Adaptation to high temperatures through macromolecular dynamics by neutron scattering

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Abstract

Work on the relationship between hyperthermophile protein dynamics, stability and activity is reviewed. Neutron spectroscopy has been applied to measure and compare the macromolecular dynamics of various hyperthermophilic and mesophilic proteins, under different conditions. First, molecular dynamics have been analyzed for the hyperthermophile malate dehydrogenase from Methanococcus jannaschii and a mesophilic homologue, the lactate dehydrogenase from Oryctolagus cunniculus (rabbit) muscle. The neutron scattering approach has provided independent measurements of the global flexibility and structural resilience of each protein, and it has been demonstrated that macromolecular dynamics represents one of the molecular mechanisms of thermoadaptation. The resilience was found to be higher for the hyperthermophilic protein, thus ensuring similar flexibilities in both enzymes at their optimal activity temperature. Second, the neutron method has been developed to quantify the average macromolecular flexibility and resilience within the natural crowded environment of the cell, and mean macromolecular motions have been measured in vivo in psychrophile, mesophile, thermophile and hyperthermophile bacteria. The macromolecular resilience in bacteria was found to increase with adaptation to high temperatures, whereas flexibility was maintained within narrow limits, independent of physiological temperature for all cells in their active state. Third, macromolecular motions have been measured in free and immobilized dihydrofolate reductase from Escherichia coli. The immobilized mesophilic enzyme has increased stability and decreased activity, so that its properties are changed to resemble those of a thermophilic enzyme. Quasi-elastic neutron scattering measurements have also been performed to probe the protein motions. Compared to the free enzyme, the average height of the activation free energy barrier to local motions was found to be increased by 0.54 kcal.mol-1 in the immobilized dihydrofolate reductase, a value that is of the same order as expected from the theoretical rate equation.

Keywords

experimental molecular dynamics, flexibility, rigidity, neutron scattering, thermostability

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MINIREVIEW

Adaptation to high temperatures through macromolecular dynamics by neutron scattering

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Work on the relationship between hyperthermophile protein dynamics, stability and activity is reviewed. Neutron spectroscopy has been applied to measure and compare the macromolecular dynamics of various hyperthermophilic and mesophilic proteins, under different conditions. First, molecular dynamics have been analyzed for the hyperthermophile malate dehydrogenase from *Methanococcus jannaschii* and a mesophilic homologue, the lactate dehydrogenase from *Oryctolagus cunniculus* (rabbit) muscle. The neutron scattering approach has provided independent measurements of the global flexibility and structural resilience of each protein, and it has been demonstrated that macromolecular dynamics represents one of the molecular mechanisms of thermoadaptation. The resilience was found to be higher for the hyperthermophilic protein, thus ensuring similar flexibilities in both enzymes at their optimal activity temperature. Second, the neutron method has been developed to quantify the average macromolecular flexibility and resilience within the natural crowded environment of the cell, and mean macromolecular motions have been measured *in vivo* in psychrophile, mesophile, thermophile and hyperthermophile bacteria. The macromolecular resilience in bacteria was found to increase with adaptation to high temperatures, whereas flexibility was maintained within narrow limits, independent of physiological temperature for all cells in their active state. Third, macromolecular motions have been measured in free and immobilized dihydrofolate reductase from *Escherichia coli*. The immobilized mesophilic enzyme has increased stability and decreased activity, so that its properties are changed to resemble those of a thermophilic enzyme. Quasi-elastic neutron scattering measurements have also been performed to probe the protein motions. Compared to the free enzyme, the average height of the activation free energy barrier to local motions was found to be increased by 0.54 kcal·mol^{-1} in the immobilized dihydrofolate reductase, a value that is of the same order as expected from the theoretical rate equation.

The study of thermophile adaptation

Organisms can thrive in what we call extreme environments on Earth and perhaps elsewhere in the Solar System. Macelroy [1] named these lovers ('philos' to Greeks) of extreme environments 'extremophiles'. They had to adapt to one or several physico-chemical extreme parameters: high temperatures for thermophiles

that live above 60 °C and temperatures above 85–90 °C for hyperthermophiles near geysers and hydrothermal vents, whereas psychrophiles grow at temperatures below 15 °C in glacier water and polar seas. Halophiles thrive in hypersaline environments such as the Lac Rose in Senegal. Other examples of physicochemical extreme parameters are high pressure, high radiation activity, high and low pH.

