flexibility for the "breathing" of critical protein functional regions (Fields and Somero, 1998; Jaenicke and Böhm, 1998).

Low temperatures tend to increase protein compactness and reduce conformational dynamics, whereas to guarantee the appropriate stability at high temperatures, thermophilic enzymes appear to have generally a very rigid and compact structure, which is often characterized by a tightly packed hydrophobic core and a maximal exposure of surface ion-pairs organized in networks (Kumar and Nussinov, 2001; Li et al., 2005). Accordingly, psychrophilic enzymes should be characterized by an enhanced flexibility of the molecular structure in order to compensate for the lower thermal energy provided by low temperature habitats (Fields, 2001; Svingor et al., 2001). However, the molecular determinants and the relationships between catalytic activity, thermal stability and flexibility in psychrophilic enzymes are still a matter of debate.

The high thermolability of psychrophilic enzymes, in addition to their increased low temperature activity, suggests a direct link between activity and stability: the maintenance of activity at low temperatures requires the weakening of intra-molecular forces which results in reduced stability. On the other hand, it has also been suggested that this high thermolability may be due to random genetic drift as a consequence of lack of evolutionary pressure for stable enzymes in the low temperature habitats (Arnold et al., 2001; Miyazaki et al., 2000). The existence of non-canonical cold-adapted enzymes characterized by both unusual thermostability

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and high catalytic efficiency at low temperatures (Fedøy et al., 2007; Leiros et al., 2007), as well as the capability to uncouple activity and stability in *in vitro* evolution studies (Wintrode and Arnold, 2000) make the definition of activity-stability-flexibility relationships even more difficult.

Proteomic and genomic analyses of sequence compositions in extremophiles have identified significant correlations with the corresponding optimal organism growth temperatures (Gu and Hilser, 2009; Zeldovich et al., 2007). Moreover, an inventory of structural and molecular characteristics related to temperature adaptation in extremophilic enzymes came from several comparative statistical studies of cold- and heat-active enzymes (Berezovsky and Shakhnovich, 2005; Gianese et al., 2002; Jahandideh et al., 2008), pointing out that general theories cannot be formulated. Gu and Hilser (2009) also showed that there is no uniform modulation of conformational flexibility and stability across the components of the proteome of organisms adapted to different environmental conditions, as well as that apparent mechanisms of thermal adaptation differ from protein to protein. Each enzyme seems to display slightly different structural strategies to adapt to low or high temperatures.

In this context, the degree of similarity between warm- and cold-adapted enzymes to compare should be relatively high to be meaningful. Single structural effects cannot be easily estimated if significant differences in the protein architecture are found. Recently, several comparative approaches of homologs adapted to different temperature conditions but belonging to the same enzymatic family (Cartier et al., 2010; Coquelle et al., 2007; Khan and Sylte, 2009; Papaleo et al., 2008; Siglioccolo et al., 2010) have been successfully applied, along with comparison of protein dynamic properties of extremophilic homologs (Aurilia et al., 2009; Chiuri et al., 2009; D'Auria et al., 2009; Heidarsson et al., 2009; Kundu and Roy, 2009; Mereghetti et al., 2010; Papaleo et al., 2006; Pasi et al., 2009) to disclose structure–function relationships in cold- and warm-adapted enzymes. Most of these studies compared mesophilic and psychrophilic enzymes, since few cases are known for which enzymes with know 3D structure are available along the whole scale of temperature conditions. It has also been suggested that enzymes sharing the same function and 3D fold may have to adopt similar strategies to optimize structural stability and flexibility in order to elicit their biologic function under the challenging conditions of extreme temperature habitats (Johns and Somero, 2004; Papaleo et al., 2008).

In light of the above observations, we present a comparative study on the whole scale of temperature adaptation of psychrophilic, mesophilic and thermophilic subtilisin-like serine proteases by all-atom molecular dynamics (MD) simulations in explicit solvent using a multiple-*replica* approach (Caves et al., 1998; Friedman and Caflisch, 2010; Loccisano et al., 2004; Monticelli et al., 2008). The results of the present investigation enforce the current view on localized flexibility in crucial functional regions for cold-adapted serine-proteases (Papaleo et al., 2008) and pointed out a different optimization of salt-bridge interactions and networks in cold- and warm-adapted enzymes. In particular, our analyses allow the identification of a subset of structural and dynamic properties associated to cold adaptation and which change from cold- to heat-active subtilisins.

