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Dynamics fingerprint and inherent asymmetric flexibility of a cold-adapted homodimeric enzyme. A case study of the *Vibrio* alkaline phosphatase

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ABSTRACT

Background: Protein dynamics influence protein function and stability and modulate conformational changes. Such motions depend on the underlying networks of intramolecular interactions and communicating residues within the protein structure. Here, we provide the first characterization of the dynamic fingerprint of the dimeric alkaline phosphatase (AP) from the cold-adapted *Vibrio* strain G15-21 (VAP), which is among the APs with the highest known k_{cat} at low temperatures.
Methods: Multiple all-atom explicit solvent molecular dynamics simulations were employed in conjunction with different metrics to analyze the dynamical patterns and the paths of intra- and intermolecular communication.
Results: Interactions and coupled motions at the interface between the two VAP subunits have been characterized, along with the networks of intramolecular interactions. It turns out a low number of intermolecular interactions and coupled motions, which result differently distributed in the two monomers. The paths of long-range communication mediated from the catalytic residues to distal sites were also characterized, pointing out a different information flow in the two subunits.
Conclusions: A pattern of asymmetric flexibility is evident in the two identical subunits of the VAP dimer that is intimately linked to a different distribution of intra- and intermolecular interactions. The asymmetry was also evident in pairs of cross-correlated residues during the dynamics.
General significance: The results here discussed provide a structural rationale to the half-of-site mechanism previously proposed for VAP and other APs, as well as a general framework to characterize asymmetric dynamics in homomeric enzymes.

1. Introduction

It is well established that bio-macromolecules are not to be considered as static entities but rather as dynamic systems characterized by a large degree of flexibility and mobility. Protein atoms are subjected to internal and external forces, which can promote conformational changes across a number of energetic minima. In this context, the protein native state can be described as an ensemble of statistically populated conformers. These motions are generally related to numerous subtle modifications in the networks of intra-molecular interactions at any given temperature over the absolute zero [1–4]. The knowledge of protein dynamics, even on short timescales, and of the mechanisms related to protein collective motions are crucial for a complete understanding of enzyme properties. In fact, conformational movements are also related to the rate-limiting step in many enzymes [5–7]. In this context, computational studies involving biomolecular simulations can act as a bridge between the smallest scales of macromolecules and the macroscopic world of molecular ensembles [8,9].

The majority of enzymes in the biosphere work at the low temperatures that prevail in the oceans, at high altitude, or in sub-polar climate regions [1,10,11]. Low temperatures are related to relatively low levels of kinetic energy and a reduced extent of molecular motions. Cold-adapted enzymes are generally characterized by a higher structural flexibility than the heat-tolerant counterparts. The enhanced flexibility is likely to provide the proper dynamics required for a fully functional enzyme at low temperatures [2,12–14]. This notion is emphasized by the folding funnel model proposed for cold-adapted enzymes [15,16]. The bottom of the funnel of a cold-adapted enzyme is wider and more rugged than in the classical enzymes. In fact, the free energy landscape of a cold-adapted enzyme is likely to be characterized by a large number of conformers separated by low energy barriers, resulting in a relatively labile and flexible protein. Moreover, results from molecular dynamics (MD) simulations supported by biophysical investigations are consistent with a scenario

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in which both rigidity in regions far from the functional sites and enhanced flexibility of regions in the proximity of the protein active site can be important factors for cold adaptation [17–23].

Intramolecular atomic interactions are only one side of the story. In fact, proteins involved in many fundamental biological processes carry out their activity and are regulated through interactions with other proteins in intermolecular complexes. Many proteins function as parts of permanent complexes, which often fold and simultaneously associate, e.g. multimeric enzymes [24,25]. The forces that keep protein complexes together are the same interactions that maintain the stability of their components in the folded state [24]. In some cases, the active site is formed by two or more of the monomers, so that the disruption of the quaternary structure would directly result in the complete loss of their activity. In the context of cold-adaptation, oligomeric psychrophilic enzymes are subjected to the same structural and dynamical adaptive strategies of non-oligomeric enzymes. In both cases, they lead to higher catalytic activity at low temperatures, lower thermal stability, and higher structural flexibility than their homologs from mesophilic organisms [13,20,26]. Thus, intermolecular interactions in cold-adapted enzymes are of particular importance for their function and are still not well characterized in a dynamics framework.

In this context, we used atomistic MD simulations to study the dimeric cold-adapted alkaline phosphatase (AP) from a *Vibrio* bacterium (VAP) [27]. Despite similar structural cores and active sites, different APs feature a broad array of remarkably dissimilar structural details [28,29]. In fact, subtle influences of key active site residues were demonstrated to influence protein activity as shown by several mutagenesis studies and natural variants [30,31].

