Molecular Determinants of Enzyme Cold Adaptation: Comparative Structural and Computational Studies of Cold- and Warm-Adapted Enzymes

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Abstract: The identification of molecular mechanisms underlying enzyme cold adaptation is a hot-topic both for fundamental research and industrial applications. In the present contribution, we review the last decades of structural computational investigations on cold-adapted enzymes in comparison to their warm-adapted counterparts. Comparative sequence and structural studies allow the definition of a multitude of adaptation strategies. Different enzymes carried out diverse mechanisms to adapt to low temperatures, so that a general theory for enzyme cold adaptation cannot be formulated. However, some common features can be traced in dynamic and flexibility properties of these enzymes, as well as in their intra- and inter-molecular interaction networks. Interestingly, the current data suggest that a family-centered point of view is necessary in the comparative analyses of cold- and warm-adapted enzymes. In fact, enzymes belonging to the same family or superfamily, thus sharing at least the three-dimensional fold and common features of the functional sites, have evolved similar structural and dynamic patterns to overcome the detrimental effects of low temperatures.

Keywords: Molecular dynamics, flexibility, stability, electrostatic interactions, cold-adapted enzyme, psychrophilic enzyme, flexibility, cold adaptation, extremophilic enzyme.

1. INTRODUCTION

It is of crucial relevance to acquire detailed knowledge on the molecular mechanisms that rule the relationships between stability, flexibility, and activity in extremophilic enzymes both for fundamental research and industrial applications [1-6].

Among extremophilic enzymes, those isolated from psychrophilic organisms have received particular attention by the scientific community only in the last 20-25 years, mainly thanks to their unique properties of high activity at low and challenging temperatures, high thermolability and unusual specificity, thus offering a broad spectrum of biotechnological applications [5-8]. In this context, for example, increasing interest has been directed to applications of cold-adapted enzymes in detergents, for low-temperatures laundry with a remarkable reduction in energy consumption [9]. Applications in food and milk industry are also numerous. In baking processes, enzymes such as amylases, proteases and xylanases can be used to reduce the dough fermentation time, as well as to improve the properties of the dough and the crumb [5]. Cold-adapted enzymes have also great potential in the field of wastewater treatment and bioremediation in contaminated cold environments [10]. Moreover, psychrophilic enzymes, and especially lipases have emerged as important biocatalysts in biomedical applications, thanks to their excellent capability to perform specific regioselective reactions in a variety of organic solvents [11].

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Evolution has allowed cold-adapted organisms not to merely survive, but also to successfully grow in the restrictive conditions of cold habitats. Psychrophiles display metabolic fluxes at low temperatures that are more or less comparable to those exhibited by closely related mesophiles, which live at moderate temperatures [12], suggesting that mechanisms of temperature adaptation are involved. Such mechanisms include a broad array of structural and physiological adjustments to cope with the reduction of chemical reaction rates induced by the low temperatures [13, 14]. In fact, temperature is one of the most important environmental factors for life; reaction rates can be reduced from 30- to 80- folds when the medium temperature decreases from 37°C to 10°C [15]. Moreover, cold denaturation of proteins is a well-established phenomenon [16, 17] with complete unfolding occurring below -15°C, a process thermodynamically favoured at low temperatures, which weakens the hydrophobic interactions crucial for protein folding and stability [1, 18]. Biological activities have been recorded in the brine veins of sea-ice at temperatures as low as -20°C, as well as psychrophilic enzymes isolated from microorganisms still active at temperatures below -10/-15°C, have been identified [19]. Thus, in the cold environments psychrophilic organisms had to evolve and optimize their enzymatic repertory.

Several biochemical studies on cold-adapted enzymes, carried out in the last decade, have identified common key features in the optimization of the kinetic and thermodynamic parameters. In particular, cold-adapted enzymes are generally characterized by lower thermal stability, higher catalytic activity, and higher catalytic efficiency (k_{cat}/K_m) at low temperatures with respect to their warm-adapted counterparts [15, 20]. In fact, psychrophiles produce enzymes

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characterized by tens folds higher specific activity at low temperatures than "canonical" enzymes. The apparent maximal activity is shifted toward low temperatures, reflecting the weak stability of psychrophilic enzymes which are generally prone to inactivation and unfolding at moderate temperatures. It has also been shown that enzyme cold adaptation is usually incomplete, since the specific activity exhibited by most of the psychrophilic enzymes around 0°C although high remains generally lower than that of the mesophilic counterparts at 37°C.

In principle, cold-adapted enzymes can optimize the k_{cat}/K_m ratio by increasing k_{cat}, decreasing K_m or altering both the kinetic parameters. A survey of the available kinetic parameters revealed two main trends; psychrophilic enzymes that increase k_{cat} at the expense of K_m and those that optimize both the kinetic parameters (increase of k_{cat} and decrease of K_m) [15, 21]. The increase in k_{cat} is also related to a decrease in activation free energy of the catalyzed reaction, and in particular to a decrease of activation enthalpy, which is likely to be structurally achieved by a decrease in the number of the enthalpy-driven intra-molecular interactions which have to be broken to reach the transition state. These aspects have also been considered an indication of low stability of cold-adapted enzymes and of a higher structural flexibility, at least of the catalytic site and its proximity [15, 22, 23]. This was the first insight suggesting that activity and stability could be linked in the process of thermal adaptation.

The increasing number of primary sequences, and above all of three-dimensional (3D) structures, of enzymes and proteins from thermophiles and psychrophiles have provided the suitable background to carry out investigations of the molecular determinants of their temperature adaptation. In this context, computational approaches and in particular biomolecular simulations have a prominent role in complementing and integrating the experimental data, but also to clarify critical issues related to cold adaptation, which are difficult to accurately and specifically address by the only mean of experiments as, for example, the role of protein flexibility and dynamics.

In fact, enzyme catalysis generally involves the "breathing" of particular regions of the enzyme structure, enabling for example the accommodation of the substrate. The ease of such molecular movements may be one of the determinants of the catalytic efficiency. At a given temperature, the optimization of enzyme function requires a proper balance between two opposing factors: structural rigidity, which allows the retention of a specific 3D conformation at the physiological temperature, and flexibility, which allows the protein to perform catalysis and substrate recognition [22, 24]. Therefore, a protein can be considered a mechanically heterogeneous 3D structure composed of locally rigid and flexible sub-structures. In this context, it has been suggested that enzymes isolated from cold-adapted organisms are generally characterized by a higher plasticity or flexibility of their molecular structure to compensate for the lower thermal energy provided by the low-temperature habitats [18, 21, 22, 24, 25]. High structural flexibility of the psychrophilic enzymes can allow better interactions with the substrate and can be related directly or indirectly to their higher catalytic rate (k_{cat}) at low temperature, high thermolability and lower activation energy (Ea) if compared with mesophilic and thermophilic counterparts [18, 22].

However, the molecular determinants and the exact relationships between the triad of catalytic activity, thermal stability and flexibility in cold-adapted enzymes are still a matter of debate. The intrinsic thermolability of psychrophilic enzymes, in addition to their increased low temperature activity, suggests a direct link between activity and stability; the activity at low temperature requires a weakening of intramolecular forces which results in reduced stability. Otherwise, it has also been suggested that the thermolability may be related to random genetic drift, as a consequence of lack of evolutionary pressure for stable enzymes in low temperature habitats [26-28]. Moreover, the existence of non-canonical cold-adapted enzymes, which feature both unusual thermostability and high catalytic efficiency has been reported [29, 30], along with evidences of the ability to uncouple activity and stability in *in-vitro* studies [28], makes the definition of activity-stability-flexibility relationships even more difficult.

It has been demonstrated that psychrophilic enzymes adopt several different structural strategies to increase molecular flexibility, such as weakening intra-molecular hydrogen bonds, optimizing protein-solvent interactions, decreasing the packing of the hydrophobic core, increasing number of hydrophobic side chains exposed to the solvent, and reducing the number of salt bridge networks [21, 31]. However, each protein family, whose members share functional residues and the 3D architecture, carries out its own adaptation strategy at the molecular and structural level [18, 22]. In this context, the optimization of the kinetic parameters k_{cat} and K_m is strictly correlated to the enhanced structural flexibility of cold-adapted enzymes, which in turn depends on their 3D architecture. In fact, as discussed in details above, most of psychrophilic enzymes are known to improve k_{cat} at the expense of affinity for substrates (K_m) [15]. Conversely, some psychrophilic intracellular enzymes are known, which operate at sub-saturating substrate concentrations and are characterized by K_m values as much as 10 times lower than that of their mesophilic homologs [15, 31], keeping their characteristic high-reaction rates. This decrease in K_m is achieved by acquiring rigidity in some protein sub-structures that restrict the number of conformational states available for the enzyme-substrate complex [22]. As a matter of fact, cold-adapted enzymes with catalytic efficiency greater than that of their mesophilic counterparts display adaptation strategies relying on local flexibility/rigidity mechanisms [22, 6-36], which are likely to cooperate, each acting on specific areas of the enzyme structure. The networks of molecular interactions and correlated motions related to these mechanisms can change in the different enzyme families and are strictly connected to specific features of the 3D fold.

In conclusion, the molecular details related to the determinants of low thermal stability, high catalytic activity and flexibility of cold-adapted enzymes are therefore still not completely achieved. However, in several cases, the cross-talk between computational and experimental data turned out to be a solid framework for structural studies of cold adaptation mechanisms. The present contribution reviews and rationalizes the data presently available in the literature on

psychrophilic enzymes achieved by computational approaches, with particular attention to the investigation of structural and dynamic properties.

2. COMPARATIVE SEQUENCE AND STRUCTURE ANALYSES OF COLD- AND WARM-ADAPTED ENZYMES

Proteomic and genomic analyses of sequence compositions, in different extremophiles, have identified a significant correlation between the amino acidic composition of the primary sequence of these enzymes and the optimal growth temperature of the corresponding source organisms [37-39].

Moreover, an inventory of structural and molecular characteristics related to temperature adaptation in cold-adapted enzymes came from several comparative statistical investigations of cold- and heat-active variants [40-42], pointing out that general theories cannot be formulated. Gu and Hilser [38] have also reported a uniform modulation of conformational flexibility and stability across the components of the proteome of organisms adapted to different environmental conditions, as well as that mechanisms of thermal adaptation can significantly vary from protein to protein. Each enzyme displays slightly different structural strategies to adapt to low or high temperature conditions, strategies which are difficult to precisely identify.

Even if an unified theory cannot be formulated for enzyme cold adaptation, comparative approaches have contributed to point out some common features [40-42], that are likely to be related to protein structural thermolability and flexibility of psychrophilic enzymes. The residues and geometry of the active site are conserved both across orthologs and along the temperature adaptation scale, fact that can be expected, considering that the catalytic mechanism has to be conserved between homologous enzymes adapted to different temperature conditions. This observation was the first suggestion that the determinants of cold adaptation, from the structural point of view, reside in residues not directly involved in the catalysis but indirectly influencing it. The location of the amino acidic substitutions to achieve this effect must therefore occurs at some distance from the catalytic center, and they will influence, directly or by indirect long-range effects, the relative flexibility of protein portions in the surroundings of the functional regions. It has also been proposed that the cold-adapted enzymes could display a broader specificity, due to the enhanced plasticity of the residues in the surroundings of the substrate binding cavity. In several cases, substrate can bind less firmly, giving rise to higher K_m values, as mentioned in the Introduction. The catalytic cleft of the cold-adapted enzymes is also generally larger and more solvent accessible than in the mesophilic counterparts, often related to deletion of residues in loops surrounding the active site or to modification in loop conformations, as well as to the replacement of bulky residues with smaller ones. The higher solvent accessibility of the catalytic pocket both allows a better substrate accommodation at lower energy cost and facilitates the release of the reaction products.

All the structural features currently known to stabilize warm-adapted proteins are generally attenuated in strength and number in cold-active enzymes, and often minor structural modifications between cold- and warm-adapted enzymes can be detected. Salt-bridges that are among the strongest stabilizing factors for protein conformations are less represented in psychrophilic enzymes [40, 43, 44]. Cold-adapted enzymes also show a lower arginines content (low Arg/(Arg+Lys) molar ratio), as arginine residues are mainly suitable in the context of stabilizing networks of interactions, such as hydrogen bonds, electrostatic interactions and cation-Π interactions.

In fact, arginine are suitable residues to enhance thermostability, more than lysine since their side chain can facilitate the formation of networks of electrostatic interactions, allowing up to two salt bridges and five H-bonds thanks to unique properties of the guanidine group [45, 46]. In an aromatic context, arginine has also higher impact than lysines, impact which is not mainly ascribable to an intrinsic higher cation-Π capability of Arg, as one could expect. In fact, the Arg side chain is larger and less water-solvated than the cognate Lys thus, likely to benefit from better van der Waals interactions with aromatic rings. Moreover, the Arg side chain can still be involved in H-bonds or salt-bridges simultaneously interacting with aromatic rings, whereas Lys typically has to relinquish H-bonds to bind to aromatic residues.

In this context, Cavicchioli and coworkers [44] demonstrated that the replacement of lysines with homo-arginine in the psychrophilic α-amylase from *Pseudoalteromonas haloplanktis* (AHA), the paradigm of cold-adapted enzymes, induces a "mesophilic-like" character in AHA, supporting the relevance of lysine residues in promoting cold-adapted properties Fig. (**1**). Experimental support for thermostabilization promoted by arginine over lysine came also from previous studies on several mammalian enzymes, which were reported to be stabilized by the introduction of guanidinium groups [47]. These studies provided remarkable insights into the role of lysines in facilitating protein cold adaptation at the expense of arginines, aspects to be considered in the attempt to convert a mesophilic enzyme into a cold-adapted one or the opposite.

