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# UNFOLDING SIMULATIONS OF COLD- AND WARM-ADAPTED ELASTASES

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## ABSTRACT

***The earth surface is dominated by low temperature environments, which have been successfully colonized by several extremophilic organisms. Enzymes isolated from psychrophilic organisms are able to catalyze reactions at low temperatures at which enzymes from mesophiles or thermophiles are fully compromised. The current scenario on enzyme cold-adaptation suggest that these enzymes are characterized by higher catalytic efficiently at low temperatures, enhanced structural flexibility and lower thermostability. In the present contribution, molecular dynamics simulations in explicit solvent have been carried out at different high temperatures in order to investigate the unfolding process of cold- and warm-adapted homologous enzymes. In particular, we focused our attention on cold-adapted elastases for which it was previously demonstrated that the psychrophilic enzyme presents higher localized flexibility in loops surrounding the catalytic site and the specificity pocket. The unfolding simulations show a slower unfolding process for the cold-adapted enzyme, but characterized by a greater loss of intramolecular interactions and α-helices than the mesophilic counterparts.***

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**Keywords:** *cold-adapted enzymes; unfolding; molecular dynamics simulations; psychrophilic enzymes; elastase; serine-protease*

## [I] INTRODUCTION

In recent years, increasing interest has been devoted to the investigation of determinants of adaptation to low temperatures of the enzymatic repertory of cold-adapted organisms [1, 2]. The study of cold-adapted enzymes attracts both fundamental researches than industrial application for the design of new biocatalysts [3, 4]. The number of reports on enzymes from psychrophilic organisms has increased over the past years, and reveals that adaptative strategies vary among different enzymes, which use different structural features for gaining increased catalytic efficiency at low temperatures, reduced thermal stability and increased molecular flexibility.

In fact, enzyme catalysis generally involves the "breathing" of particular protein regions, enabling the accommodation of the substrate [1]. The optimization of enzyme function at a given temperature requires a proper balance between two opposing factors: structural rigidity, allowing the retention of a specific conformation at the physiological temperature, and flexibility, allowing the protein to perform its catalytic function [5].

Whereas heat-adaptation seems to be generally related to protein rigidity, cold-adaptation, at the opposite side of the temperature scale, should be characterized by enhanced flexibility of crucial protein regions in order to compensate for the lower thermal energy in low temperature habitats [6, 7]. This plasticity has been suggested to enable a good complementarity with the substrate at a low energy cost. In return, this flexibility would be responsible for the high thermolability of the psychrophilic enzymes. The low stability of psychrophilic enzymes has been demonstrated by the drastic shift of their apparent optimal temperature of activity, the low resistance of the protein to denaturating agents and the high propensity of the structure to unfold at moderate temperatures [1].

High temperature MD simulations turned out a valuable tool to study unfolding process of several protein systems [8-12]. In particular, all-atom explicit solvent MD simulations provide insights into biomolecules dynamics thanks to the continuity of the trajectory in the phase space and are a useful tool to complement experimental investigations. MD can provide further information on the protein folding and unfolding processes along with elucidation of intermediates and factors affecting stability of folded and unfolded forms [8, 13-14].

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Protein unfolding studies by atomistic simulations are carried out generally increasing the temperature necessary to overcome the enthalpic forces stabilizing the three-dimensional structure. It has been demonstrated that high temperature simulations accelerate the unfolding pathway without affecting the pathway itself [15]. The use of higher temperatures over the unfolding experimental temperatures is necessary considering the differences in the time scales accessible by experiments and calculations. In MD simulations at high temperatures, protein unfolding can occur within a few nanoseconds [15-16].

In light of the above observations, we decided to investigate the thermal unfolding process of a mesophilic and cold-adapted elastases by multiple-*replica* MD simulations. The aim of this study is not only to analyze specific features of the unfolding mechanisms of the two homologous elastases, but also to gain a deeper understanding of molecular basis of cold-adaptation of psychrophilic serine proteases, which at low temperatures are known to be characterized by higher catalytic activity [17] and higher local structural flexibility in the proximity of the functional sites [6], with respect to the mesophilic counterpart.