Research on extremophiles has intensified in recent years due to both their practical and fundamental significance. On the practical side, the high thermostable thermophiles and their enzymes have an important economic potential in multiple areas, such as detergent manufacturing, production of high-fructose corn syrup, or biochemistry with the invention of PCR to amplify genes, either by direct applications for catalysis under high temperature, or by tapping them as sources of ideas to modify mesophile enzymes (adapted to 'normal' temperature conditions), with the aim of improving their properties and stability at high temperature. On the fundamental side, a comparison of thermophilic proteins with their mesophile counterparts can help to increase our understanding of the physicochemical basis of protein stability. The study of thermophile adaptation has also broad implications for exobiology. Evaporite minerals such as Jarosite have been identified in the Meridiani region on Mars [2,3]. Terrestrial Jarosite forms in deeper high temperature magmatic hydrothermal environments, in near-surface high temperature magmatic steam, and in steam-heated environments. On Earth, fossils are often found in evaporite deposits [4,5]. If traces of life exist in Martian Jarosite evaporite, the corresponding organisms will likely be adapted to a high temperature environment and will fall into the thermophiles category. In this minireview, we focus on the study of the dynamic basis of thermophile adaptation.

The role of sequence and structure in thermostability

Sequence comparison studies and structural analyses carried out on thermophilic proteins and their mesophilic homologues have shown that thermal stability is associated with multiple factors. A correlation has been demonstrated between adaptation to high temperature and the average charged minus noncharged polar (asparagine, glutamine, serine and threonine) amino-acid percentage (Ch-Pol) in protein structures [6]. The charged residues are likely to favor ion pairs, hydration interactions and hydrogen bonds. Ion pairs have been proposed to play a key role in the maintenance of enzyme stability at high temperature [7,8] in thermophilic and hyperthermophilic proteins. Furthermore, an

increase in α-helix or β-sheet content accompanied by a reduction in the lengths of the external loops and an increase in the number of hydrogen bonds [9,10] has also been associated with higher thermal stability in hyperthermophilic proteins. The decrease of denaturation entropy due to shortening of the polypeptide chain also plays an important role in extremely high temperature stability in hyperthermophilic proteins [11]. For thermophilic and hyperthermophilic proteins, the reduction in number and volume of cavities [9,11,12] has also been associated with protein stabilization. Cavities are energetically unfavorable due to a loss of van der Waals contacts [13]. Filling or creating cavities by site-directed mutagenesis can increase or decrease the thermostability of proteins [14–16] and the most destabilizing replacements tend to occur in the most rigid parts of a protein structure [17]. The water accessible surface area (WASA) allows quantification of the extend to which atoms on the protein surface can form contacts with water. The WASA has energetic significance in that it is directly related to hydrophobic free energies. The reduction of each square angström of WASA yields a gain in free energy of 25 cal·mol^{-1} [18] and was shown to improve the stability of thermophilic proteins [16]. The available data clearly indicate that the intrinsic thermal stability of thermophilic proteins cannot be explained by a single mechanism. Moreover, solvent conditions [19] or ligand binding [20,21] may induce changes in protein thermal stability. Note that all the forces responsible for the higher thermostability of thermophiles and hyperthermophiles are the same as the forces associated with protein stabilization in general. They are weak forces associated with energies similar to thermal energy.

Experimental methods used to study the role of dynamics in thermostability

Forces arising from the structural modification discussed above will necessarily lead to modification in protein dynamics.

Various experimental methods have been applied to explore protein dynamics adaptation to high temperature. Hydrogen/deuterium (H/D) exchange [22], fluorescence quenching [23], high-resolution NMR [24] and neutron scattering [25] are probably the major ones. The flexibility of protein molecules is reflected in conformational fluctuations. These reversible local rearrangements expose buried segments of the polypeptide chain to solvent. In D$_2$O, H/D exchange occurs during such exposure and the probability distribution of the accessibility of protein hydrogens can be determined by H/D exchange experiments providing a measure of protein