The data were also consistent with computational analysis that demonstrated that AHA has a compositional bias favoring decreased conformational stability and increased flexibility [44], allowing the authors to point out a parallel between cold-adapted enzymes and intrinsically unfolded proteins. In the last year, several intrinsically disorder proteins (IDPs) have been identified, which are flexible and functionally versatile [48-50], stimulating the scientific community to extensively investigate the effects of amino acid composition on their unique structural properties [51-53]. Statistical analyses revealed that natively unfolded proteins or protein domains are significantly different from intrinsically ordered proteins. Disordered regions are often characterized by low sequence complexity coupled with a compositional bias; a low content of bulky hydrophobic amino acids (as long aliphatic or aromatic residues) and cysteines; and a high proportion of polar and charged residues (Q, S, P, E, K and less often G) which are disorder-promoting. Charged residues in particular emerged as a key factor of intrinsic disorder [54-55]. It has to be considered that, although high flexibility often correlates with low conformational stability, the relationship between flexibility and intrinsic disorder is still not well clarified. Some proteins are

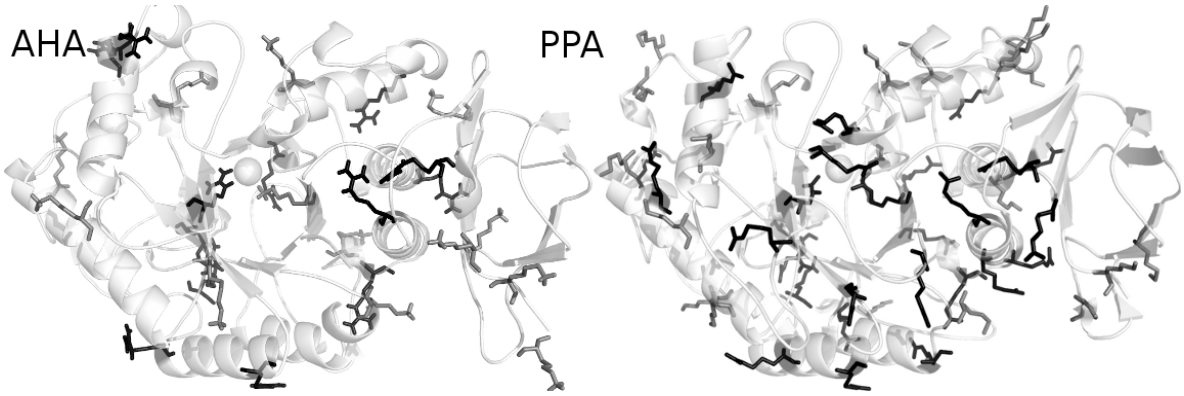

Fig. (1). Localization of lysine and arginine residues on the 3D structure of cold-adapted (AHA, pdb entry 1AQH) and warm-adapted (PPA, pdb entry 1PIF) α-amylases. The 3D-structures and calcium and chloride cofactors are shown as cartoon and spheres, respectively. Lysine and arginine residues are shown as grey and black sticks, respectively.

well-known to be characterized by multiple metastable states, rather than being dynamically disordered, as well established by Nuclear Magnetic Resonance (NMR) and Small-Angle X-ray Scattering (SAXS) experiments [48, 56]. Proteins may also have relatively rigid overall structures and increased dynamics and flexibility of specific regions [35, 36, 57], whereas other proteins may be intrinsically disordered, lacking of any rigid structures and displaying highly dynamic ensembles of rapidly interconverting structures. Moreover, Cavicchioli and coworkers [44] point out that AHA is also depleted in the major order-promoting residues (C, W, F and I) and enriched in several disorder-promoting amino acids (A, T, S and Q), as well as it has a lower number of bulky hydrophobic side chains, which might decrease the total number of weak interactions. These characteristics of AHA composition are likely to reflect a requirement for proper solvation of the enzyme at low temperatures, achieved by the relaxation of the AHA structure and related to the compositional bias favoring decreased conformational stability and increased flexibility. Interestingly, the cold adaptation biases in amino acid composition are not the only aspects resembling properties of intrinsically disordered proteins. In fact, cold-adapted enzymes and intrinsically disordered proteins, thanks to their conformational flexibility, are likely to interconvert in the native-state among different conformations separated by low energy barriers [57] aspect which will be extensively addressed in the next section (3.2).

Long disordered segments were also successfully predicted in the linker region that separates catalytic and cellulose binding domains in a cellulase from *Pseudoalteromonas haloplanktis* [58], as well as it has been demonstrated that the shortening of extra-loop in domain B of bacterial α-amylases affects thermostability and reinforces the architecture of domain B and active site conformation, reducing flexibility at this region and abolishing tight hydrogen bonds networks [59]. Independently from the nature of protein flexibility, prediction of disorder has been suggested as a potential guide to plan studies on cold-adapted, or more in general, extremophilic enzymes [44, 60]. However, several details cannot be successfully identified from the only mean of disorder predictors, and more accurate calculations have to be employed, as discussed in the Section 3.2. In this context, a suitable example is the application of disorder prediction tools to the case of cold- and warm-adapted α-amylases Fig. (**2**), for which several data from biochemical characterizations, biophysical measurements and atomistic simulations are available as a comparison [61-65]. A disorder predictor, based on the analysis of the primary sequence and which employs a residue type-based contact order index to discriminate among ordered and disordered protein regions [60], has been applied to cold- and warm-adapted α-amylases, calculating a per-residue profile of degree of protein disorder. In the per-residue disorder profile of α-amylases, there is only partial consistency between the identified disordered regions and the secondary structure elements and also partial correlation can be identified with the estimation of protein flexibility derived from root mean square fluctuations (rmsf) calculated with atomistic molecular dynamics simulations Fig. (**2**). The disorder prediction tools are designed to identify protein area with disordered propensity, whereas rmsf has been often used to quantitatively evaluate differences in flexibility, which are generally subtle and related to peak intensity when cold- and warm-adapted homologs are compared, since they share a common 3D fold [32, 34, 35, 66].

Intra-molecular hydrogen bonds can also contribute to the stability of the proteins, and are expected to be less represented in cold-adapted proteins [31]. On the contrary, cold-adapted enzymes often feature increased protein-solvent hydrogen bonds, which are often related to a higher number of polar solvent accessible groups [21] and a higher number of non-compensated charges on the protein surface [67]. These two elements are thought to be involved in achieving higher structure flexibility, allowing the optimization of the interactions with the solvent and disruption of the ordered structure of the hydration water shell at low temperatures, thus increasing conformational entropy. The aspects mentioned above, related to characteristics of the solvent-accessible surface of cold-adapted enzymes, can also account for the existence of poly-extremophilic enzymes, which are not only adapted to low temperature conditions but also are characterized by halophilic properties [68-70].

Cold-adapted enzymes also feature a low binding affinity for metal cofactors [21, 71, 72], such as calcium or zinc ions,

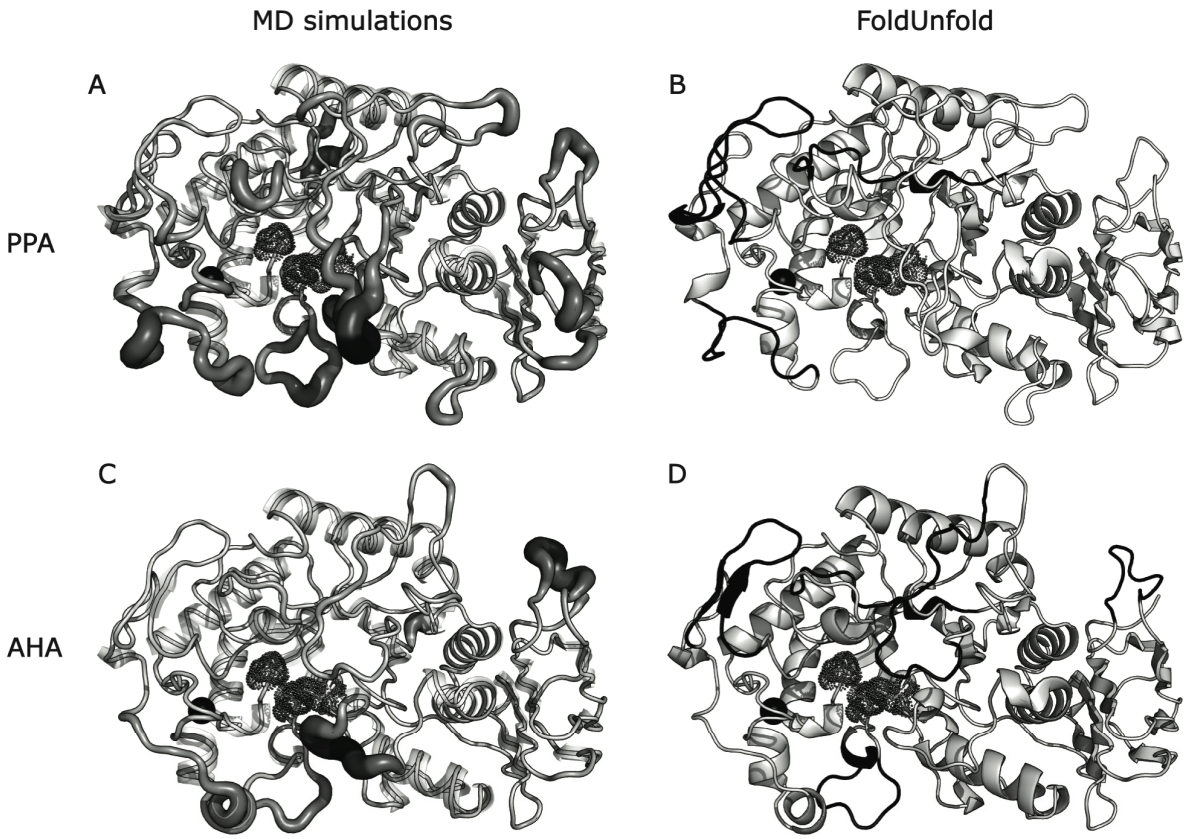

Fig. (2). Comparison between flexibility degree calculated from MD simulations [65] and disordered regions identified by the Fold Unfold disorder prediction server [60]. A-C. cartoon darkness and thickness are proportional to the C-α flexibility values. B-D. Regions predicted as disordered are colored in black.

and a less effective dependence of thermostability from structurally bound metal ions, as discussed in details in Section 5.2. They also show a less packed and hydrophobic internal core, which reflects in a decreased stabilization of the internal scaffold, along with an observed lower fraction of large aliphatic residues ((Ile+Leu)/(Ile+Leu+Val) ratio) respect to the warm-adapted counterparts [21]. Conformational freedom of cold-active enzymes seems also to be related to a decrease of the number of prolines in the loops and turns connecting the secondary structure elements and to the presence of glycines cluster near functionally important regions [34, 73-78].

In summary, structural and computational comparisons carried out so far on cold- and warm-adapted organisms, as well as proteomic approaches, allowed the identification of trends in the intra- and intermolecular networks characterizing differently temperature-adapted enzymes, even if a general and unified mechanism cannot be rationalized.

3. PROTEIN FLEXIBILITY AND DYNAMICS IN COLD-ADAPTED ENZYMES: COMPARATIVE MOLECULAR DYNAMICS SIMULATIONS AND EXPERIMENTAL EVIDENCES

Proteins and in general biological macromolecules are not static entities but they experience a large degree of internal mobility and flexibility. By the interactions with the solvent and with other molecules, at any given temperature over the absolute zero, protein atoms are subjected to internal and external forces that promote conformational changes across a number of energetic minima. The behavior of proteins in solution is biologically relevant and the protein native state prevailing under physiological conditions can be well described as an ensemble of statistically populated conformers. In this context, flexibility of the protein structure can refer to the fast interconversion among several conformations of comparable energy, achieved by a combination of small and subtle changes. The larger the flexibility, the larger is the population of conformers and the lower is the energy barrier for interconversion between them [57, 67, 79]. Micro-unfolding of confined regions of the protein determines the so-called "breathing" of the protein structure. The transitions between different conformations are concerted motions of groups of residues involving hinge and rocking motions in timescales from 10^{-8} to 10^{3} s. Several fluctuations, involving side chains or backbone motions and originating from rotations, stretching and torsional motions (10^{-12} s), underlie to the large structural motions [67].

As detailed before, the protein dynamics have a relevant impact on its functional aspects, such as substrate binding or catalysis, thus relating its internal motions to high-order properties that are routinely measured on enzymes. Nonetheless, these dynamics have been proved hard to investigate in atomic details and on large systems, because they can involve a different timescale (from pico- to microseconds) as

well as can be strongly influenced by the conditions in which the protein acts (see above). It is well established that the knowledge of protein dynamics and the mechanisms beneath to such events is crucial for a complete understanding of enzyme catalysis, also since conformational movements have been suggested to represent the rate-limiting step [80, 81]. As the forces acting on each protein atom strictly depend on temperature, the rigidity/flexibility of each enzyme should be tuned in order to obtain the optimal breathing level, depending on the temperature the enzyme usually experiences. Low temperatures are related to relatively low levels of kinetic energy and a reduced extent of molecular motions; thus, the structure of psychrophilic enzymes should be more flexible to allow the proper protein dynamics required for a fully functional enzyme.