## [II] MATERIALS AND METHODS

MD simulations were performed using the GROMACS software and GROMOS96 force field (www.gromacs.org) implemented on a parallel architecture. The enzymes selected for this study have been the mesophilic pancreatic porcine (PE) and psychrophilic Atlantic salmon (SE) elastases (PDB entries 1LVY and 1ELT, respectively). For these enzymes various MD simulations were previously carried out at 283 and 310K [6]. In particular, for both the warm and cold-adapted elastase, we selected two structures from two different independent simulations at 310 K. These structures are representative of the conformational space explored at this reference temperature. To reach the desired unfolding temperatures, solvent have been equilibrated in 4 steps increasing the temperature at each step (310→350, 350→400, 400→450, 450→500 K), during which the protein atomic positions were restrained using a harmonical potential. In the simulations, the structures were soaked in a dodecahedral box of SPC (Simple Point Charged) water molecules and simulations were carried out using periodic boundary conditions.The ionization state of the 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. $Cl^-$ counterions were added to neutralize the system.

Productive MD simulations were performed in the isothermic-isobaric ensemble (NPT ensemble) at 400, 450 and 500K, applying periodic boundary conditions and using an external bath with a coupling costant of 0.1 ps. Pressure was kept constant at 1 atm and the time-constant for the pressure coupling was set to 1 ps. The LINCS algorithm was used to constraint bond lengths, allowing to use a 2 fs time step. Electrostatic interactions were calculated using the Particle-mesh Ewald (PME) summation scheme. Van der Waals and Coulomb interactions were truncated at 1.0 nm. The nonbonded pair list was updated every 10 steps and conformation stored every 2ps.

To improve the conformational sampling, independent simulations (*replicas*) obtained initializing the MD runs with different Maxwellian distributions of initial velocities, were carried out for each protein system at each temperature. In particular, two and four 3 ns simulations have been carried out at 400K and 450-500K, respectively. The different simulations have been identified according to different codes.

The two letters (PE-SE) identify the enzyme, the number (400-450-500) is referred to the simulation temperature, the roman number (III-IV) defines the two different initial structures whereas the last letter (a-b) discriminates between the different initial velocities.

For example, PE450IIIa and PE450IIIb refer to simulations of the porcine elastase at 450K performed starting from the same initial structure but with a different initial random seed (a and b), whereas PE450IIIa and PE450IVa refer to simulations of the porcine elastase at 450K performed starting from two different initial structures (III and IV).

## [III] RESULTS

Unfolding simulations of a cold-adapted elastase (SE) and its mesophilic counterpart (PE) have been carried out in order to shed light on molecular characteristics of cold-adaptation.

Generally, each protein molecule may unfold via its own pathway and, though the majority of the pathways are probably similar, some of them may differ from other considerably. Therefore, to ensure the validity of unfolding simulations and that the main traits of the trajectories are reproducible and representative, it is necessary to analyse multiple trajectories related to the same system.

### *3.1. Unfolding profiles*

A measure to identify the unfolded state is the RMSD (root mean square deviation) with respect to a reference structure. It is generally accepted that mainchain RMSD > 5 Å, calculated using as reference structure the native structure, defines that the protein is in an unfolded state [18]. RMSD values were calculated on mainchain atoms using as a reference the starting structure of the simulations **[Figure-1]**.

The most evident changes in the elastase native structure are detected at 500K, even if structural changes can be already identified at 400K simulations. At 500K, the mesophilic enzyme undergoes a greater structural rearrangement, presenting a fast unfolding of the structure, after which seems to reach an equilibrium state. In SE the trend is more linear: the increase in RMSD is slower and on-going.