A better understanding of this variability and how it serves each organism in different temperature regimes is going to contribute to basic understanding in enzymology. Although the reaction mechanism of APs is relative simple, a one substrate hydrolysis where specificity for the organic part of the substrate is broad, there is still debate on how this group of enzyme works [32–35]. The role of key conformational change has been suggested in conjunction with cooperativity, with only one of the potential active sites being reactive at any one time [36–40]. The AP active site has the ability to recognize different transition states [41] and is known to have promiscuous activities [34,42]. As APs belong to a wider family of hydrolases, it is also of general interest to apply MD investigation to this archetypal dimeric enzyme.

In this contribution particular attention has been devoted to the study of structural flexibility, networks of coupled motions and intra-intermolecular interactions, which characterize the native VAP conformational ensemble.

2. Materials and methods

2.1. Molecular dynamics (MD) simulations

The X-ray structure of alkaline phosphatase from the cold-adapted *Vibrio* strain G15-21 (VAP, pdb entry 3E2D [27] was used as a starting structure for the simulations. MD simulations were performed using Gromacs 4.5.3 software (www.gromacs.org) implemented on a parallel architecture and the Gromos96 43a1 force field.

The starting structure was soaked in a dodecahedral box of SPC (Simple Point Charge) water molecules [43], using periodic boundary conditions, with a minimum distance between the solute and the box edges of 0.8 nm. To neutralize the overall charge of the system, a number of water molecules equal to the protein net charge were replaced by Na^+ ions. The system was initially relaxed by molecular mechanics (steepest descent, 10000 steps) followed by 50 ps of solvent equilibration at 303 K (time step 1 fs and thermal coupling constant of 0.006 ps), while restraining the protein atomic positions using a harmonical potential. Subsequently, the system was submitted to a series of thermalization and pressurization simulations of 50 ps each, in order to slowly target the simulation temperature and pressure to 303 K and 1 bar, respectively.

Productive 40/60 ns MD simulations were carried out in the isothermal-isobaric (NPT) ensemble, using an external bath with a coupling constant of 0.1 ps at 303 K, whereas pressure was kept constant (1 bar) by modifying the box dimensions. The time-constant for pressure coupling was set to 1 ps. The LINCS algorithm [44] was employed to constrain heavy atom bond lengths, allowing the use of a 2 fs time-step. Long-range electrostatic interactions were calculated using Particle-Mesh Ewald (PME) summation scheme. Van der Waals and Coulomb interactions were truncated at 0.8 nm. The non-pair list was updated every 10 steps and conformations were stored every 4 ps. To identify recurring features and to avoid simulations artifacts, four independent simulations were carried out for the full-length VAP (Fig. 1S), collecting 200 ns overall. One of these simulations was carried out, as a control, at a physiological salt concentration ([NaCl] = 100 mM) (Fig. 1S).

2.2. MD analyses

The time evolution of the main chain root mean square deviation (rmsd), which is an important parameter to evaluate the stability of MD trajectories, was computed using the starting structure of the MD simulations as a reference. The first 2 ns of each replica were discarded in order to assure stability of the trajectories for further analyses (Fig. 3S). Moreover, the stability of metal ions at their binding sites was assessed by monitoring the distances between each coordinating residues and the metal ion (*data not shown*). The stability of secondary structural elements was also monitored using DSSP, along with calculations of the most frequently attained secondary structure for each residue by a profile of residue-dependent persistence degree of secondary structure.

The dynamic properties described by the four independent simulations were compared by the calculation of root mean square inner product (rmsip) for the first 10 eigenvectors derived by the Principal Component Analysis (PCA) on the C-alpha (Cα) covariance matrix of atomic fluctuations [45]. Rmsip is an index of similarity between the essential subspaces sampled by the different MD trajectories. In our MD simulations, rmsip was in the range of 0.6–0.7, indicating an overall agreement in the dynamics fingerprints described by the different simulations.

The per-residue Cα root mean square fluctuation (rmsf) was calculated using as a reference the average structure. To properly evaluate the description on protein flexibility achieved by rmsf profiles, rmsf was computed on the whole trajectories, along with average rmsf profiles over different timescales (1 ns, 2 ns, 3 ns, 5 ns and 10 ns). Moreover, the time-evolution of rmsf was calculated comparing rmsf profiles obtained by sliding windows of different time lengths (1 ns, 2 ns, 3 ns, and 5 ns).

To further validate the congruence between the information accounted by the four independent simulations, the Pearson correlation coefficient between rmsf profiles calculated on 1 ns time-windows of each trajectory was calculated, providing correlation values higher than 0.75.

Under periodic boundary conditions, the protein molecule might interact with its image across the boundary, which is clearly an artifact. Since the simulation time that can be sampled by an MD run decreases rapidly with the increase in the box size, it is often not possible to use a very large box and, thus, make impossible any kind of interaction between the protein image and the box. A good compromise is to use a box large enough to make self-interaction a very rare event. In light of these observations, to validate our MD simulations, the time-series of the minimum distance between the molecule and its image was monitored in each trajectory. None of the trajectories showed any close approaches between them. The minimum separation between the molecule and its image was always higher than