One hypothesis often accredited in the literature, but still not experimentally or computationally proved, is that thermophilic and psychrophilic homologs should in principle feature similar molecular motions at the physiological temperatures of their source organisms [18, 82]. The hypothesis of similar flexibility patterns of cold-adapted enzymes in low temperature conditions and heat-adapted enzymes in high temperature conditions is hard to test both by experiments and simulations due to several intrinsic difficulties. It is complex to precisely define the "optimum temperature" for the extremophilic enzymes under comparison, since to use the optimum growth temperature of the organism can be a too simple approximation, whereas conditions in which the enzyme acts in its physiological intracellular/extracellular environments have to be considered. It is also difficult to find enough data on biochemical characterization of enzymatic activity of cold- and warm-adapted homologs in comparable experimental conditions. Dealing with molecular simulations, another degree of complexity has to be considered, as discussed in the following.

It has been shown by hydrogen/deuterium exchange [82] and neutron scattering studies [83, 84] that thermophilic and mesophilic enzymes present similar pattern of flexibility under optimal working conditions. Similar evidences for the cold-adapted enzymes are still missing even if simulations at temperatures close to the optimal temperatures of cold- and warm-adapted subtilisins respectively, have been carried out and their properties have been compared [66]. Further studies, which can bring to successful results only if experimental and computational investigations suitable to determine differences in details in protein dynamics will be integrated, are still necessary to disclose aspects related to protein flexibility at the optimal temperature of extremophilic homologous proteins. In the context of classical molecular dynamics (MD) simulations, it is difficult to quantitatively define differences in flexibility profiles (for example calculated according to root means square fluctuation (rmsf) or B-factor values) among proteins simulated in different temperature conditions; thus the results should be taken with extreme caution, at least in absence of experimental biophysical validation. In fact, it has to be considered for example that at low temperatures (as 283 K, often employed in MD studies on cold-adapted enzymes [34, 35, 85-88]) in a classical MD simulation, the motions are constrained and a wide simulation ensemble might be necessary to quantitatively compare fluctuations. Some studies have been recently reported, in which a successful correlation between simulations and experiments at different temperatures was achieved [90]. However, it has to be considered that increasing temperatures, in MD simulations, is mainly a strategy to accelerate some conformational changes or to allow a faster exploration of the conformational landscape. Higher MD temperatures often allow the identification of effects on a shorter timescale than the timescale necessary to identify the same event at lower simulation temperatures. In fact, several simulations of cold- and warm-adapted enzymes have been carried out to a reference MD temperature of 300 K [32, 35, 65, 91]. This approach turns out to be a reasonable compromise to compare differently temperature-adapted homologous proteins by classical MD to identify distinctive characteristics of the cold-adapted variants. Indeed, 300 K is generally a temperature at which the cold-adapted enzyme is still active and stable and in several cases even more active than the warm-adapted counterparts. Moreover, it is a temperature close to the temperatures generally used in most of the *in vitro* biochemical assays for the comparison between cold- and warm-adapted enzymes. From a computational perspective, it is a simulation temperature that in classical MD provides a reasonable quality of the conformational sampling and flexibility profiles so that can be compared with higher confidence.

It is well-established that the flexibility "fingerprint" of cold-adapted enzymes should not be necessarily spread on the whole structure (global flexibility) but it can be localized in small regions that affect the mobility of active-site structures (local flexibility) Fig. (**3**). In fact, two main cold adaptation mechanisms related to flexibility are emerging: the enzyme could have evolved towards the lowest possible stability of its native state, as exemplified by a cold-active α-amylase [61]; on the other hand, only few well-localized regions of the enzyme may have acquired a higher flexibility, which in some cases results in the appearance of a distinct heat-labile thermodynamic domain, as exemplified by a psychrophilic phosphoglycerate kinase (EC 2.7.2.3) [92]. The latter strategy has been proposed to be more favorable for proteins which require movements of great parts of their structure to function properly (e.g. hinge-bending mechanism), while the former would be suitable for more compact enzymes [25].

Although the low thermal stability of psychrophilic enzymes supports the flexibility hypothesis, its verification is not straightforward due to intrinsic difficulties in defining and measuring flexibility [66, 67, 84, 93]. If flexibility can be described in terms of a dynamic motion that is related to a specific timescale, it can also be considered as a concept related to the amplitude of the protein structure deformation at a given temperature.

3.1. Experimental Techniques

In fact, the experimental assessment of protein flexibility in solution is a challenging task [81]. Movements of single atoms are difficult to measure in real time, except in few special cases. Protein dynamics must, therefore, be inferred from various biophysical measurements performed on an ensemble of biomolecules containing various sub-states in

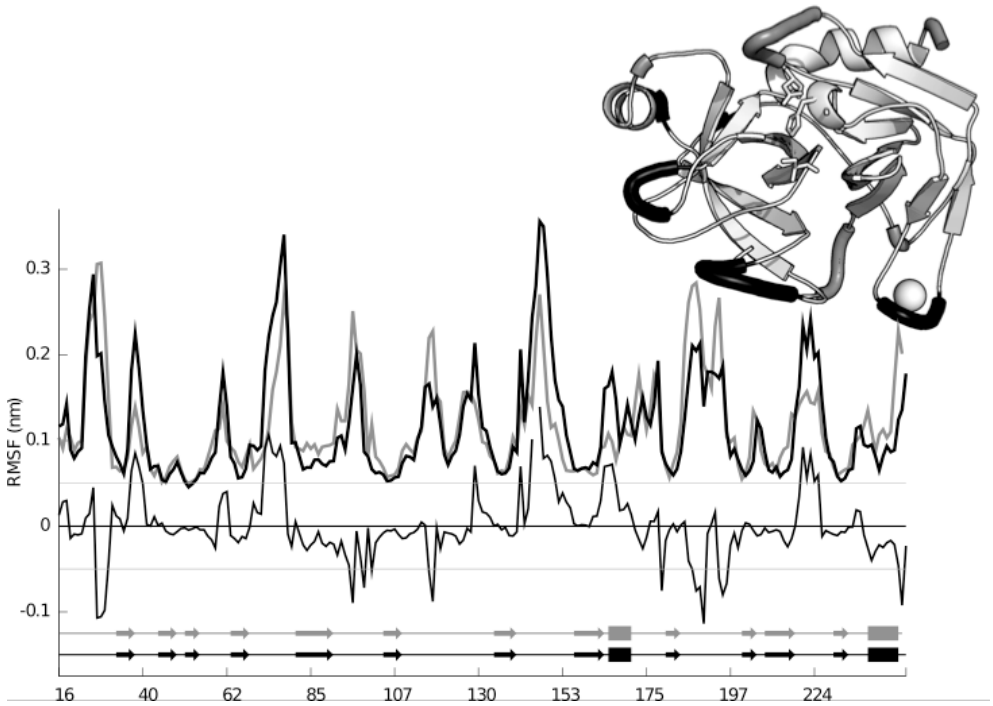

Fig. (3). Localized flexibility in cold-adapted serine-proteases. The Cα rmsf profiles of cold- (grey) and warm-adapted (black) trypsins from multi-replica MD simulations [35]. The regions characterized by higher flexibility in the cold-adapted or warm-adapted variants are mapped on the 3D structure as grey or black thicker cartoons, respectively. Residues of the catalytic triad are shown as sticks.

equilibrium, with the relative population of each state depending on the experimental conditions.

Techniques that provide a relative estimation of structural flexibility exist, but they in several cases do not directly measure flexibility, but some properties correlated to it, as relative resistance to proteolysis [94-96], fluorescence of tryptophan or tyrosine residues [97-100], hydrogen/deuterium (H/D) exchange experiments monitored by NMR [101, 102], Fourier Transform Infrared Spectroscopy (FT-IR) [103, 104] or ElectroSpray Ionization-Mass Spectrometry (ESI-MS) [105, 106], experiments with fluorescent ANS dye [36-107], Electron Paramagnetic Resonance (EPR) [108-110], Single Molecule Foster Resonance Energy Transfer (FRET) [111]. Therefore, these techniques are often unable to identify specific but relevant regions of localized flexibility, because their estimations describe, in many cases and with few exceptions, overall properties. As example, hydrogen/deuterium exchange experiments suffer of both these disadvantages, as they measure the degree of accessibility of residues and not atomic fluctuations. In dynamic fluorescence quenching experiments the decrease of fluorescence arising from diffusive collisions between the quencher (acrilamide) and the fluorophore (tryptophan) reflects the ability of the quencher to penetrate the structure and can consequently be considered as an index of protein permeability, thus giving a low structural resolution measure of flexibility.

The concept of localized flexibility was also experimentally evaluated for some cold-adapted enzymes, by carrying out comparison between thermal inactivation and thermal unfolding (as summarized in refs. [18, 21]). The loss of activity is not necessarily correlated with the unfolding of the enzyme, since enzyme inactivation precedes the modification of the structure in the case of the cold-adapted enzymes, so that mechanisms of enhanced flexibility of the active site have been proposed for psychrophilic enzymes. It has to be mentioned that the decoupling between thermal inactivation and thermal unfolding is not a peculiar characteristic of cold-adapted enzymes, since it has been highlighted also for mesophilic and even thermostable enzymes [112, 113]**.** In fact, for many enzymes the increase of activity and structural unfolding cannot be explained by a simple two states model. It has been proposed an "Equilibrium Model" in which folded active state (E_{act}) is in rapid equilibrium with a folded but inactive form (E_{inact}) that could irreversibly lead to denatured state (X) [114-116]

$$E_{act} \leftrightarrow E_{inact} \rightarrow X$$

In this model, activity is influenced by temperature dependence of the equilibrium between E_{act} and E_{inact}

In fact, k_{cat} is influenced on $[E_{act}]$ that in equilibrium model is given by

$$[E_{cat}] = ([E_{tot}]-[X])/(1+K_{eq})$$

Where K_{eq} is the equilibrium constant between the active and inactive forms of the enzyme and it is given by

$$\ln(K_{eq}) = (\Delta H_{eq}/R)[(1/T_{eq})-(1/T)]$$

Where ΔH_{eq} is the enthalpy change associated with conversion of the active to the inactive form and T_{eq} is the temperature of transition between the two forms, which is correlated with the environmental temperature of the enzyme.

ΔH_{eq}, the enthalpic change associated with the equilibrium, governs the temperature range over which the equilibrium occurs and thus the ability of the enzyme to function at different temperatures and temperature ranges: a small ΔH_{eq} indicates that the enzyme will function at relatively high activity over a wide range of temperatures whereas a large ΔH_{eq} indicates activity in a small range of temperatures [117]. ΔH_{eq} for $E_{act} \leftrightarrow E_{inact}$ transition is much smaller (at least one fold) than enthalpic change associated to $E_{inact} \rightarrow X$ transition [115]. Analysis of more than 20 bacterial enzymes from different organisms showed that there are small differences in optimal growth temperatures of thermophilic organisms respect to T_{eq} (7.3±6.6 K), whereas in psychrophilic and mesophilic organisms at low temperatures the difference is larger (27.8±15.1 K). This tendency is difficult to explain, but a hypothesis is that microbial evolution has proceeded from thermophile down to lower temperatures adapted organisms, bequeathing an unnecessary wide gap between the organism environmental temperature and its T_{eq} [117]. Interestingly, it has been recently demonstrated that thermal structural changes associated to the transition from E_{act} to E_{inact} are located in or near the active site of the protein [115].

In the context of biophysical investigations of protein dynamics and conformational ensembles, the most suitable experimental techniques, able to provide fine atomistic and local details, are EPR in conjunction with site-directed spin-labeling and NMR spectroscopies. EPR spectroscopy has emerged as a powerful tool for studying protein structures and dynamics in conjunction with site-directed spin-labeling (SDSL) [109, 118]. A cysteine side chain is introduced into the protein structure with site-directed mutagenesis, or free cysteine residues already available are employed, and they are subsequently chemically modified. EPR spectroscopy, performed after site-directed spin-labeling, mutating serine residues to cysteine, was used to study structural dynamics in a cold-adapted alkaline phosphatase (EC 3.1.1.1) and on its mutant variants in the proximity of the active-site [108]. Differences in the structural environments of six spin labels allowed them to be classified to specific secondary structural elements and to define the degree of contacts and the solvent accessibility, according to mobility maps. The EPR technique allowed the identification in mutants with reduced activity and increased thermal stability, of a concomitant reduction in spin-label mobility in the proximity of the mutations, suggesting that the movement of a specific loop, close to the catalytic residues, is interconnected with catalytic events.

NMR is the primary technique for the study of protein structures in solution and of properties related to protein dynamics [81, 119, 120]. In a typical NMR experiments, the simultaneous detection of signals from several "reporters" (NMR active nuclei) within a protein is possible, providing a comprehensive and detailed description of internal motions at atomic level, over a wide range of timescales. The great advantage of NMR spectroscopy is the ability of coupling the structure determination of polypeptide chains in solution with the description of their internal dynamics, over a wide range of timescales, ranging from picoseconds to several hours, mainly by the mean of spin relaxation and residual dipolar couplings measurements, as well as slow exchange motions [121-124].

As far as we know, no NMR studies of cold-adapted enzymes have yet been proposed in the literature, probably due to the intrinsic difficulty to manipulate extremely labile cold-adapted enzymes, with few exceptions [125]. More efforts in these directions, coupled to atomistic simulations, will increase our knowledge on mechanisms of cold adaptation.