fluctuations. These experiments do not provide total protein dynamics and must be performed in D_2O, which may affect the activity, stability and dynamics of proteins [19,26]. Fluorescence quenching is well established in the investigation of structural flexibility and integrity for protein structures. This technique requires the use of a quencher (e.g. acryl-amide) for the quenching of tryptophan fluorescence. In general, however, different tryptophan residues have a different accessibility to the quencher and the quenching behavior is only weakly coupled to structural fluctuations. NMR permits the study of backbone dynamics in proteins. The method usually requires isotope enrichment of the protein by ^{15}N labeling and provides information essentially in terms of order parameters and entropy values. There is an overlap between the time scales of neutron scattering experiments and those of NMR. Neutron scattering experiments can be performed under close to physiological conditions on proteins of any size and do not require isotope labeling, although D_2O solvent or deuterium labeling can be used to explore specific effects. The method provides direct information on atomic fluctuation amplitudes in a given time-scale and in absolute units. Furthermore, neutron absorption is low for protein atoms and the radiation penetrates deeply into the sample with negligible radiation damage.

Neutron scattering to study macromolecular dynamics

The length and time scales of atomic motions examined in a neutron scattering experiment are in the angstrom and 10^{-12}–10^{-9} s ranges, respectively. Because of their large incoherent scattering cross-section, the motions of ^{1}H nuclei dominate the observations [27]; the experiments provide information on global protein dynamics because, on the timescale examined, the H atoms reflect the dynamics of the side chains and backbone atoms to which they are bound [28]. So-called elastic scattering experiments yield mean square fluctuation values, $<u^2>$, as a function of temperature (Fig. 1) [29]. The value of the root mean square fluctuation $\sqrt{<u^2>}$ in absolute units (Å), quantifies the global flexibility of the system studied. An effective mean force constant, $<k'>$, defining mean resilience (rigidity), can be calculated from the derivative of $<u^2>$ plotted versus temperature, T [30,31] (Fig. 2). Using the elastic neutron scattering approach, the combined analysis of neutron data on dynamics, on the one hand, with activity and stability data, on the other, has revealed a strong adaptation of resilience and mean square fluctuations to physiological temperature and suggested mechanisms that govern the high
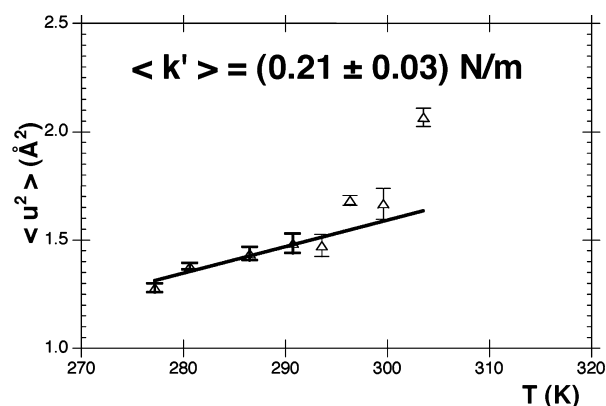

Fig. 1. Mean square fluctuations $<u^2>$ of *Aquaspirillum arcticum.* The $<u^2>$ values were plotted versus absolute temperature T; the effective mean force constant $<k'>$, describing mean macromolecular resilience, was calculated from the slopes of the straight-line fits in the temperature region where the bacteria macromolecules are stable. Figure modified from [29].
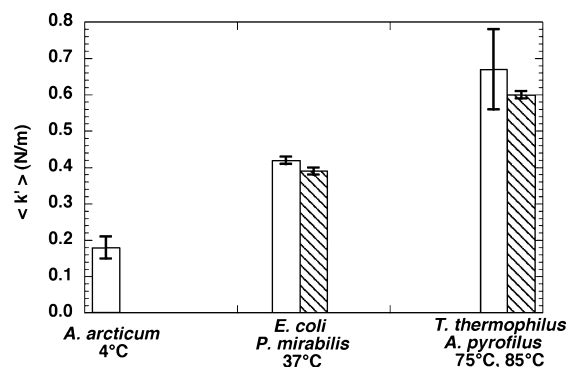

Fig. 2. Mean macromolecular resiliences $<k'>$ measured *in cellulo* for each bacteria type. The $<k'>$ values were plotted against adaptation temperature: 4 °C for *Aquaspirillum arcticum* (blank), 37 °C for *Escherichia coli* (blank) and *Proteus mirabilis* (hatched), 75 °C for *Thermus thermophilus* (blank) and 85 °C for *Aquifex pyrofilus* (hatched). Figure modified from [29].

thermal stability of thermophiles [9]. So-called quasi-elastic neutron scattering is due to diffusive motions. The scattering is described by Lorentzian functions, from whose energy width, Γ, as a function of scattering vector Q, it is possible to calculate, mean square displacement of high frequency vibration, diffusion coefficients (diffusive motions) and residence times for the scattering particle. Quasi-elastic neutron scattering also provides the average height of the potentiel barrier to the motions [27], which is higher for a more thermostable enzyme, and therefore suggests how dynamics determines the adaptation to high temperature.