Another parameter reflecting structural changes is the protein radius of gyration (Rg), which is a measure of compactness of the protein structure. However, it is not sufficiently informative if considered alone, since in the unfolded state a protein could still retain some degree of compactness. Therefore, two-dimensional plot of mainchain RMSD versus protein radius of gyration have been carried out, also in order to remove the differences due to the fact that corresponding events could occur at different times in different trajectories **[Figure-2, 3]**. Different colors indicate three different parts of the simulations: black, red and green correspond to the first 1ns, from 1 to 2 ns, and from 2 to 3 ns, respectively. Some trajectories show an approximately simultaneous increase in Rg and RMSD in the first part of the unfolding process and much more complexity after partial denaturation, whereas others have a complex behavior starting from the initial stages of the unfolding process.

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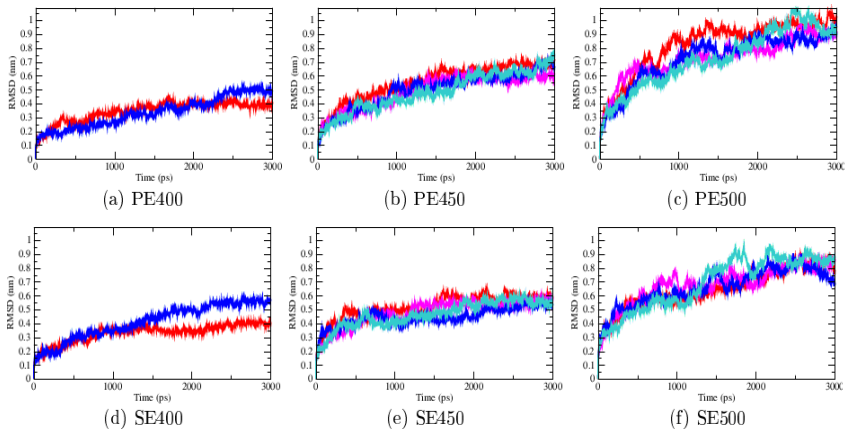


**Fig 1. RMSD as a function of simulation time.** The different colors indicate the different independent simulations for each system.

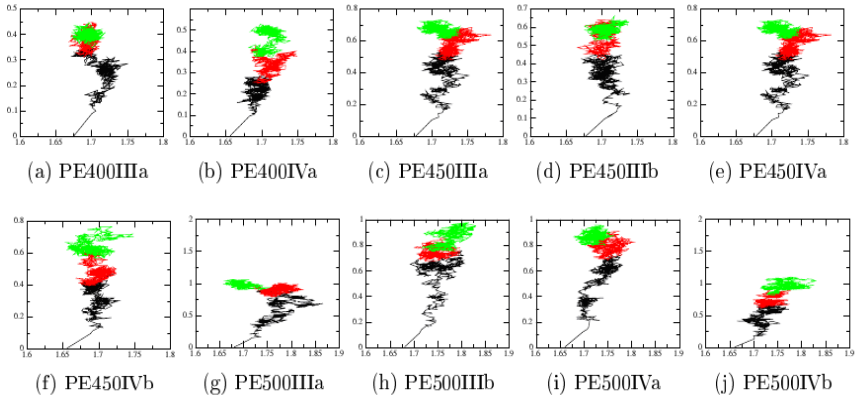


**Fig 2. Rg (x-axis) versus Rmds (y-axis) in PE simulations.** The different colors indicate different simulation time windows (black 0-1ns, red 1-2 ns and green 2-3 ns).

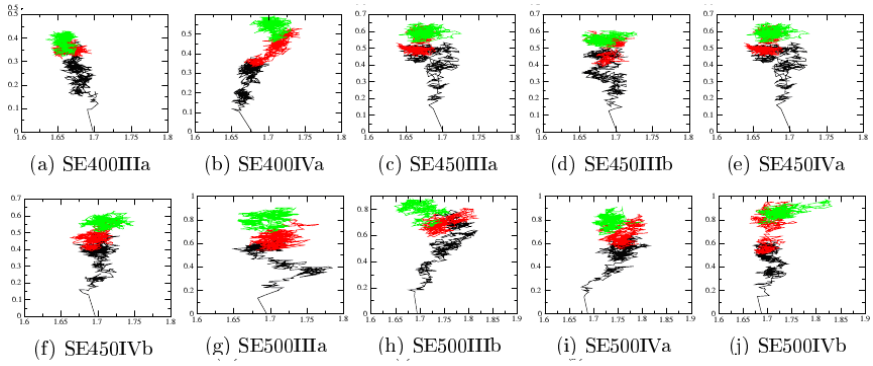


**Fig 3. Rg (x-axis) versus Rmds (y-axis) in SE simulations.** The different colors indicate different simulation time windows (black 0-1ns, red 1-2 ns and green 2-3 ns).