On the contrary, crystallographic temperature factors (B-factors) derived by crystal structures, which are an indirect estimation of atomic fluctuations in the crystal, have often been used to investigate properties of cold-adapted enzymes (see for a summary ref. [21]), but are not suitable for detailed comparisons between individually refined protein structures. The main reason for this is that the crystallographic temperature factors can be biased by external factors such as data collection methods, data processing and scaling, as well as the refinement protocols. In addition, atomic fluctuations in the crystal are highly influenced by intermolecular close contacts, and long-range effects from such interactions are difficult to estimate. In this method caution has to be taken considering where the analyzed atoms are located in the structure, as previously mentioned.

At last, on the experimental side, neutron scattering analysis gives insights into the fast atomic thermal motions in macromolecules on the picoseconds to nanoseconds timescale and allows the identification of large conformational changes [84, 126, 127]. Neutron scattering can provide a direct method to measure static and dynamic flexibility, as well as versatility in terms of sample preparations.

Despite their limitations, some of the aforementioned biophysical techniques managed to detect higher flexibility in psychrophilic enzymes with comparative approaches between differentially temperature-adapted homologs, whereas in other cases the results are still controversial (see ref. [18, 21, 67] for a summary).

3.2. Molecular Dynamics Simulations

In this context, computer simulations of biomolecules can act as a bridge between the microscopic scales of macromolecules and the macroscopic world: simulations can be used to understand the protein motions at the atomic level and link their low-level characteristics to overall properties, such as thermodynamic stability, within the boundaries given by the approximations introduced in computational simulations [128]. The most accurate theoretical description for chemical systems, i.e. quantum-mechanics, is still not applicable to large biomolecules. This method is still not affordable for the current computing resources, if the biomolecule under study includes more than hundreds of atoms. The application of classical mechanics, considering all electrons to be in their quantum-mechanical ground state, yields a suitable description of the system and allows the investigation of large macromolecules on adequate timescales. However, it has to keep in mind that a classical description of protein motions also presents a higher degree of approximation, since some processes as bond breaking and formation or fine conformation-dependent atomic polarization cannot be represented in classical implementations.

Pioneering MD studies of cold-adapted enzymes have been carried out on short timescales (lower than 1000-1500 ps) and often comparing single MD runs of cold- and warm-adapted counterparts, due to the computational efforts required [91]. These first MD investigations were fundamental from the methodological point of view, since they showed that while the stability of secondary structure elements and solvent accessibility were reproducible, the loop motions were more randomized. This observation was likely to be related to the rather short timescale accessible to MD simulations and the use of single run per systems, which strongly affect the conformational sampling [129-131]. The entity of the deviation amplitudes for extremely high flexible regions was probably determined by factors which were difficult to monitor in short timescales, but the consistency can be increased with the increase of the simulation time and the availability of several independent trajectories (replicas) for each system, as well as thanks to the possibility of new approaches to increase the conformational sampling [132, 133], as replica exchange MD simulations (REMD). Even if some technical aspects need to be clarified for the application.

In less than ten years, the optimization of computer power has allowed the necessary computational resources to carry out MD simulations on large scales to be acquired. As a consequence, the study of structural and dynamic properties of cold-adapted enzymes has taken great advantages, pointing out several crucial and punctual details on differences in protein dynamics and flexibility of cold- and warm-adapted enzymes [32, 34, 35, 65, 66]. Results from comparative MD simulations of cold- and warm-adapted enzymes are consistent with a scenario in which both local rigidity in regions far from the functional sites and improved flexibility of regions not directly involved in catalysis and in the proximity or communicating with the active sites can be a positive factor in cold adaptation of different enzyme families, as serine-proteases [34, 35, 57, 66] or uracil-DNA glycosylases [32].

In recent MD investigation of cold-adapted enzymes, the application of long and multi-replica MD simulations guarantees a wider conformational sampling, which in turn provides a detailed description of protein flexibility and of flexibility differences between cold- and warm-adapted counterparts. The achievement of an extensive conformational sampling and of several multiple simulations of the same system are of crucial relevance to avoid simulation artifacts and misleading conclusions [134, 135]. This is a key aspect, especially when Essential Dynamics (ED) techniques are employed. ED is a well-documented application of Principal Component Analysis (PCA) to MD data [136], aimed at extracting informative directions of dynamics in a multi-dimensional space, by reducing the overall complexity of the trajectories and isolating the relevant motions with low frequency of the system. In this context, an insufficient sampling can lead to misinterpretations of the results, masking diffusive motions in random directions within the shape of the essential motions. Effective indexes of sampling quality have to be monitored as the conformation re-sampling in the phase space (for example by root mean square deviation (rmsd) matrices and structural cluster analysis). For instance, the overlap values of increasing portion of the simulations with the total trajectory, the cosine content of the first principal components (PCs) derived from the covariance analysis and an overall correspondence between structural clusters and basins in the free energy landscape (FEL) described by the PCs can be employed [131, 134, 135, 137]. In fact, in several cases it turns out that single ns simulations cannot extensively describe protein structural and dynamics features. Thus, the use of multi-replica approaches can be successful to guarantee a suitable and efficient sampling with acceptable use of computational resources. Multi-replica simulations rely on the merging in a macro-trajectory of several independent simulations of the protein system obtained starting from the same initial structure but with different sets of atomic velocities, or even different initial protein structures.

In order to evaluate flexibility and dynamic fingerprints from comparative MD simulations of extremophilic enzymes, a successfully strategy should employ more than a single flexibility index from the trajectories, and should also include the analyses of properties related to protein dynamics and networks of interactions. In fact, suitable flexibility indexes are per-residue root mean square fluctuations (rmsf) [32, 34, 35, 65, 66, 88, 91], anisotropic temperature factors [65] from the trajectories, carefully filtered by ED techniques, or generalized order parameters of the backbone motions [34, 138]. In particular, rmsf values have to be carefully validated to ensure a consistent description of protein flexibility in the simulations in order to infer relevant biological conclusions. In fact, the per-residue rmsf indicates the intensity of fluctuations of each residue with respect to the average structure. The average structure and consequently the rmsf profiles strongly depend on the simulation time considered, since the average structure can change if different time intervals are considered. Moreover, since that cold- and warm-adapted counterparts share the same 3D architecture and are homologs, often close homologs, most of the rmsf differences are in the intensity of fluctuations and have to be carefully checked to avoid artifacts or erroneous conclusions. In order to verify their consistency, the rmsf profiles of a protein can be computed considering different sets of protein conformational ensemble derived by the simulations, as well as different timescales for the calculations. The Pearson correlation coefficient can also be evoked to define the convergence and the overlap between rmsf profiles, which have to capture flexibility properties of the proteins under analysis. The correlation coefficients among the different rmsf profiles tend to decrease if the number of sampled conformations in the MD-ensembles is low and related to an insufficient conformational sampling, whereas when a sufficient number of conformations are collected, the correlation coefficients tend to 1, strongly indicating the achievement of a suitable conformational sampling. Moreover, the possibility to monitor rmsf profiles over different timescales from picoseconds to few or several nanoseconds, allows also defining differences in the protein motions of diverse nature.

The comparison of protein dynamics can be also achieved by ED techniques, for example, by the calculation of the root mean square inner product (rmsip), which is a measure of subspace overlap (i.e. the overlap among sampled regions of the essential subspace defined by the first PCs). This allows to estimate the overall similarity among motions described by the protein simulations under compari-

son [139], and it is generally calculated on the first 10 PCs. Rmsip can range from 0 to 1: the value is 1 if sampled subspaces are identical, and 0 if the sampled subspaces are completely orthogonal.

Moreover, the investigation of cross-correlated motions, which has still been applied only to a few cold-adapted enzymes, can be very informative in order to trace relevant differences among differently temperature-adapted enzymes. In fact, it can be achieved by calculation of Dynamic Cross-Correlation Matrices (DCCM) [65, 140, 141] or using other indexes of communication among protein residues in the structure, as communication propensity [142]. In fact, it has been recently demonstrated that coevolving residues with crucial role in function and fold stability are interconnected by intramolecular interactions, as well as they control the most important and conserved correlated and anti-correlated motions governing the fold and function of the investigated proteins [143-146].

Results from atomistic simulations are also expected to drive site-directed mutagenesis to better clarify correlation between flexibility, kinetic parameters and thermal stability. In fact, a direct proof that a fine balance between flexibility and rigidity cooperate in determining cold-adapted features has been provided by fluorescence spectroscopy studies of the cold-adapted carbonic anhydrase II Ice-CA (EC 4.2.1.1) [36]. In Ice-CA, it has been observed, if compared to the mesophilic homolog, an enhanced flexibility in the upper part of the enzyme that controls the correct folding of the catalytic architecture, along with an increased rigidity in the lower part of the enzyme, which anchors the catalytic β-strands into the hydrophobic core, stabilizing the 3D architecture.

3.3. QM/MM Calculations

Active sites of homologous enzymes adapted at different temperatures often show similar structures, but different thermodynamic activation parameters [15, 20]. This is mainly due to the differences in the active site environment, strictly dependent on different networks of intramolecular interactions and residue communications. In this context, it is crucial to investigate the modulation mechanisms of the thermodynamic activation parameters in differently temperature-adapted enzymes. Although MD is a useful tool to investigate dynamic properties of enzymes, it is not able to reproduce the chemical changes occurring during catalysis, which require a quantum mechanics (QM) description. However, the great computational cost of QM calculations allows, nowadays, only the description of few atoms. A nice compromise is to describe by QM a few number of atoms, directly involved in the reaction, whereas the rest of the system can be adequately represented by classical force fields, as in QM/MM (Quantum Mechanics/Molecular Mechanics) methods. QM/MM is a collective term that embodies many techniques at different levels of accuracy and it is based on different approximations of the QM theory. The QM/MM methods have been extensively used during the last decades to study chemical reactions in enzymes [147-149]; despite that, this approach has been rarely applied to cold-adapted enzymes.

Recently, Aqvist and coworkers [150] investigated the mechanisms of cold adaptation in the citrate synthase enzyme family, using a QM/MM scheme in which the QM part was modeled with the Empirical Valence Bond approach [151]. This method describes reactions by mixing resonance states corresponding to classical valence-bond structures, which represent the reaction intermediates, as well as the product states. These diabatic states are represented by MM classical force fields, calibrated to reproduce the reaction of interest and lacking of a full explicit treatments of electrons. This description is used to compute the potential of mean force along a generalized reaction coordinate by free energy perturbation, with MD used to sample the energy difference between the reaction states. In particular, the authors compared a psychrophilic citrate synthase (CSp) with its mesophilic (CSm) and hyperthermophilic (Csh) homologs, also including a control reaction in water. Several replicas were carried out at different temperatures to obtain accurate Arrhenius plots, which were used to compute activation enthalpies ($\Delta H^{\ddagger}$) and entropies ($\Delta S^{\ddagger}$). Classical MD simulations in periodic boundaries condition (PBC) were also performed for each enzyme, to better sample the dynamic properties of the system. The calculated activation energies were in excellent agreement with the activation barriers estimated from experimental reaction rates, at least in CSm and CSp, for which experimental data were available. As expected, $\Delta H^{\ddagger}$ of the enzymes was lower than values calculated for the water reaction, as well as $\Delta H^{\ddagger}$ of CSp was lower than in CSm, in line with the expectations. Moreover, a more negative $\Delta S^{\ddagger}$ was found for CSp than for CSm, as well as a lower temperature-dependence of the reaction rate for the psychrophilic homolog, which successfully reproduced the available experimental data. The flexibility of few residues located at the active site, in terms of ensemble-averaged rmsf has been evaluated both in QM/MM and MD-PBC simulations. These residues share the same flexibility in the three extremophilic homologues, with a rigid active site characterized by low rmsf values. These data support the notion that an increased flexibility at the active site is not a determinant factor in cold adaptation, at least in the citrate synthase enzyme family. Furthermore, it has to be considered that also evidences from classical simulations, biophysical experiments and structural comparisons, summarized in Sections 3, are in agreement with the identification of similar features in the active sites of extremophilic homologous enzymes, with differences in flexibility dislocated in regions different from the active site even long-range, which can communicate with the functional residues.

In the attempt to understand the source of the observed differences in activation thermodynamic parameters among extremophilic citrate syntheses [150], the authors examined the energy components associated with the activation energy barrier. Only minimal differences have been identified. In particular, the simulations show that the transition state of the psychrophilic enzymes is less stabilized by electrostatic interactions. However, the loss of electrostatic stabilization is effectively counterbalanced by more favorable changes in internal energy in the cold-adapted enzyme during the reaction.

These data are in line with the notion of a relationship between activity and flexibility: the rigidity of thermophilic

enzymes, which is required to prevent unfolding, would require higher degree of pre-organization toward the transition state. On the contrary, the requirement for a more flexible structure has, as a counter effect, the loss of some pre-organization. A correlation between flexibility, activation enthalpy and entropy thus exists, and it is dictated by distal regions and long-range effects transmitted by residues not directly involved in the active site, in agreement with the hypothesis derived by classical simulations and spectroscopic investigations (see Section 3.1).

Moreover, an accurate study of reaction energy components and structural properties underlying the fine mechanisms of cold adaptation will be provided by QM/MM [149, 152] and above all by QM/MD [153] when a wider sampling of the conformational space will be achievable with these techniques.