This minireview focuses on three areas of research investigating the dynamic basis of macromolecular

adaptation to high temperature, using neutron scattering.

First, the enzyme malate dehydrogenase from the hyperthermophile Archaeon *Methanococcus jannaschii* (*Mj* MalDH) [9]. Using neutron spectroscopy, we have demonstrated that macromolecular dynamics presents one of these molecular mechanisms of adaptation to high temperature in this enzyme. We present results for *Mj* MalDH and a mesophilic homologue, lactate dehydrogenase from *Oryctolagus cunniculus* (Rabbit) muscle (*Oc* LDH). Thermoadaptation appears to have been achieved by evolution through selection of appropriate resilience to preserve specific macromolecule structure while allowing the conformational flexibility required for activity.

Second, the whole live psychrophile, mesophile, thermophile, and hyperthermophile bacteria [29]. We characterized the mean motions of the entire cellular macromolecular population *in vivo* to establish how adaptation to high temperature occurred through dynamics acting to optimize average macromolecular stability and flexibility. The flexibilities are in fact maintained within narrow limits around 1.2 Å, independent of physiological temperature for all cells in their functional state, whereas the macromolecular resilience of bacteria increases with adaptation to high temperature.

Third, the immobilized *Escherichia coli* dihydrofolate reductase (DHFR) [21]. DHFR is an essential enzyme required for normal folate metabolism in prokaryotes and eukaryotes and it is recognized as a drug target for inhibiting DNA synthesis in rapidly proliferating cells, such as cancer cells [32]. One of the main advantages concerning immobilized enzymes is their enhanced stability [33–35]. The covalent immobilization of the mesophilic DHFR through external lysine reduced the activity rate by a factor of approximately seven and increased its stability [21,36], so that its properties are changed to resemble those of the thermophilic enzyme. The comparative study of the native and the immobilized DHFR showed that the average height of the potential barrier of the enzyme to achieve the local motions is increased in the immobilized DHFR, which may increase the activation energy for the enzyme catalysis, decreasing the observed rate. These results suggest that the local motions on the picosecond time scale may act as a lubricant for those associated with DHFR activity occurring on a slower millisecond time scale. Experiments indicate a significantly slower catalytic reaction rate for the immobilized *E. coli* DHFR. The phenomenon has broad implications for the action of drugs binding to an enzyme far away from the active site; DHFR is in fact immobilized via a site on the exterior of the enzyme and essentially distal to the active site [21].

Neutron scattering explored the dynamical basis of macromolecular adaptation to high temperature

In vitro: the hyperthermophile *Mj* MalDH

Activity and stability data, combined with elastic neutron scattering results for hyperthermophilic and mesophilic enzymes of the malate lactate dehydrogenase family, established a strong adaptation of root mean square fluctuation (global flexibility) and resilience (rigidity) to physiological temperature. The value of the global flexibility (1.5 Å) observed for *Oc* LDH and for *Mj* MalDH at their respective temperatures of optimal activity (37 °C for the mesophile and 90 °C for the hyperthermophile) is essentially identical. The observation suggests that the enzymes have conformational flexibility adjusted to the optimum working temperature, in accordance with the hypothesis that adaptation of proteins to different physiological temperatures tends to maintain enzymes in 'corresponding states', characterized by similar conformational flexibility [37]. The stability data show that the hyperthermophilic protein is significantly more stable with a temperature of optimal activity higher [9]. The mean resilience (rigidity) is an order of magnitude larger for *Mj* MalDH (1.50 N·m^{-1}) than for *Oc* LDH (0.15 N·m^{-1}). The higher stability of *Mj* MalDH is therefore correlated with higher resilience. By performing comparative analysis using the 3D crystal structures and the sequences, we suggested mechanisms that govern the high thermal stability of *Mj* MalDH through increased resilience. The structural bases of thermophilic stability in the (LDH-like) malate dehydrogenase group have been discussed previously [10,12]. Enhanced stability arises from a combination of different mechanisms. In the present study, the results indicate that several factors, such as increased average charge (lysine, aspartate, arginine and glutamate) minus noncharged polar (asparagine, glutamine, serine and threonine) amino acid percentage (Ch-Pol) value of protein sequence, increased packing density, as well as a reduction of number and total volume of internal cavities, increased ion pairs and increased hydrogen bond interactions, are responsible for a more stable protein and contribute to an observed increase in protein resilience, suggesting the dominance of enthalpic contributions to the free energy landscape in thermoadaptation. For proteins in which entropic effects are dominant, a less resilient macromolecule