### *3.2. RMSD matrices*

In order to understand if there are common features in the unfolding pathway, all the structures of each simulations at a given temperature have been compared, measuring mainchain RMSD, with the structures of other simulations at the same temperature (for example, simulations a versus b) **[Figure-4]** and with the simulations at the other temperatures but performed from the same starting structures (for example simulation at 400K versus 500K) **[Figure-4]**.

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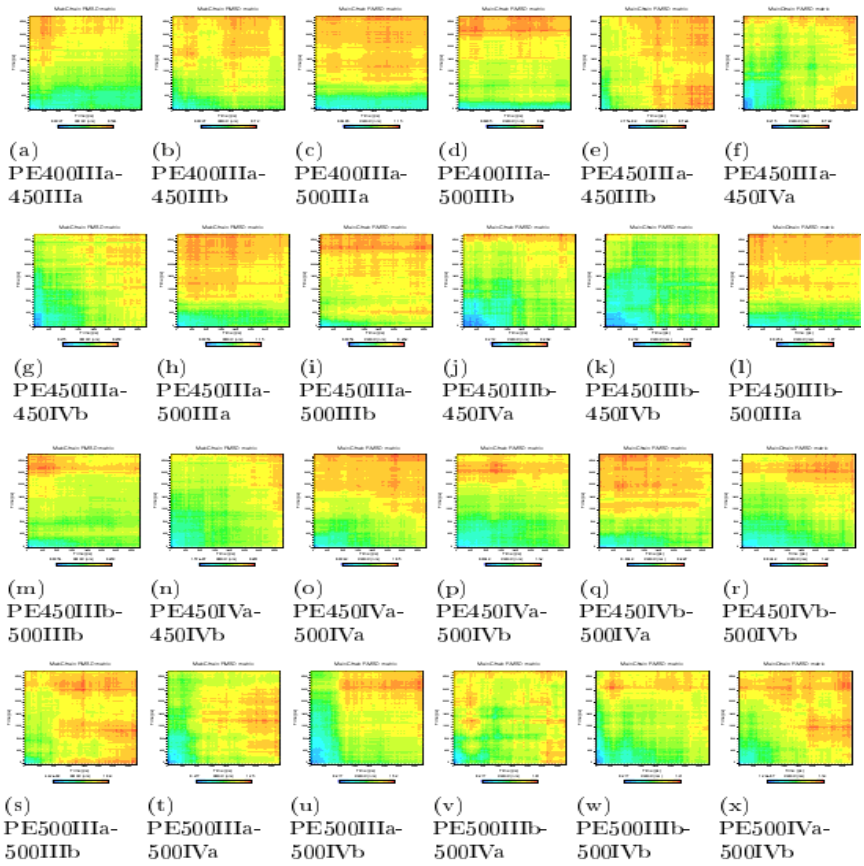


**Fig 4. RMSD matrices of PE.** The matrices present the RMSD obtained from the comparison of each structure of a simulations with the structures collected from another simulation. Both comparisons of independent replicas of the same system at the same temperature and of replicas of the same system at different temperatures are shown. The RMSD matrices of SE simulations present a comparable trend (*data not shown*). The color bar indicate increasing RMSD values from blue to red.

In particular, the RMSD matrices are computed calculating the mainchain RMSD between all the structures of one simulation (x-axis) against all the structures of another simulation (y-axis). A color scale from blue to red indicates increasing values of RMSD. A blue area means that corresponding structures in the two simulations have an high degree of similarity.