3.4. Cases of Study Integrating Experimental and Molecular Dynamics techniques

The cross-talk between molecular simulations and experimental data is crucial for a complete understanding of protein structure-function relationships, being well documented also in the studies on cold adaptation. In fact, a successful integration of biochemical characterization of cold-adapted enzymes and their mutant variants in a mesophilic-like direction, along with subsequent MD investigation, has provided crucial molecular details on the mechanisms involved. In particular, a linear correlation between flexibility of the DNA recognition loop of cold- warm- and mutant uracil-DNA-glycosylases (UDGs, EC 3.2.2.3) and their k_{cat}/K_m values has been demonstrated [32], as well as, in the case of AHA and its mesophilic-like mutants, a direct link has been observed between the persistence of particular salt-bridge networks and cross-correlated motions and their catalytic efficiency [154].

Experimental biophysical techniques are also a suitable instrument to integrate MD simulations, in particular to assess the stability and flexibility issues. A successful interconnection between classical MD simulations at different temperature conditions and circular dichroism, ESI-mass spectrometry and fluorescence studies has been demonstrated useful to determine the effects induced by calcium-ion removal on thermal stability of a bacterial lipase and can be used as a reference strategy for this kind of investigation [90].

A valuable integration of MD simulations and FT-IR spectroscopy was successfully applied to the investigation of the cold-adapted esterase from *Pseudoaltermonas haloplanktis* PhEST [155], which provides molecular details on protein thermal stability and discloses the regions mainly involved in changes induced by the increased temperatures on protein fluctuations around the native state. In particular, MD simulations indicate a limited conformational freedom for a region surrounding two tryptophan residues, and a flexible region for other Trp residues. The authors followed the dynamics properties as a temperature function also using protein intrinsic tryptophan phosphorescence spectroscopy [156], demonstrating an unusual phosphorescence emission largely dominated by intramolecular quenching interactions, well fitting computational hypotheses.

4. FOLDING AND UNFOLDING

4.1. Characterization of Unfolding of Cold-Adapted Enzymes by Computational Approaches

High temperatures MD simulations can successfully describe the unfolding process of several proteins [157]. Protein unfolding studies by MD simulations are usually carried out by increasing the temperature necessary to overcome the enthalpic forces stabilizing the 3D structure. In particular, all-atom explicit solvent MD simulations provide insights into biomolecules dynamics thanks to the continuity of the trajectory in the phase space.

Considering the differences between the time-scales accessible by experimental and computational techniques, higher temperatures are needed when performing simulations respect to those observed in experimental unfolding studies. A MD study of CI2 has shown that the unfolding pathway is not significantly affected by the simulation temperature, indicating that the increase of temperature during the simulations only accelerates the pathway itself [157]. In fact, in MD simulations at high temperatures protein unfolding can occur within a few nanoseconds. Generally the temperature can be increased up to 500K/600K to speed up the process, even if simulations at lower temperature and for longer simulation times have to be carried out for control, in particular when a new protein fold is under investigation.

Unfolding MD can provide further information on the protein unfolding processes, along with elucidation at the atomic level of intermediates and factors affecting stability of the folded and unfolded forms [158-161]. As in any reaction, protein unfolding/folding processes present multiple states and the pathway across which a protein moves from the unfolded to the folded native state is one of the primary interests of protein biochemistry. In this field, protein folding and unfolding simulations are a suitable alternative to experiments, since currently no experimental techniques can provide a level of atomic details and dynamics which are inherent to MD simulations. Due to timescale limitations (microseconds or generally even less), the study of protein folding by MD simulations is still not overspread. Thermal denaturation simulations can be a valuable alternative, since they describe the unfolding pathway of a protein in a computationally reasonable timescale (nanoseconds) and have also been demonstrated to be reversible [162].

The unfolding MD simulations have been applied, as far as we know, to the study of unfolding pathway of a few cold-adapted proteins in comparison with the mesophilic counterparts; UDGs [163], elastases [164] and antifreeze proteins [165]. In all these studies, simulations have been carried out at different high temperatures and several properties have been monitored to obtain either an overall or a detailed picture of the unfolding process. In the UDG case, an implicit solvent model has been employed to increase the computational efficiency. The authors showed that the mesophilic UDG requires higher temperatures to reach the same unfolding rate of the cold-adapted counterpart. However, once the thermal energy of the system is sufficient (i.e. at temperatures higher than 425 K) rapid unfolding is detectable for both the enzymes.

In the elastase case, at 500K the unfolding process was clearly visible. After 0.4 ns, the mesophilic enzyme loses 30% of the native structure. The unfolding process in the cold-adapted counterpart occurs later in agreement with a higher flexibility and a lower number of intramolecular interactions localized in the proximity of the functional site, along with a higher rigidity of the rest of the structure with respect to the mesophilic homolog [33]. Interestingly, even if the cold-adapted enzyme undergoes toward a slower unfolding process, the regions in the proximity of the catalytic site are the first to be affected by the increasing temperatures and to promote the local unfolding in the first steps of the process. This scenario well fits with the notion of decoupling in protein activity and thermal structural stability. The mechanism of local flexibility could be a general rule underlying this phenomenon.

Usually, 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 others considerably. Therefore, it is necessary to analyze multiple trajectories related to the same system in order to ensure the validity of unfolding simulations, as well as that the main traits of the trajectories are reproducible and representative.

One of the principal difficulty in the analyses of unfolding/folding trajectories is the description of the protein conformations along the so called "reaction coordinates". A limit of the mentioned investigation on cold-adapted unfolding is that they generally employ one or two reaction coordinates, i.e. one or two collective properties to describe the unfolding pathway. The radius of gyration (Rg), Cα or main chain rmsd, the fraction of native secondary structures, and the fraction of native contacts are usually employed as reaction coordinates in unfolding simulations. However, these properties can be misleading. For example, Rg should be expected to increase during unfolding, but it cannot be exclude that during the unfolding pathway lower Rg are observed due to rearrangements of the protein structure in a non native-like conformation. Furthermore, two structures can be characterized by the same number of contacts, even when they feature both native and non-native contacts. Therefore, the description of the folding/unfolding pathway by the only mean of global single properties can be inadequate, without revealing the full complexity behind unfolding process, which a MD investigation has the potential to detect.

Daggett and coworkers developed a suitable alternative, to represent the folding/unfolding process of several proteins from their Dynameomic database [166-168]. They developed a property space-based description of the unfolding process, which includes analytical and physical properties derived from the time-dependent cartesian coordinates of the protein. The principal aim is to filter out the MD data and to reduce the information to a set of properties, which alone capture the most important features of the unfolding process, meanwhile creating a simple but still informative multidimensional-embedded, one-dimensional reaction coordinate.

Recently, other methods suitable to infer molecular details from unfolding simulations have been proposed, which could be insightful if applied to the study of unfolding pathways of cold- and warm-adapted homologs [169, 170]. The correlation in dynamics among several structural fragments and the contribution of single residues, during the unfolding pathway of model proteins, has been elucidated by applying PCA to atomistic unfolding simulations. The cross-correlation matrix obtained from MD simulation trajectories provided relevant information regarding the anisotropy of backbone dynamics which trigger unfolding [169]. One of the critical issues in unfolding simulations is also the definition of the transition state ensemble, for which several approaches have been proposed [158, 170, 171]. The most useful approach still is to monitor, over the simulation times, the evolution of native contacts, which have been estimated from simulations at standard temperatures.

Nowadays, MD unfolding simulations for the study of cold-adapted enzymes have not been extensively employed and there is still a gap to fix between the number of known 3D structures of cold-adapted enzymes and the ones for which unfolding pathways have been clarified. Moreover, the continuous improvement in the analysis of unfolding trajectories, with the specific aim of disclosing atomic details of the unfolding pathways, can provide the right framework for the application of these techniques to cold-adapted enzymes.

4.2. The Folding Funnel Model of Cold-Adapted Enzymes

D'Amico and coworkers [79] have proposed the integration of biochemical and biophysical data available on enzymes adapted to low temperatures in a folding funnel model [172, 173], which describes the folding-unfolding reaction of cold-adapted enzymes. They proposed that differentially temperature-adapted enzymes also possess differently shaped funnels Fig. (**5**). The height of the funnel, which represents the free energy of folding, corresponds to the conformational stability, whereas the upper edge of the funnel is occupied by the unfolded state in a random coil conformation. In this model, the edge of the funnel for the psychrophilic proteins is slightly larger respect to the one of thermophilic proteins, corresponding to a broader distribution of the unfolded state. When the folding of the polypeptide chain occurs, the free energy levels decrease, as well as the broadness of the conformational ensemble accessible to protein structures. The walls of the funnel present a different amount of roughness in psychrophilic and thermophilic enzymes. Thermophilic proteins feature intermediate states corresponding to local energy minima due to the reduced cooperativity of the folding reaction, resulting in a more corrugated funnel. Since the structural elements of psychrophilic proteins generally unfold cooperatively without intermediates, due to fewer stabilizing interactions, the funnel slopes are steeper and smoother. The bottom of the folding funnel for thermophilic proteins can be represented as a single global minimum, or only a few minima with high energy barriers between them, with little conformational freedom. The bottom for psychrophilic proteins is wide and rugged, as it represents a large collection of conformers separated by low energy barriers, resulting in a more labile and flexible protein.

It has been argued that, upon substrate binding to the protein sub-population competent for the interaction, the equilibrium between all conformers is shifted towards this sub-population, leading to an "active" conformational en-

semble. In the case of the rugged bottom of the folding funnel of psychrophilic enzymes, the aforementioned equilibrium shift requires only modest free energy changes for interconversion of the different conformations, thus explaining the role of increased flexibility in facilitating the binding of the substrate.

Dynamic and functional properties of proteins are strongly related to the fluctuations between different local minima on the protein free energy landscape (FEL). Therefore, the comprehension of the FEL shape and of how a native protein explores its free energy landscape, in terms of both the potential energy and the entropic contributions, is an important requirement for a complete microscopic description of its function and of the complicate relationships between folding, stability, and dynamic properties [174-176]. In protein structures, the shape and the ruggedness of the FEL is the result of fulfilling physical and evolutionary constraints [177, 178].

In light of the above observations, the validation of the aforementioned cold-adapted funnel model is a challenging task, requiring proper sampling and representation of the free energy surface accessible to the cold-adapted proteins. In fact, extensive sampling of the FEL, as well as a suitable reduction of the multidimensional phase space to two or three coordinates, are necessary. The variables describing the free energy landscape have to be collective variables, which capture the main features of the conformational ensemble and they are generally named "reaction coordinates" [179, 180]. The FEL description strongly depends on the selection of the reactions coordinates, which have to be properly identified. Suitable variables can be main chain rmsd of specific protein regions, Rg or principal components from PCA. In an equilibrated thermodynamic system at constant pressure, the free energy can be estimated from the probability density function of one or more reaction coordinates [181].

For small proteins, several strategies for describing and visualizing the FEL have been developed, such as disconnectivity graphs (DG) [182]. However, DG methodologies can nowadays be usefully applied to small protein systems and efforts are still required in this field, which represents one of the most suitable strategies to describe FEL. An alternative to investigate large protein systems close to their native-state, for which a full sampling of the conformational space is not necessary, is a qualitative representation of the FEL, using a few reaction coordinates [137]. In general, given a collection of states and a reaction coordinate q_α , the probability of finding the system in a particular state α, described by the reaction coordinate, is proportional to $(e^{-G(q\alpha)/kT})$, where k is the Boltzmann constant, T the absolute temperature, and $G(q_\alpha)$ is the Gibbs free energy of the state α. Therefore, the free energy landscape can be computed from $G(q_\alpha)=-kT\ ln\ [P(q_\alpha)]$, where T is the simulation temperature and $P(q_\alpha)$ is an estimate of the probability density function obtained, for example, from a histogram of the data. Considering two different reaction coordinates q and p, the two-dimensional FEL representation is obtained from the joint probability distributions $P(q,p)$ of the considered variables [137, 183] .

According to the folding funnel model of psychrophilic enzymes, a cold-adapted enzyme in its native-state consists of a large population of conformations separated by low energy barriers, as described in details above. A complete validation by simulations of the folding funnel model is still challenging even for coarse-grained simulations, since it would require a simulation of the whole folding process. However, the description related to the native state, can be verified by comparing the near native-state free energy landscape of cold-adapted enzymes with their mesophilic counterparts, belonging to different families.

Attempts in this direction have been recently carried out [57] by extensive MD simulations, at 283 and 310K, to sample the conformational space of psychrophilic and mesophilic homologs belonging to elastase and uracil-DNA glycosylase families. In this contribution, the FEL has been described by different reactions coordinates, as cartesian principal components from PCA, rmsd of loops in the surrounding of the catalytic site, and the protein Rg. Using these two-dimensional representations of the substates distribution of cold- and warm-adapted enzymes, the authors identify the most densely populated regions in the conformational subspace. These regions are likely to be basins in the conformational landscape, since they suggest a spontaneous tendency of the protein system to adopt structures associated to those basins [137]. Even if the whole protein dynamics is not restricted to those basins, due to the limit of the conformational sampling achievable from atomistic simulations, they can represent a significant portion of the native protein landscape. In particular, common features have been identified in the near native-state FEL of psychrophilic enzymes belonging to different enzymatic families when compared to the mesophilic counterparts. The data achieved by the simulations allow the validation of the cold-adapted folding funnel model previously proposed. In fact, the mesophilic enzyme has a funnel-like landscape, whereas the psychrophilic homologue is characterized by a rugged flat-bottom landscape with low barriers, which favors the interconversion among several metastable states.