will be more thermostable [9]. This feature was already observed for BSA. The protein is more stable in D_2O compared to H_2O and its resilience is lower in heavy water [19]. The solvent properties of D_2O differ from those of H_2O. For protein, this difference in properties between these two solvents leads to stronger hydration–bond interactions (enthalpic terms) in D_2O, as well as to the solubility of apolar groups being lower in D_2O than in H_2O, which favours the hydrophobic interactions (entropic terms) [19,38]. The more stable BSA structure is less resilient, suggesting that the stabilizing effect of D_2O is dominated by entropic terms (i.e. an increase in conformational freedom). Thermoadaptation appears to have been achieved by evolution through selection of appropriate structural rigidity to preserve specific protein structure, while allowing the conformational flexibility required for activity.

In vivo: whole bacteria adapted at different temperatures

During the elastic neutron scattering experiments on whole live bacteria, the instrument space-time window essentially selected motions of atoms (in the Q range used) that are anchored to macromolecules (proteins, nucleic acids and their complexes). The overall macromolecular composition of the bacteria cells examined is not expected to vary significantly from one cell type to another [39]. Macromolecules constitute 96% of the total dry weight of an *E. coli* cell. DNA represents 3%, and lipids and polysaccharides approximately 17%; the majority, more than 75% of the dry weight, consist of proteins and ribosomes, themselves made up of approximately 50% protein and 50% RNA by mass [40,41]. Within a given bacterium, differences in protein expression due to metabolic modifications in unstressed cells affect a few hundred proteins out of approximately 5000 [42]. It is reasonable to assume therefore that the neutron scattering data described here are dominated by the dynamics of the proteins, making up the cellular proteome, in association with their natural environment. We compared the macromolecular dynamics on different whole live bacteria: the psychrophile *Aquaspirillum arcticum*, the mesophiles *E. coli* and *Proteus mirabilis*, the thermophile *Thermus thermophilus* and the hyperthermophile *Aquifex pyrofilus*. The $<u^2>$ values of *A. arcticum* showed a striking transition above 20 °C reflecting macromolecular denaturation (Fig. 1). We note that 17 °C is the maximum temperature at which *A. arcticum* can maintain net growth. We found that the flexibilities are in fact maintained within narrow limits of around 1.2 Å, independent of physiological

temperature for all cells in their functional state. Mean effective force constant values, $<k'>$, of 0.21 ± 0.03, 0.42 ± 0.01, 0.39 ± 0.01, 0.67 ± 0.011 and 0.60 ± 0.01 N·m^{-1} were found for *A. arcticum*, *E. coli*, *P. mirabilis*, *T. thermophilus* and *A. pyrofilus* cells, respectively (Fig. 2). Therefore, the macromolecular resilience of bacteria increases with adaptation to high temperatures. Macromolecules in hyperthermophile and thermophile cells are, on average, the most resilient followed by those in mesophile, whereas those in psychrophiles present the softest structures [29]. The resilience values, which increased with stabilization temperature, indicated the dominance of enthalpy terms in the stabilization free energy differences. Thus, we show *in vivo* that thermoadaptation appears to have been achieved through increasing of resilience with adaptation temperature, while maintaining the flexibility within narrow limits, independent of physiological temperature for all cells in their functional state.