Comparing simulations at the same temperature, the highest similarity is gathered in the left-low corner. Following the simulation course, simulations performed using different starting structures and different initial atomic velocities, drift away from each other.

In **Figure-4**, cyan indicates low RMSD values and structural similarity is concentrate in the lower rows of the matrices. The x-axis corresponds to the simulation at the lower temperature, indicating that at 500K the protein follows the same pathway explored at 400K, but employing less computational time. The high temperature seems not affect the unfolding pathway itself since the structures collected in the initial steps of 500K simulations resemble structures from 400K simulations, but only speed up the process.

### *3.3. Intramolecular interactions and secondary and tertiary structure content.*

The numbers of intramolecular hydrogen bonds (H-bonds) have been monitored along the simulation time. Even if the total number is not informative in terms of individual residues involved in the interactions, it can give an estimation of the compactness of the structures and the lack of native structures. It turns out that at 500K the number of H-bonds is clearly reduced. In fact, at 500K PE and SE has an average value of H-bonds of 110 and 100, respectively, whereas the native structure is characterized by 185 and 182 H-bonds, respectively [6]. In particular, in PE the H-bonds decrease till 2 ns and then increase again at the end of the simulations, suggesting the breaking of native intramolecular interactions and formation of non-native intramolecular interactions in the unfolded state. Whereas in the native structure SE and PE present a almost comparable number of total intramolecular H-bonds, SE tends to lose a higher number of H-bonds that PE in the early step of the unfolding process. In fact, average H-bonds in the first 500 ps of simulation at 500K are reduced to 104 in SE and 120 in PE.

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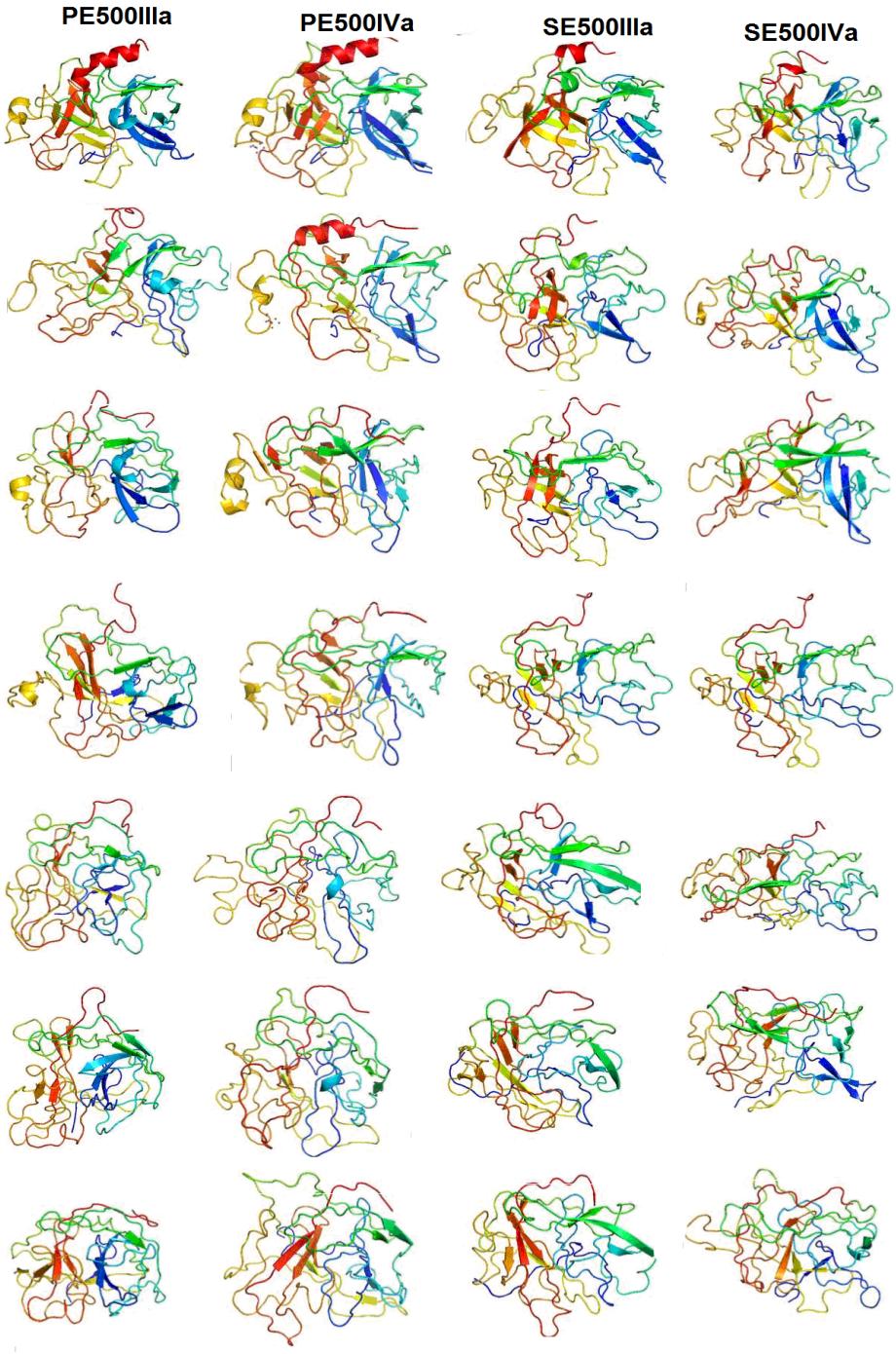