Recently, engineered proteins in conjunction with conformationally gated electron transfer (ET) methods were used to assess the response of the kinetics at the bottom of a folding funnel to global stability [184]. It was concluded that the funneled landscape evolved such that loss of global stability lowers barriers at the bottom of a folding funnel, still allowing for efficient folding. The authors found analogies with the current view on cold-adapted enzymes, which is supported by the aforementioned computational results, according to which the strongly unstable cold-adapted enzymes present enhanced dynamics, allowing the catalytically active state to still be readily accessible at low temperatures.

A similar computational study [185], in the attempt to describe FEL of thermophilic proteins, has been performed on the other side of the temperature adaptation scale, comparing two rubredoxins from a hyperthermophilic and a mesophilic organism and using the conformational distance D_4 and the gyration radius as reaction coordinates. Although these results should be taken with care due to the small timescale of the simulations (10 ns per simulation), the authors point out different protein behaviors. The energy surface of the thermophilic protein is slightly modified by the shift to higher temperatures whereas the mesophilic pro-

tein features a broader distribution of states and a more rugged surface minimum. The former also presents a deeper and more distinct energy minimum, as expected by a thermostable protein.

The computational data described in this section strongly enforce the current view on stability and flexibility relationship in cold-adapted enzymes. These studies have to be extended in the future, for a deeper comprehension of cold adaptation, to other more computationally challenging known structures of cold-adapted enzymes and to proteins for which both psychrophilic and thermophilic variants are available. The evidence of differences in the shape and whole properties of the conformational landscape accessible to psychrophilic, mesophilic and thermophilic enzymes, pointed out in the present investigation, also opens the possibility of further investigations by other computational techniques, such as metadynamics [186, 187], suitable to capture and describe details of the free energy surface and of energetic barriers dividing the different sub-states which populate the native-state.

5. THE EFFECTS OF STRUCTURAL ELEMENTS AND ELECTROSTATIC INTERACTIONS ON THE STABILITY OF COLD-ADAPTED ENZYME

5.1. The Role of Electrostatic Interactions and Charged Residues in Thermal Adaptation

The stability of a protein results from a delicate balance between different weak intramolecular interactions. Electrostatic interactions and in particular charge-charge interactions have been shown to play a crucial role in determining protein stability [188]. Charge-charge interactions have distal effects and occur between charged protein groups, even if they are located several angstroms apart.

Electrostatic interactions between charged groups have highly variable effects in protein structure, related both to their attractive or repulsive nature, as well as to the fact that salt-bridge formation requires ordering of the protein structure and a desolvation of the charged residues. Ordering and desolvation are costly in entropy and enthalpy and counteract favorable electrostatic interaction between opposite charged residues. In fact, in the unfolded state the charged residues of a protein are fully solvated by water molecules. Solvation of charged residues is energetically favorable. As the protein folds, these solvated charged residues must desolvate. Hence, depending upon their location in the protein each charged residue incurs in energetically unfavorable desolvation penalty of different entity, which often remains uncompensated [189]. Therefore, in term of thermodynamic stability of the folded state the contribution of electrostatic interactions can be described as follow:

$$\Delta G_{elec}=\Delta G_{brd} + \Delta G_{prt}+\Delta G_{hb} +\Delta G_{ds}$$

where ΔG_{brd} is the contribute of salt bridge formation, ΔG_{prt} the contribution of long range electrostatic interactions, ΔG_{hb} the contribution of hydrogen bonds to the charged residues, and ΔG_{ds} the contribution of desolvation. Many authors found salt bridges to be stabilizing, but their contribution to stability has always been argued [190, 191]. There are plenty of examples where predicted mutations designed to improve electrostatic interactions in protein resulted in a reverse effect in the experimental validation [192]. This is mainly due to two factors: first, the energy contribution of bridge formation strongly depends on the unfavorable desolvation penalty, which is estimated to be 10-16 kcal/mol, and hinges on the burial of the charged residue in the folded protein. Second, electrostatic interactions in the unfolded state are often underestimated. In its seminal work, Pace and collaborators demonstrated for RNase Sa that the difference between empirical results and calculation on contribution of electrostatic optimization derived from more favorable interactions in the denatured ensemble than the native state [193]. Solvent denaturation curves showed a pH-dependent increase in the m-value explainable only if the denatured state ensemble expands at low pH due to electrostatic repulsions among the excess of positive charges increasing the accessibility of the denaturant. The m-value decreases at pH 7 due to more favorable electrostatic interactions in the denatured state becoming more compact. This experimental approach has been widely used to investigate the crucial influence of the electrostatic interactions in the denatured state in a deep cross-talking with computational studies able to depict at a molecular level interactions in the denatured state [194-196]. Briefly, the approach relies on the relationship between the pH dependent ΔG_0 and the protons bound to native and denatured state:

$$\delta\Delta G_0/\delta pH=2.303RT\Delta Q$$

where ΔG_0 is the free energy of unfolding and ΔQ is the difference between the number of protons bound in the native state (Q_N) and in the denatured state (Q_D) as a function of pH. In native conditions, Q_N can be directly measured, but not Q_D and thus ΔQ could not be determined. Nevertheless, one model for the titration behavior of the denatured state can be assumed in order to calculate the expected values of ΔQ. Comparison of the model with experimentally determined curves of pH vs. stability can determine if the model account for the data. If a discrepancy exists, interactions in the denatured state influence Q_D to values different from the predicted model. Therefore electrostatic interactions in the denatured state can be probed and a model tested. This approach proved that electrostatic interactions can contribute up to 4 kcal/mol to the stability of the denatured state [197].

In light of the above observations, it turns out that the identification of salt-bridge interactions and networks from the analysis of 3D protein structures is not a trivial task. In fact, the definition of salt bridges (or ion pairs) using a simple distance cutoff, assuming that the main stabilization arises from attraction between two oppositely charged residues within a certain distance (often 4-6 Å), from a static experimental structure cannot be the suitable strategy to compare extremophilic enzymes. In fact, most extremophilic protein structures have been solved by X-ray crystallography and it has to be considered that ion-pairs are composed of hydrophilic charged residues and that the majority of ion-pairs are found on the surface of protein structures, which makes the residues susceptible to contacts with neighboring molecules in the crystal lattice. Therefore, it becomes unclear whether the formation of ion-pairs is native or affected by crystal artifacts and packing. The intrinsic flexible nature of the charged residues involved in the salt bridge also makes their identification difficult. Highly solvent exposed

and long side chains of the charged residues often result in high flexibility and consequently poorly-defined electron density with high crystallographic B-factors. However, it is not desirable to exclude residues having high B-factors in the search for ion-pairs since two residues can be flexible and form a tight ion-pair at the same time by having concerted motions.

A dynamic framework, as the one provided by MD simulations can help in solving these problems. Residues under the influence of crystal contacts can be relaxed during minimization and equilibration, allowing to mime their behavior in solution during the simulations [198]. The flexibility issue is also overcome thanks to the possibility to monitor the motions of all the atoms during the timescale of the dynamics. Therefore, it is more straightforward to discern ion-pairs by selecting those that are maintained during the simulations over certain persistence rather than using simple distance cutoff calculated on average static structures.

A major limitation of this strategy is that MD simulations only provide information about ion-pairs geometries but not about their contribution to the overall stability of the protein, since it is not possible to accurately calculate protein stability in terms of thermodynamic quantities derived from MD simulations. In fact, it is proposed that ion-pairs can be tightly maintained without contributing to overall protein stability [198]. However, useful indications can be provided if solvent accessibility of the salt-bridges is included. In fact, it has been shown that solvent-exposed salt-bridges can be stabilizing for the protein structure, since the desolvation penalty they encounter is only marginal and generally counteracted by the formation of the intramolecular electrostatic interactions. Nevertheless, it has been shown, in a study on the conservation of salt bridges in different protein families, that buried salt bridges are more likely to be conserved than the surface exposed ones and are crucial in the maintenance of protein structure [199]. For several years, it has been considered that a charged residue in the interior of a protein, even if contributing to the formation of a salt bridge, was always destabilizing due to large desolvation penalty, idea supported by experimental works [200]. This evidence led to the assumption that burial of charged residues was an evolutionary strategy for keeping protein not too much stable. However, this is not always true, since different experimental works showed that buried salt bridges can be highly stabilizing [201], in the order of 4 kcal/mol. In 2006 Pace's group showed that the two most buried carboxyl groups in RNase Sa, Asp33 and Asp79, are the former stabilizing (6 kcal/mol) and the latter destabilizing (3 kcal/mol). The different contribution is mainly due to three intramolecular hydrogen bonds formed by Asp33 [202].

In addition to pairwise salt bridges, more complex associations of charged residues in proteins are also observed. A statistical analysis of complex salt bridges involving at least three charged residues has been carried out [199], demonstrating the tendency of the charged residues to form cooperative networks. These salt-bridge networks are more often found at subunit-subunit interfaces. Arginine, which contains a guanidinium group in its side chain, often acts as a connector in such networks.

The definition of complex clusters and networks of salt-bridges from a conformational ensemble can be extremely insightful to disclose aspects related to protein stability and function. However, before the clustering of electrostatic interactions along the structure, it has to be defined a cut-off of salt-bridge persistence during the dynamics or in the conformational ensemble, due to the dynamic nature of salt-bridges themselves. The persistence cut-off can be defined selecting a value which best divides the interactions dataset into two well-separated groups of signal and noise by the application of supervised classification algorithms [66]. Once a significant cut-off has been selected, a suitable method to identify not only salt-bridge pairs or short-range networks, but also wider clusters of electrostatic interactions, has been recently applied to a comparison along the whole scale of temperature adaptation to psychrophilic, mesophilic and thermophilic subtilisins [66]. To identify clusters of interactions, the residues involved in salt-bridges can be represented as nodes of an unrooted unoriented graph, in which two nodes were connected by arcs if a salt-bridge was identified between them or if they were at less than five residues of distance in the sequence. An exhaustive search procedure can be subsequently carried out on the graph to isolate networks of electrostatic interactions Fig. (**4**).

While the role of electrostatic interactions in enhancing thermal stability has been extensively investigated [190, 203], their possible contribution toward flexibility and proper solvation in cold-adapted enzymes at low temperature is still not well clarified. It has been proposed that, in the case of citrate synthase, protein electrostatics play a crucial role also in cold adaptation [43]. The hyperthermophilic homolog employs electrostatic interactions to avoid thermal denaturation and to preserve its dimeric state and active site at high temperatures. On the other hand, the psychrophilic enzyme optimizes electrostatic interactions to ensure a proper solvation at low temperatures and to confer greater flexibility, particularly in the active-site region. A similar trend has been observed, by the study of salt-bridges distribution and persistence during molecular dynamics simulations at different temperatures in extremophilic serine-proteases [66, 85, 204] and uracil-DNA-glycosylases [205].

5.2. Effects of Metal Cofactors on Protein Stability of Cold-Adapted Enzymes

Metal ion binding, frequently involving calcium and zinc ions, can provide extra stability superior to any other weak interactions and even higher than that of a disulphide bridge. Zinc is one of the most important proteins cofactors, not only for its key role in assisting enzymatic catalysis, but also for its structural relevance [206]. It usually shows a tetrahedral coordination, with a preference for hystidine and cysteine residues. Coordination geometry is so well defined that attempts to develop a computational approach to predict protein structure starting from the position of the zinc coordinating residues successfully occurred [207]. Role of zinc ions in protein stability is often associated with zinc finger motif (Cys_2His_2) originally found in transcription factors involved in nucleic acid binding [208]. Zinc fingers are self-existing domains and their conformation is closely related to the coordination of the ion, assuming a relevant role in proper folding of transcription factors [209]. Moreover, structural role

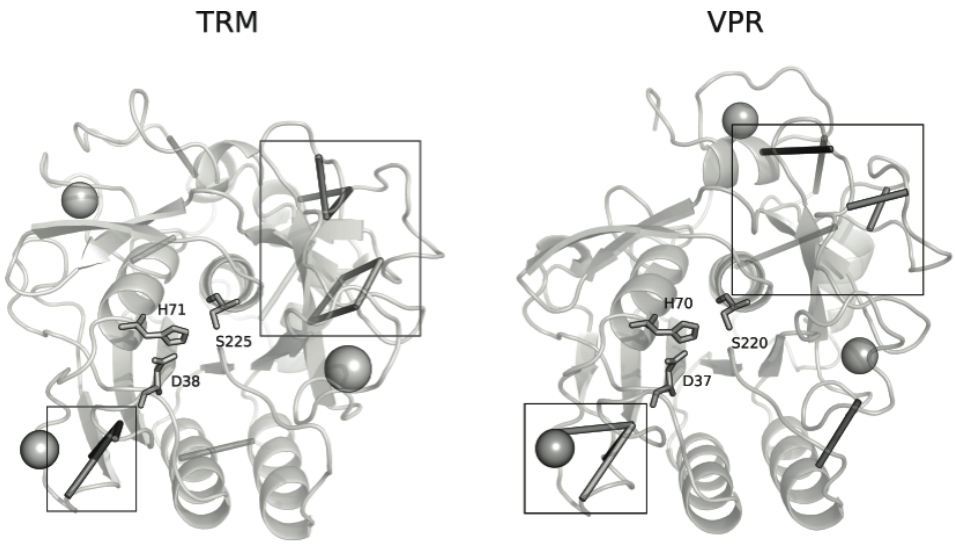

Fig. (4). Salt bridge networks of a thermophilic (TRM, thermitase from *Thermoactinomyces vulgaris*) **and of a psychrophilic** (VPR, *Vibrio* proteinase from *Vibrio* sp. PA-44) **subtilisins**, as calculated by molecular dynamics simulations. [66] salt-bridges are shown as cylinders connecting Cα atoms on the respective 3D structure; the main clusters of salt-bridges are included in black boxes.

of zinc ions has been demonstrated for complex proteins not associated to nucleic acid binding, such as superoxide dismutase 1 and carbonic anhydrase [210, 211]. Although in some cold-adapted enzymes zinc ion binding has been characterized [212], investigation on a possible active role of the metal on cold adaptation properties is still lacking. In the case of Ca^{2+} ion, contribution to structure stability is certainly related to the eight possible coordination bonds that are able to bridge several secondary structure domains of a protein. All Ca^{2+} binding psychrophilic proteins investigated so far are characterized by low binding affinity for the metal ion (see ref. [21] for a summary). On the other hand, residues forming the calcium binding sites of cold- and warm-adapted enzymes [72, 213-215] are often identical to that of mesophilic homologs, indicating that the low affinity for Ca^{2+} probably results from the flexible structure of the cold-adapted enzyme or from long-range effects.