Biotechnology: immobilized dihydrofolate reductase

The mean square displacement of high frequency vibration values are 0.23 ± 0.04 Å^2 and 0.19 ± 0.02 Å^2 for the native and the immobilized DHFR, respectively. The amplitude is slightly smaller for the immobilized DHFR but they are similar within error. The Γ width of the Lorentzian in is given as a function of Q^2 in Fig. 3. The Γ-values of the immobilized and native DHFR increase with Q^2 and asymptotically approaches a constant value Γ_∞ at large Q. The linewidths follows the well-know jump diffusion behavior, given by [43]:
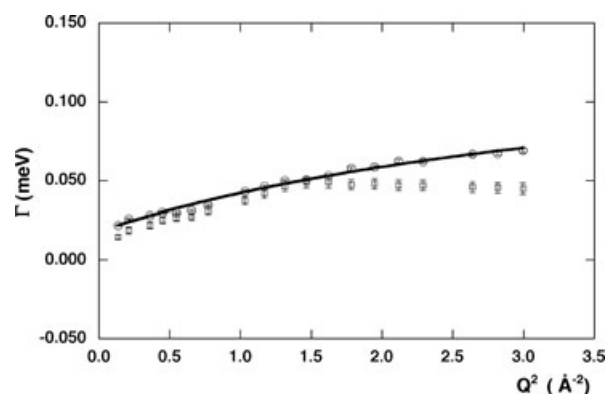

Fig. 3. Half widths Γ of the quasi-elastic Lorentzian function. The Γ values were plotted as a function of Q^2 for the immobilized (squares) and the native (circles) dihydrofolate reductase from *Escherichia coli* at $T = 285$ °K. The line results from the fit of $\Gamma(Q)$ using Eqn (1). Figure modified from [21].

$$\Gamma(Q) = \frac{DQ^2}{1 + DQ^2\tau} \qquad (1)$$

At low Q, the diffusion coefficients D of internal diffusive motions are also comparable for the native and the immobilized DHFR. In the high Q region, one observes motion on short length scales. On such scales, the average local jump motion of all the protons in the protein becomes dominant. The residence time of a hydrogen atom on one site between jumps is $\tau = 1/\Gamma_\infty$ where is Γ_∞ obtained from the asymptotic behavior at high Q when Γ approaches a constant value. The resulting residence times are $\tau = 7.95 \pm 1.02$ ps and $\tau = 20.36 \pm 1.80$ ps for the native and immobilized DHFR, respectively. With respect to a H atom that can occupy two locations separated by an activation free energy, residence time is the mean time spent before jumping. Free activation energy barrier and residence time are related by:

$$\tau = \tau_0 e^{(E_a/k_B T)} \qquad (2)$$

The activation energy difference between the two systems is 0.54 ± 0.12 kcal·mol^{-1}. Recall that the catalytic rate of the immobilized enzyme is seven-fold slower than for the native. According to the Arrhenius relation, the theoretical rate may be written as $k = \frac{k_B T}{h} \exp(-\frac{E_a}{RT})$, where E_a is the activation energy barrier for the catalyzed reaction, k_B is Boltzmann's constant, h is Planck's constant, R is the gas constant and T is the absolute temperature. Using this theoretical rate equation, we can calculate that the activation energy difference required to reduce the rate by a factor of seven, at the same temperature, between the native and the immobilized DHFR is 1.10 kcal·mol^{-1} [21]. The observed activation energy difference between the immobilized and the native DHFR was 0.54 kcal·mol^{-1}, which is of the same order but less than expected from calculation. The rate of DHFR catalysis is expected to be influenced more by motions occurring on the millisecond time scale [44]. Thus, this result suggests that average local motions occurring on the picosecond time scale may have an influence on the much slower millisecond time scale of the catalytic activity. The results presented here show that, although the immobilization of the DHFR is on the exterior of the enzyme and essentially distal to the active site, experiments indicate a significant decreasing of catalytic reaction rate. It is possible that the binding of ligands generally may exert a similar effect. In any event, this phenomenon has broad implications for protein engineering, drug design and effect of pharmacaphores distal to the active site.