2. Methods

2.1. Molecular dynamics (MD) simulations

MD simulations were performed using the GROMACS 3.3.3 software package (www.gromacs.org) implemented on a parallel architecture, using the GROMOS96 43a1 force field.

The X-ray structures of the psychrophilic subtilisin-like protease from *Vibrio* sp. *PA-44* (VPR, pdb entry 1SH7 (Arnórsdóttir et al., 2005), thermophilic thermitase from *Thermoactinomyces vulgaris* (TRM, pdb entry 1THM (Teplyakov et al., 1990)) and mesophilic Proteinase K from *Tritirachium album* (PK, pdb entry 1IC6 (Betzel et al., 1988)) have been used as initial structures for the simulations. The crystallographic water molecules at more than 0.1 nm of distance from the structures were retained during simulations, as well as the bound calcium ions. Generally subtlisin enzymes bind conserved calcium ions with different affinities (Siezen and Leunissen, 1997). To obtain the starting structures of TRM simulations, the sodium ion that occupied the Ca3 bindig site of TRM in the crystal structure was removed (TRM simulations) and replaced by calcium (TRM-Ca3 simulations), respectively (Table 1) .

The starting structures, including the crystallographic water molecules, were soaked in a dodecahedral box of SPC (Simple Point Charge) water molecules and simulations were carried out in periodic boundary conditions. The total number of water molecules was of 6282 for TRM, 6280 for TRM-Ca3, 6783 for PK and 7347 for VPR. All the protein atoms were at a distance equal or greater than 0.6 nm from the box edges. The ionization state of residues was set to be consistent with neutral pH and tautomeric form of histidine residues was derived using GROMACS tools and confirmed by visual inspection. In order to neutralize the overall charge of the system, a number of water molecules equal to the protein net charge were replaced by Cl- or Na+ ions.

Initially, the system was relaxed by molecular mechanics (steepest descent, 10,000 steps). The optimization step was followed by 50 ps of solvent equilibration at 300 K (time step 1 fs), while restraining the protein atomic positions using an harmonical potential. During equilibration the coupling constant to the external bath was set to 1 fs. The system was slowly driven to the target temperature (300 K) and pressure (1 bar) through a series of short thermalization and pressurization simulations of 50 ps each.

The same preparation procedure was carried out for TRM and VPR simulations at 343 and 283 K, respectively. 343 and 283 K have been selected as representative of the optimal temperatures of the two extremophilic enzymes. However, it has to be mentioned that a comparison of flexibility and dynamic properties of cold- and warm-adapted homologs at their optimal temperature by MD simulations, in absence of an experimental validation of the simulated temperatures by biophysical approaches, has to be taken with caution.

While PK and VPR both belong to the Proteinase K subfamily, Thermitase does not (Siezen and Leunissen, 1997) and it could be argued that it is not the best candidate to carry out a comparative MD study even if it is the only thermophilic subtilisin with a known 3D experimental structure. To validate the use of thermitase in the forthcoming analyses and to enforce conclusions derived by our investigation, the 3D structure of the thermophilic Aqualysin I from *Thermus aquaticus* (AQUI) was predicted through homology modeling and multi-*replica* MD simulations have been collected. AQUI's two closest homologs, VPR itself and Proteinase K from *Serratia Sp.* GF96. were used as input templates for the homogy modelling software package MODELLER (Eswar et al., 2008). Aqualysin I features two calcium-binding sites of unknown position (Lin et al., 1999), with high and low binding affinity for the metal, respectively. The location of these sites was defined by similarity, evaluating the conservation of the calcium-coordinating residues with respect to the template structures. The calcium ions were modelled in correspondence of VPR's Ca1 and Ca3 sites. MODELLER performance scores and the structure validation tool AIDE (Mereghetti et al., 2008) were employed to assess the model quality.

Productive MD simulations have been carried out in the isothermal-isobaric ensemble, using an external bath with a coupling constant of 0.1 ps for each protein (PK, VPR, TRM, AQUI) at 300 K and