MD simulations has proved effects induced by Ca^{2+} binding in cold- and warm-adapted trypsins [72] with particular attention to effects induced on autoproteolytic sites. The removal of Ca^{2+} ion affects the structural and dynamic properties of specific regions of trypsins. The effects are more pronounced in the N-terminal domain where the calcium-binding site is located and which lacks disulphide bridges, but the fluctuations are channelled also to sites in the C-terminal domain, in agreement with a general mechanism by which the signal induced by a metal ion is transmitted to remote regions in the 3D structure. In particular, the long interdomain loop of trypsin, which connects the two globular protein domains, has been proposed to be involved in the transmission of the signal correlated to Ca^{2+} binding. A decrease of autoproteolysis rate at R177 has been shown in mammalian trypsins [216] upon Ca^{2+} binding and the MD investigation has shown that R177 and K188 are the only primary autolysis sites which are mainly affected by Ca^{2+} binding or removal in bovine mesophilic trypsin, disclosing the molecular relationship connecting Ca^{2+} binding to autoproteolysis propensity in mesophilic trypsins. Remarkably, R177 is not conserved in cold-adapted trypsins known so far and the flexibility of K188 is not significantly affected by Ca^{2+} removal in agreement with a weak dependence of autolysis propensity on Ca^{2+} binding in cold-adapted trypsins.

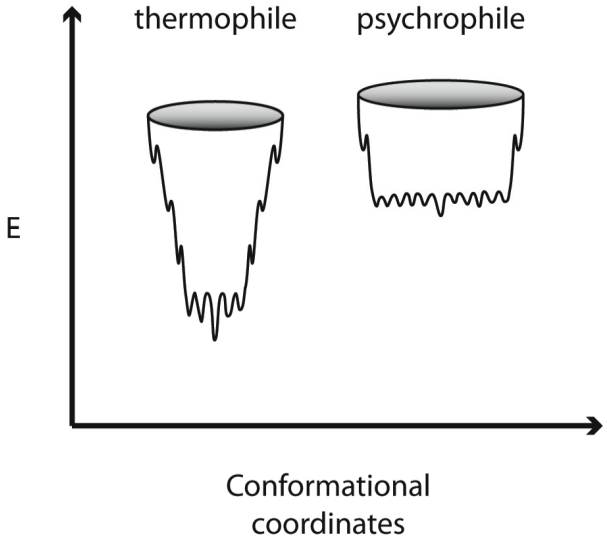

Fig. (5). Schematic representation of the folding funnel model for a thermophilic and a psychrophilic enzyme.

A combined approach of simulations and experimental data can be a powerful method to determine the molecular mechanisms involved in Ca^{2+} binding and its role in determining structural integrity of proteins. In fact, it has been demonstrated in the *Burkholderia glumae* lipase that Ca^{2+} ion is not solvent accessible unless local flexibility on loops coordinating the ion, in particular the one harbouring Asp241, is gained through an increase of temperature. Loss of the ion causes a highly cooperative protein unfolding with a consequent increase in β-structures, which trigger protein aggregation. This dramatic effect could be accounted for loss of in-

teractions mediated by Ca^{2+} ion not only on the proximity of the binding site but also on the distant N-terminal of the protein [90, 217]. This kind of work suggests that experimental data coupled with fine MD simulations could be a powerful approach to investigate the role of Ca^{2+} ion on cold adaption of many proteins.

5.3. Molecular Determinants of Cold Adaptation in Multimeric Proteins

Proteins involved in many fundamental biological processes carry out their activity and are regulated through interactions with other proteins. Many proteins function as parts of permanent obligate complexes, such as multimeric enzymes, which often fold and bind simultaneously [218]. The forces that keep protein complexes together are the same interactions at the basis of the stability of their components folded state. At moderate temperatures, protein-protein interactions are in fact largely driven by the hydrophobic effect. Hydrogen bonds and electrostatic interactions are extremely important, and covalent bonds also play a relevant role [219]. In some cases, the active site is formed by two or more of the monomers, so that the disruption of the quaternary structure of these multimeric enzymes would directly result in the complete loss of their activity. In the context of cold adaptation, the monomers of oligomeric psychrophilic enzymes are subject to the same structural and dynamical adaptive strategies which characterize non-oligomeric enzymes, which in general lead to higher catalytic activity at low temperatures, lower thermal stability and higher structural flexibility with respect to their mesophilic homologs [21]. The features of the interaction interfaces are often of particular importance for these properties, so that the question of whether the same adaptive strategies apply to them is naturally of great interest [220-234].

The issue was put forward by Russell *et al*. [220], in the comparison between the crystal structures of homodimeric bacterial psychrophilic and hyperthermophilic citrate synthases. The authors found that cold adaptation of the dimerization interface entails a reduction of the number of ion pairs and ion pair networks, in agreement with the studies presented in Section 5.1. A similar trend was identified in the comparison of homologous psychrophilic and thermophilic malate dehydrogenases (MDH) [221], of psychrophilic and mesophilic lactate dehydrogenases (LDH) [222], and of the alkaline phosphatase (AP) from the *Antarctic bacterium* TAB5 with its mesophilic homologs from *E. coli* and human placenta [223].

An opposite trend was suggested in a recent systematic study of the interfaces of the available psychrophilic oligomeric enzymes performed with the aim of identifying statistically significant differences in those characteristics of the interfaces that are most likely to be related to structural stability [224]. In this study, the interfaces of 35 psychrophilic oligomeric enzymes from 5 different families were compared on a per-family basis, and the identified differences were tested for significance against a non-redundant reference dataset of 148 mesophilic oligomeric proteins from 43 different families.

This study detected a significant increase in the number of interface ion pairs in psychrophilic enzymes when compared with their mesophilic homologs. A similar trend was identified in the analysis of homology models of serine hydroxymethyltransferases (SHMT) adapted to different temperatures [77]. The authors argue that an increase in the number of ionic interactions could balance the cold-induced weakening of the hydrophobic effect, especially since the formation of ion pairs is an exothermic electrostatic interaction, and therefore particularly strong at low temperatures. In agreement with these observations, they find a significant decrease in the interface hydrophobicity of psychrophilic oligomers, especially when compared to their thermophilic homologs [224]. The strengthening of the hydrophobic effect was found to be one of the most important factors in the temperature adaptation of the interfaces of oligomeric thermophilic and hyperthermophilic enzymes [226]. On the other hand, the dimeric interface of a psychrophilic AP has been found to be more hydrophobic than that of a mesophilic homolog: the authors suggest that the substitution of specific hydrogen bonds by less specific van der Waals contacts may promote interface flexibility [223].

The systematic analysis of Tronelli *et al*. [224] also identified a significant decrease in the number of interface hydrogen bonds in psychrophilic enzymes with respect to their mesophilic and (hyper)thermophilic homologs. Similar trends were described in the aforementioned comparative studies of MDH and LDH. For the AP from *Antarctic bacterium* TAB5 the decrease is less pronounced; the authors point out that in the psychrophilic enzyme a higher proportion of interface hydrogen bonds are between backbone atoms (77%) than in the homologous enzyme from *E. coli* (59%) [223]. The same is true for the triosephosphate isomerase (TIM) from the cold-adapted bacterium *V. marinus* (63% of backbone interactions [227]) with respect to its mesophilic homolog from *E. coli* (59% of backbone interactions) [228]. The authors suggest this could be functional to cold adaptation since backbone atoms have less freedom in the solvated conformation than the side chains, and therefore the entropy cost of dimerization would already have been paid upon folding, also making the stability of the complex less temperature-dependent [223].

A common trend in the adaptation of the interfaces of psychrophilic oligomeric enzymes seems to be the reduction of inter-monomer interactions. This must provide the increase in the structural flexibility of the oligomers to sustain their activity at low temperatures without disrupting their quaternary structure.

Nevertheless, other strategies may be available, and some oligomeric enzymes may not require any specific adaptation of the interface. It may also be the case that cold-adapted enzymes have weakened inter-monomer interactions due to lack of evolutionary pressure to enhance temperature stability [229].

6. THE REQUIREMENT OF A FAMILY-CENTERED POINT OF VIEW IN COMPARATIVE STRUCTURAL AND DYNAMIC STUDIES OF COLD- AND WARM-ADAPTED ENZYMES

The refined 3D experimentally solved structures of cold-adapted enzymes known up to now Table **1** have been extremely useful in compiling the inventory of structural factors associated to cold adaptation, mentioned above. It turns

out that subtle modifications are sufficient to adapt a mesophilic homolog to cold temperatures and that a unified theory for cold adaptation cannot be formulated. Therefore, there are no structural characteristics that can be uniquely related to cold adaptation or thermal adaptation in general, and it is extremely hard to identify tendencies at the level of primary sequence for increased or decreased occurrence of particular amino acids in cold-adapted enzymes. In fact, the location, on the 3D structure, of the residues which differentiate the cold- from the warm-adapted enzymes, is the most important determinant of properties of the cold-adapted enzymes.

In comparative studies aimed to disclose molecular determinants of cold adaptation, the degree of similarity between warm- and cold-adapted enzymes to use for the comparison should be relatively high to be effectively meaningful. Single structural effects cannot be easily estimated if significant differences in the protein architecture are found. In order to overcome this problem, comparative studies among differently temperature-adapted homologous enzymes are strongly encouraged. Several comparative investigations of homologues adapted to different temperature conditions but belonging to the same enzymatic family are presently available in the literature, allowing also pointing out specific characteristics related to thermal adaptation (see Section 2).

Due to the relatively low availability of structural data on cold-adapted enzymes, the choice of the cold-adapted protein for comparative purposes is the main constraint. Moreover, differentially temperature-adapted homologs with known high resolution 3D structure must exist to allow a suitable comparison. Most of these studies compared mesophilic and psychrophilic enzymes, since few cases are known for which enzymes with a known, experimentally determined, 3D structure are available along the whole scale of temperature conditions. However, some cases are available in the literature of comparison, on the whole scale of temperature adaptation, including either thermophilic, mesophilic or psychrophilic homologous enzymes [66, 77, 234-236]. In one of these studies, since the thermophilic variant does not share with the psychrophilic homolog a higher sequence similarity, also a homology model of a closer thermophilic enzyme has been successfully included in the comparison, allowing the evidences collected by the study to be confirmed.

In fact, in order to overcome the lack of 3D structures of extremophilic enzymes, homology modeling and other modeling techniques could be useful to provide a subset of enzyme structures available for further investigations. An experimental validation of this model could be the best approach to pursue, considering the fine details which make the difference among cold- and heat-adapted enzymes.

In this context, serine hydroxymethyltransferase (EC 2.1.2.1), a pyridoxal-5-phosphate (PLP)-dependent enzyme with a crucial role in one-carbon unit metabolism and a potential target for cancer therapy, was extensively studied as a model of adaptation to extreme environments. It is a suitable case of study since a large number of orthologous sequences are available from the three domains of life thanks to genomic projects. It is a highly conserved enzyme with few amino acids substitution tolerated during evolution, due to its crucial role in metabolism. Moreover, sequences and crystal structures are available for the cold-adapted variants but also for the thermophilic counterparts. Moreover, a dimeric protein it is a suitable target to study structural adaptation at the subunit interface. A comparative study has been carried out for mesophilic, psychrophilic and hyper/thermophilic SHMT, allowing a picture of SHMT evolution over the full temperature spectrum to be drawn [77], using homology modeling for the different variants and a comparative approach previously applied to extremophilic proteins but belonging to different families [40]. The good potential of SHMT relies on the fact that there are 10 unique crystal structures of SHMT deposited in the PDB and hundreds primary sequences deduced from genome projects worldwide, as well as on the knowledge of the enzyme catalytic mechanisms and its structural determinants. The high sequence similarity among cold- and warm-adapted SHMT allows the definition of accurate homology models, useful to define property variations by statistical analysis in different temperature-adapted proteins. Cold adaptation in SHMT turns out to be achieved through an increase in polarity and flexibility of the protein core, surface and interfaces, a higher positive electrostatic potential at the cofactor binding site, with a coherent trend identifiable from low to high temperature adaptation. A similar methodology was previously applied by Altermark, with valuable indications on the cold adaptation of bacterial endonucleases from *Vibrionacee* species [69].

However, the detailed identification of structure-derived properties from the analysis of homology models along has to be taken with extreme caution. The precision with which these properties are determined from homology models drastically decreases with the decrease in sequence identity between target and template, as inherent in the homology modeling procedure and particular attention has to be devoted to properties of the protein surface and disordered regions. It has also to be considered that, since the differences between cold- and heat-active enzymes are generally small and subtle, homology models are not sufficient alone to disclose structure-function relationships of cold-adapted enzymes, and a dynamic approach is strictly required.