Complex relationship among dynamics and adaptation to high temperature

The relationship between macromolecular dynamics, stability and activity is complex. Furthermore, there is confusion regarding this relationship, which stems from several sources. Flexibility is monitored by different techniques, which are sensitive to very different aspects of macromolecular fluctuations. Native proteins are structured by a delicate balance of noncovalent forces and they unfold when high temperatures disrupt these interactions. The free energy of stabilization of a protein is the difference between the free energies of its folded and unfolded states. It directly measures the thermodynamic stability of the folded protein. In many cases, enzyme inactivation at high temperature is concomitant with global loss of tertiary and secondary structures. Thermodynamics provides a quantification of how a macromolecule unfolds globally in response to thermal energy. In order to correlate stability and dynamics, it is therefore of interest to measure global rather then localized motions, and to define global flexibility. The forces that maintain biological molecular structure and govern atomic motion, and that are also responsible for higher thermostability of hyperthermophiles, are 'weak' forces because their associated energies are similar to thermal energy ($\approx$ meV). The time scale of corresponding molecular motions is in the picosecond to nanosecond range. Measurements in this timescale therefore are the most appropriate for clarifying the relationship between dynamics and thermodynamic stability. Neutron spectroscopy is the appropriate method for such characterization because it allows the measurement of global macromolecular internal motions on the picosecond to nanosecond timescale. Enzyme activity occurs on the much slower millisecond timescale. Fast atomic thermal fluctuations, however, act as a lubricant for larger conformational changes associated with enzyme activity [45] and allow the protein to achieve the necessary flexibility to perform their biological function [46]. In addition to enzyme motions having been probed using different techniques, the various studies of thermophile and hyperthermophile dynamics have used different protein models, with quite distinct native topologies. Different protein structures may have their dynamics altered in different ways by adaptation to high temperature.

A few experiments have shown that thermostable enzymes exhibit reduced structural flexibility at room temperature with respect to their mesophilic homologues [22,47], whereas others, investigating alpha-amylase [48] and rubredoxin [49,50], have shown the

opposite effect in that the thermostable homologues were found to be more flexible, suggesting stabilization through entropic effects. The relationships between flexibility and stability are therefore complex. Atomic fluctuations were only measured in these experiments and interpreted in terms of flexibility. However, it is important to note that reduced structural 'flexibility' does not necessarily imply a more 'rigid' structure [9]. Atoms are maintained in a structure by forces that link them to their neighbours. In terms of a force field, the width of the potential well in which an atom moves is a measure of its flexibility in terms of a root mean square fluctuation amplitude ($\sqrt{<u^2>}$, whereas the detailed shape of the well and how the flexibility increases with the thermal energy $k_B T$ reflects the rigidity of the structure, in terms of an effective force constant ($<k>$) (Fig. 4). The energy landscape of protein dynamics is complex. Each macromolecule has in general two globally distinct macrostates, the folded (F) and the unfolded (U) state. The unfolded state is clearly represented by a wide variety of different unfolded conformations. According to Frauenfelder's conformational substate model, however, the folded state itself is made up of a large number of conformations with small differences between them [51]

(Fig. 4A). As temperature increases, a transition from one conformational substate to another generally occurs within the folded state by overcoming a micro activation free energy barrier (Ea_m). The protein moves by breaking and reforming 'weak' bonds and changing structure continuously. When high temperature disrupt these interactions and the protein loose its integrity, transition occurs from the folded state to the unfolded state by a macro activation free energy barrier (Ea_M) (Fig. 4A). It has been demonstrated that thermophilic proteins pass through many conformational substate intermediates, which reduce the protein cooperativity of the folding–unfolding reaction. By contrast, it has been suggested that psychrophilic proteins generally unfold cooperatively without intermediates, and therefore the shape of the well is smooth [52].