**Fig 5. Snapshots taken from simulations at 500K every 0.5 ns.** Data relative to simulations a at 500 K are indicate. The different columns correspond to PEIIIa, PEIVa, SEIIIa and SEIVa, respectively.

3D structure of elastases folds into two juxtaposed domains characterized by an antiparallel β-type fold, including some α-helices. Secondary structure content over the simulation time have been also monitored, according to the DSSP program definition [19] as well as snapshots from the simulations [Figure-5]. The starting conformations of the mesophilic enzyme are always more structured in β-sheet, for at least 20 residues more, than the psychrophilic counterpart.

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At 400K, both in PE and SE the changes in the structures are quite slow, in agreement with the RMSD analysis. Interestingly, the reduction in residues involved in α-helices is a common feature of the two enzymes at both temperatures. However, SE tends to lose α-helices in the earlier steps than PE. In fact, in SE α-helix content is reduced of more of more that 75% just after 0.1 ns, whereas PE retains native α-helices up to 0.5 ns. At this temperature systems are not completely unfolded. More interestingly, at 500K PE unfolds rapidly, in less than 0.4 ps the decrease of all structured residues is about 30% with respect to the native structure. After this early event, the secondary structure composition remains quite stable, in agreement with the previous indications by RMSD analysis. The psychrophilic enzyme presents a slower unfolding process with the main transition appearing in the range between 0.4 an 1 ns. In both systems, β-sheet conformations, which are the main component of the native elastase 3D fold, seem to be fairly stable in the first step of the unfolding process and decrease in the last ns.

## [IV] CONCLUSION

In the present contribution, we present an investigation at the molecular level of the unfolding process of two homologous enzymes sharing a common fold and a high sequence similarity (more than 68% of sequence identity) but adapted to different temperature conditions. Both enzymes present in the first steps of the unfolding process a loss of native α-helices. At 500K the unfolding process is clearly visible: after only 0.4 ns the mesophilic enzyme has lost 30% of the native structures. The unfolding process in the psychrophilic enzyme occurs later, in agreement with a higher flexibility and lower number of intramolecular interactions localized in the proximity of the functional site but a higher rigidity of the rest of the protein structure with respect to the mesophilic counterpart [6]. Interestingly, even if SE seems from our simulations to undergo toward a slower unfolding process, the regions in the proximity of the catalytic triads are the first to be affected by increased temperatures and promote the local unfolding in the first step of the process.

### FINANCIAL DISCLOSURE

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