MD investigations on cold-adapted serine-proteases, clarifies how distinct members of this superfamily have faced the negative effects of low temperature on protein activity and stability [34-35, 66]. Generally, evolution divergence is functionally constrained so that properties more relevant for function diverge more slowly [237]. Thus, protein dynamic properties which are relevant for function have to be evolutionary constrained and several evidences of evolutionary selection for specific dynamical characteristics have been recently reported [143, 238, 239]. In particular, backbone protein flexibility profiles diverge slowly and are conserved at the protein family and superfamily levels, providing indirect evidences of the conservation of protein dynamics, along with the high robustness of these conserved motions [240]. In this context, the observation that the evolutionary separation between psychrophilic and mesophilic trypsins, as well as between psychrophilic and mesophilic elastases, is successive to the separation of trypsins from elastases, indicates that psychrophilic trypsins and elastases independently discovered the same solution to optimize protein flexibility at low temperatures, and is a remarkable example of molecular evolutionary convergence [35].

Table 1. Summary of Experimentally Solved 3D Structures of Cold-Adapted Enzymes. The 3D Structures of Cold-Adapted Enzymes have been Retrieved by Literature Searches in Pubmed and IsiWeb of Knowledge, Along with Searches with Blast in the Protein Data Bank (PDB). The Searches are Updated at the 15th February 2011

Enzyme Family	EC Number	Organism	PDB Code	Cofactors	Resolution (Å)
Adenylate kinase	2.7.4.3	*Bacillus globisporius*	1S3G	ZN	2,25
Adenylate kinase	2.7.4.3	*Marinibacillus marinus*	3FB4	ZN	2
Alkaline phosphatase	3.1.3.1	*Pandalus borealis*	1K7H	ZN	1,92
Alkaline phosphatase	3.1.3.1	*Pandalus borealis*	1SHN	ZN	2,15
Alkaline phosphatase	3.1.3.1	*Pandalus borealis*	1SHQ	ZN	2
Alkaline phosphatase	3.1.3.1	*Antarctic bacterium TAB5*	2IUC	ZN;MG	1,95
Alkaline phosphatase	3.1.3.1	*Vibrio sp. G15-21*	3E2D	MG, ZN	1,4
Alkaline phosphatase		*Shewanella*	3A52	MG, ZN	2,2
Alpha-amylase	3.2.1.1	*Pseudoalteromonas haloplanktis*	1AQH	CA;CL	2
Alpha-amylase	3.2.1.1	*Pseudoalteromonas haloplanktis*	1B0I	CA;CL	2,4
Alpha-amylase	3.2.1.1	*Pseudoalteromonas haloplanktis*	1G94	CA; CL	1,74
Alpha-amylase	3.2.1.1	*Pseudoalteromonas haloplanktis*	1G9H	CA;CL	1,8
Alpha-amylase	3.2.1.1	*Pseudoalteromonas haloplanktis*	1JD7	CA;CL	2,25
Alpha-amylase	3.2.1.1	*Pseudoalteromonas haloplanktis*	1JD9	CA;CL	2,5
Alpha-amylase	3.2.1.1	*Pseudoalteromonas haloplanktis*	1L0P	CA;CL	2,1
Alpha-amylase	3.2.1.1	*Pseudoalteromonas haloplanktis*	1KXH	CA;CL	2,3
Alpha-amylase	3.2.1.1	*Pseudoalteromonas haloplanktis*	1AQM	CA;CL	1,85
Amidase	3.5.1.4	*Nesterenkonia sp*	3HKX		1,66
Aminopeptidase	3.4.11.-	*Colwellia Psychrerthraea*	3CIA	ZN	2,79
Aspartate carbamoyl-transferase	2.1.3.2	*Moritella profunda*	2BE7	ZN	2,85
Beta-galactosidase	3.2.1.23	*Arthrobacter sp C2-2*	1YQ2	MG	1,9
Beta-lactamase class C	3.5.2.6	*Pseudomonas fluorescens*	2QZ6		2,26
Catalase	1.11.1.6	*Vibrio salmonicida*	2ISA	HEM	1,97
Cellulase	3.2.1.4	*Pseudoalteromonas haloplanktis*	1TVN		1,41
Cellulase (endoglucanase)	3.2.1.4	*Pseudoalteromonas haloplanktis*	1TVP		1,6
Chitinase	3.2.1.14	*Arthrobacter Tad20*	1KFW		1,74
Citrate synthase	2.3.3.1	*Antarctic bacterium DS2-3R*	1A59	COA	2,09
Dihydrofolate reductase	1.5.1.3	*Moritella profunda*	2ZZA	FOL, NAP	2
Elastase	3.4.21.36	*Salmo salar*	1ELT	CA	1,61
Endonuclase I	3.1.21.1	*Vibrio salmonicida*	2PU3	MG	1,5
Iron superoxide dismutase	1.15.1.1	*Aliivibrio salmonicida*	2W7W	FE	1.70
Iron superoxIde dIsmutase	1.15.1.1	*Pseudoalteromonas haloplanktis*	3LIO	FE	1,5
Iron superoxIde dIsmutase	1.15.1.1	*Pseudoalteromonas haloplanktis*	3LJ9	FE	2,1
Iron superoxIde dIsmutase	1.15.1.1	*Pseudoalteromonas haloplanktis*	3LJF	FE	2,1
IsocItrate dehydrogenase		*Desulfotalea Psychrophila*	2UXR	MG	2,3

(Table 1) contd….

Enzyme Family	EC Number	Organism	PDB Code	Cofactors	Resolution (Å)
IsocItrate dehydrogenase		*Desulfotalea Psychrophila*	2UXQ	MG	1,75
Lactate-dehydrogenase	1.1.1.27	*Champsocephalus gunnari*	2V65		2,35
Lipase	3.1.1.3	*Photobacterium lipolyticum*	2ORY		2,2
Lipase B	3.1.1.3	*Candida antarctica*	1LBT		2,5
Lipase B	3.1.1.3	*Candida antarctica*	1TCA		1,55
Lipase B	3.1.1.3	*Candida antarctica*	1TCB		2,1
Lipase B	3.1.1.3	*Candida antarctica*	1TCC		2,5
Lipase B	3.1.1.3	*Candida antarctica*	1LBS		2,6
Lysozyme g		*Salmo salar*	3MGW	CO, SO4	1,75
Malate dehydrogenase	1.1.1.37	*Aquaspirillium arcticum*	1B8P	NAD	1,9
Malate dehydrogenase	1.1.1.37	*Aquaspirillium arcticum*	1B8U	NAD	2,5
Malate dehydrogenase	1.1.1.37	*Aquaspirillium arcticum*	1B8V	NAD	2,1
Metallo-protease	3.4.24.40	*Pseudomonas tac II*	1O0T	CA;ZN	2,5
Metallo-protease	3.4.24.40	*Pseudomonas tac II*	1OM7	CA;ZN	2,8
Metallo-protease	3.4.24.40	*Pseudomonas tac II*	1OM8	CA;ZN	2
Metallo-protease	3.4.24.40	*Pseudomonas tac ii*	1OMJ	CA;ZN	2,38
Metallo-protease	3.4.24.40	*Pseudomonas tac ii*	1O0Q	CA;ZN	2,2
Metallo-protease	3.4.24.40	*Pseudomonas tac ii*	1G9K	CA;ZN	1,96
Metallo-protease	3.4.24.40	*Pseudomonas tac ii*	1H71	CA;ZN	2,1
Metallo-protease	3.4.24.40	*Pseudomonas tac ii*	1OM6	CA;ZN	2
Parvulin-like peptidyl-prolyl isomerase	5.2.1.8	*Cenarchaeum symbiosum*	2RQS		(NMR)
PePsin	3.4.23.1	*Gadus morhua*	1AM5		2,16
Phenylalanine hydroxylase	1.14.16.1	*Colwellia Psychrerythraea*	2V27	FE	1,5
Phenylalanine hydroxylase	1.14.16.1	*Colwellia Psychrerythraea*	2V28		1,95
Protein-tyrosine-phosphatase	3.1.3.48	*Shewanella sp.*	2Z72	ZN	1.10
Protein-tyrosine-phosphatase	3.1.3.48	*Shewanella sp.*	2ZBM	ZN	1.50
Proteinase K like enzyme	3.4.21.	*Serratia species*	2B6N		1,8
S-formylglutathione Hydrolase	3.1.1.-	*Pseudoalteromonas haloplanktis*	3LS2	CL	2,2
Sphericase		*Bacillus sphaericus*	3D43	CA	0,8
Sphericase subtilisin-like	3.4.21.62	*Bacillus subtilis psycrophile*	2GKO	CA	1,4
Subtilisin-like Serine Protease	3.4.21._	*Pseudoalteromonas sp.*	1Y9Z	CA	1,4
Subtilisin-like Serine Protease APA1	3.4.21._	*Pseudoalteromonas sp.*	1WVM	CA	1,6
Subtilisine-like serine-protease	3.4.21._	*Vibrio sp*	1S2N	CA	2,44
Subtilisine-like serine-protease	3.4.21._	*Vibrio sp*	1SH7	CA	1,84
Subtilisine-like serine-protease/Alkaline serine-protease	3.4.21._	*Pseudoalteromonas sp.*	1V6C	CA	1,8
Triose-phosphate isomerase	5.3.1.1	*Vibrio marinus*	1AW1		2,7

(Table 1) contd….

Enzyme Family	EC Number	Organism	PDB Code	Cofactors	Resolution (Å)
Triose-phosphate isomerase	5.3.1.1	*Vibrio marinus*	1AW2		2,65
Trypsin	3.4.21.4	*Salmo salar*	2TBS	CA	1,8
Trypsin	3.4.21.4	*Salmo salar*	1BIT	CA	1,83
Trypsin	3.4.21.4	*Salmo salar*	1HJ8	CA	1
Trypsin	3.4.21.4	*Salmo salar*	1UTM	CA	1,5
Trypsin	3.4.21.4	*Salmo salar*	1UTL	CA	1,7
Trypsin	3.4.21.4	*Salmo salar*	1UTK	CA	1,53
Trypsin	3.4.21.4	*Salmo salar*	1UTJ	CA	1,83
Trypsin	3.4.21.4	*Salmo salar*	2STB	CA	1,8
Trypsin	3.4.21.4	*Salmo salar*	2STA	CA	1,8
Trypsin	3.4.21.4	*Salmo salar*	1BZX	CA	2,1
Trypsin	3.4.21.4	*Oncorhynchus keta*	1MBQ	CA	1,8
Trypsin	3.4.21.4	*Atlantic cod*	2EEK	CA	1,85
Tyrosine phosphatase	3.1.3.48	*Shewarella sp*	1V73	CA	1,82
Uracil DNA-Glycosylase	3.2.2.3	*Vibrio cholerae*	2JHQ		1,5
Uracil-DNA-glycosylase	3.2.2.3	*Gadus morhua*	1OKB		1,9
Xylanase	3.2.1.8	*Pseudoalteromonas haloplanktis*	1H12		1,2
Xylanase	3.2.1.8	*Pseudoalteromonas haloplanktis*	1H13		1,3
Xylanase	3.2.1.8	*Pseudoalteromonas haloplanktis*	1H14		1,5
Xylanase	3.2.1.8	*Pseudoalteromonas haloplanktis*	1XW2		1,76
Xylanase	3.2.1.8	*Pseudoalteromonas haloplanktis*	1XWQ		1,88
Xylanase	3.2.1.8	*Pseudoalteromonas haloplanktis*	1XWT		1,3
Xylanase	3.2.1.8	*Pseudoalteromonas haloplanktis*	2A8Z		3,2
Xylanase	3.2.1.8	*Pseudoalteromonas haloplanktis*	2B4F		1,95

In a broader context, these studies show that, even if a general theory for cold adaptation cannot be postulated, enzymes sharing the same function and 3D architecture adopted similar strategies to optimize structural stability and flexibility in order to elicit their biological function under the challenging conditions of extreme temperature habitats, point out an evolutionary convergence on structural and dynamical properties of homologous cold-adapted enzymes.

7. CONCLUDING REMARKS

In conclusion, this review summarized and discussed the scenario gained by the efforts of the last two decades in the investigation of enzyme cold adaptation mechanisms under a structural and molecular perspective. Results achieved in the field of cold adaption were also reviewed in the context of the emerging general view on protein dynamics and function relationships, as well as on conformational flexibility of the native ensemble of natively unfolded and psychrophilic proteins. A pivotal role turns out for electrostatic interaction and their organization in cooperative networks, which capture most of the protein dynamics signature of a protein around its native state.

A continuous cross-talk between biophysical techniques, suitable to describe protein motions in atomistic details and on different timescales, and atomistic simulations in a multiscale context will be the key to successfully clarify mechanisms of cold adaptation. It will also provide a solid framework for applicative purposes and investigation aimed to design new and optimized variants of cold-adapted enzymes for industrial applications.

Moreover, it turns out from the present scenario that, even if common structural strategies in cold adaptation cannot be formulated, a family-centered point of view will be extremely useful and necessary in the comparative analyses of cold- and warm-adapted enzymes. In fact, enzymes be-

longing to the same family or superfamily, sharing at least the 3D fold and common features of the functional sites, have evolved similar structural and dynamic features to overcome the detrimental effects of low temperatures.

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