Two types of potential wells are illustrated in Fig. 4B,C. In the case of harmonic motion (Fig. 4B), flexibility ($\sqrt{<u_1^2>}$) and rigidity ($<k_1>$) are related, as would be expected intuitively. The potential is given by $V(u) = \frac{1}{2} <k> u^2$, and the atomic mean square fluctuation is related to the force constant by $<u^2(T)> = k_B T / <k>$ [30,31]; a less rigid harmonic structure will have indeed a higher increasing of the flexibility with the thermal energy. Protein structures,
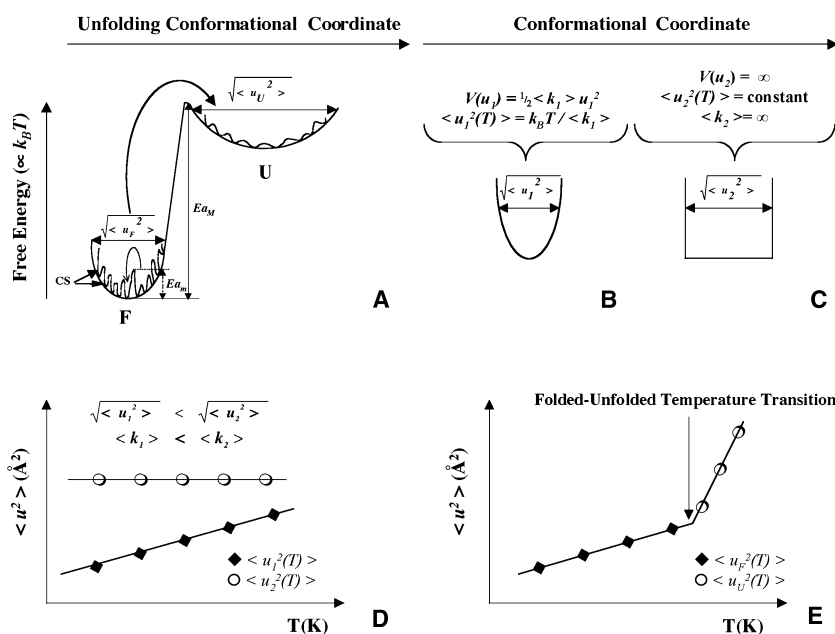

Fig. 4. Schematic diagrams of the free energy versus conformational coordinate and mean square fluctuations $<u^2>$ versus temperature. (A) Free energy versus unfolding conformational coordinate showing conformational substates (CS) in the folded state (F), atomic flexibilities ($\sqrt{<u^2>}$) in the folded state and in the unfolded state (U), micro activation free energy barrier (Ea_m) for transition between conformational substates and macro activation free energy (Ea_M) for structure loss to give the unfolded state. (B) Free energy versus conformational coordinate showing harmonic well potential. (C) Free energy versus conformational coordinate showing square well potential. (D) Mean square fluctuations $<u^2>$ versus temperature of the two types of potential wells illustrated in Fig. 4B,C. (E) Dynamical folded–unfolded temperature transition.

however, are not harmonic at physiological temperature, and atoms move in different types of potential. In the extreme case, an atom can move quite freely in a 'box' formed by its neighbours, but cannot go out of the box (Fig. 4C). Mathematically, this can be described by the square well potential. The flexibility ($\sqrt{<u_2^2>}$) in this case is a temperature-independent constant value, whereas the effective force constant ($<k_2>$) stopping the atom from leaving the box is infinitely high. The evolution of the mean square fluctuations with the temperature of the two types of potential wells (Fig. 4B,C) is shown in Fig. 4D. The $\sqrt{<u_1^2>}$ values are less important than the $\sqrt{<u_2^2>}$ values, however, the $<k_1>$ value is lower than $<k_2>$ value, which illustrates the fact that reduced structural 'flexibility' does not necessarily imply a more 'rigid' structure, and was observed experimentally using neutron scattering to examine the dynamics protein adaptation to high temperatures [9]. Flexibility and rigidity are therefore independent parameters. The folded–unfolded transition is shown in Fig. 4E. When the temperature increases, the mean square fluctuations values increase regularly until a critical temperature is reached where there is a striking transition reflecting macromolecular unfolding [29].

Conclusion

The commonly accepted hypothesis is that increased thermal stability is due to enhanced conformational rigidity of the molecular structure [53]. Thermophilic and hyperthermophilic enzymes are characterized by a high temperature of maximum activity [22,53]. The flexibility in enzymes plays an important role in their activity but the macromolecular flexibility can also lead to the loss of native structure and function if it becomes too large. Because the magnitude of the flexibility experienced by an enzyme will depend on the available thermal energy, evolution has modified the force and the type and number of stabilizing interactions in enzymes to achieve the optimal balance of stability and flexibility at a given temperature. As a result, large flexibility changes in temperature will disrupt this balance, causing psychrophiles or mesophiles to denature at high temperature, and the more rigid thermophilic enzyme would then require higher temperatures to achieve the requisite conformational flexibility for activity. Thus, thermoadaptation appears to have been achieved by evolution through selection of appropriate rigidity, in order to preserve specific macromolecule structure, while allowing the conformational flexibility required for activity.

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