Towards new thermostable proteins

Dr Fabio Sterpone describes an intriguing project in which unconventional and multi-scale in silico approaches are being applied to unravel the secrets of thermophilic proteins.

What are the aims of the THERMOS project?

Our main goal is to understand how the molecular machinery of thermophilic organisms can resist the high temperature regime in which they thrive; in some cases up to 100 °C. In particular, we are interested in the stability and function of their proteins. This class of proteins is a ‘natural template’. Understanding the elementary factors that guarantee their extreme stability could help to engineer enzymes for biotechnological purposes.

What biochemical factors enable thermophilic proteins to have thermal resistance?

Thermophiles are generally enriched in charged amino acids and therefore cross-linked by extended networks of ion pairs (strong hydrogen bonds formed between oppositely charged groups) and hydrogen bonds. It has also been proposed that improved packing of the hydrophobic core could be a key stabilising factor. Finally, it has been observed that thermophiles have shorter highly flexible regions known as ‘loops’.

Why is it important to study protein unfolding?

A protein is like a pearl chain, with each pearl being a specific chemical group (amino acid).

Unfolding is the disruption of the functional state. Disruption occurs because of temperature, the presence of chemical denaturants, pressure or mechanical induction. The unfolding mechanism can occur in different ways depending on the perturbation. Studying unfolding may help to localise a protein’s weak spots and therefore design mutations, by changing one or more amino acids, to make it more stable and still functional.

Could you elaborate on what is known about the relationship between thermophilic protein flexibility and functionality?

Thermophilic proteins function well at high temperature, but generally lack activity in ambient conditions. Protein functionality is, to some extent, related to the flexibility of the structure: the possibility of a protein accommodating a substrate in the internal binding site, or propagating conformational changes upon enzymatic reaction. So a lack of activity in ambient conditions has been considered proof of enhanced structural rigidity that will guarantee resistance to thermal stress. According to this common belief, thermophiles recover functionality at high temperature because the necessary flexibility is activated. However, recent experiments and computational work indicate that thermophiles show comparable and even enhanced flexibility compared to their homologues: stability is enhanced via entropy because the difference between the highly entropic unfolded state and a floppy native configuration is smaller.

How does water influence protein stability?

For a protein in aqueous solution, the hydration shell – the layer of water that is in direct contact with the protein – forms a network of hydrogen bonds that envelops the structure of the protein and is pinned to its surface by forming hydrogen bonds with amino acids. This network is disrupted when the protein unfolds or when the temperature increases. We are looking for a correlation among the weak spots of the protein – for instance, where unfolding takes place – and the local structure of the hydration network.

Water also hydrates internal cavities. These buried molecules act as cohesive bricks in the folded state and can very favourably contribute to protein stability. We are currently exploring how internal hydration contributes to extreme stability in thermophiles.

How might thermophilic proteins lead to new applications?

Let’s dream. First, assuming we are good and fortunate enough to design an original in silico strategy for improving the stability of proteins, the methodology could be used to optimise enzymes able to work in harsh conditions and of chemical or biotechnological interest. Even now, many chemical and biotechnological processes use thermophilic enzymes; the most famous example is the use of Taq polymerase to boost the performance of the polymerase chain reaction, a key technology for the biotechnology industry. Second, making a protein stable is important for medical applications, as in the case of antibodies that are very promising for cancer therapy. Finally, simulating the behaviour of proteins in a crowded environment makes it possible to elicit the stability problem in a context more similar to the cellular, and tackle topics like protein misfolding/aggregation in neurodegenerative diseases.
Virtual alchemy

In the THERMOS project underway at the Laboratoire de Biochimie Théorique in France, original and diverse computational approaches aim to determine new strategies for bioengineering thermostable proteins for medical and industrial purposes. Interim findings point to new design paradigms.

**FUNDAMENTAL TO THE** design of biotechnological solutions for practical problems is knowledge of the molecular mechanisms that stimulate a particular biological function, and so confer specific characteristics to an organism. Knowing what triggers a certain transformation that elicits a particular biochemical or biophysical reaction, and at what point in space and time, opens up the possibility of designing changes to the mechanism that will result in desired attributes and thus functions.

Thermophiles and their more extreme counterparts, the hyperthermophiles, are organisms that are able to function and thrive at very high temperatures – typically from 50-70 °C for thermophiles and from 80-100 °C for hyperthermophiles. Their protein molecules can maintain stability and function correctly at high temperatures, whereas those of their homologous counterparts usually found at lower temperatures rapidly unfold, become denatured and so dysfunctional under such conditions.

Although their promise for future industrial and medical applications is immense, the elemental factors that account for the ability of Thermophiles to resist high temperatures, and in particular those factors that enhance the stability of their proteins and preserve their functionality in extreme conditions, have so far eluded discovery. Thus, understanding the molecular mechanisms that underpin the stability exhibited by extreme thermophile proteins – X-proteins – is key. In a five-year project funded by the European Research Council named THERMOS, Dr Fabio Sterpone of the Laboratoire de Biochimie Théorique (LBT) in Paris, France, which is part of the Centre National de la Recherche Scientifique (CNRS), is exploring this question at the interface between biophysics and physical chemistry.

**THERMOS**

The LBT deploys expertise in theoretical and computational biochemistry to unravel molecular or conformational determinants of the biological properties and functions of living systems and disease. To support this endeavour, the LBT team devises simulation and predictive algorithms and informatics solutions that extend beyond the state of the art. In THERMOS, Sterpone’s group has developed novel frameworks and computer-based tools to explore thermophilic proteins, in collaboration with Dr Simone Melchionna of the Consiglio Nazionale delle Ricerche in Italy and Professor Philippe Derreumaux, the head of the LBT. The project benefits from strategic collaborations with experimentalists, Drs Dominique Madern and Eric Girard from the Institut de Biologie Structurale in France, Dr Marco Maccarini from the University Joseph Fourier in France and Dr Alessandro Paciaroni of the University of Perugia in Italy.

This interdisciplinary and international collaboration is designed to ensure that all possible elements are explored: “There is probably not one unique mechanism that helps thermophiles withstand high temperatures,” Sterpone muses. “In our project, we wish to gain a deep understanding of as many unambiguous and directly applicable routes as possible.”

**NEW IDEAS**

Whilst Melchionna began studying X-proteins in 2006, Sterpone’s background centred on investigating the behaviour of water at biological interfaces. Sterpone and Melchionna decided to join forces in order to approach the question of X-protein stability from a new perspective: “We asked whether water plays a crucial role in enhancing the stability of thermophiles,” explains Sterpone. “From this initial question, I started appreciating the subtleties of the issue.” In fact, these proteins are generally enriched in charged amino acids that form a higher number of salt bridges and hydrogen bonds on the protein surface which reinforce its structure and so integrity. The highly ionic character of the surface should also create a strong coupling with the water molecules of the solution in which the thermophile resides. “As a first result of our investigation, we interestingly observed that the stability of the hydrogen-bonds network formed by water around proteins correlates with their thermal stabilities,” Sterpone adds.

As a second step, Sterpone and collaborators used molecular dynamics simulations and theoretical modelling to explore the role of water on X-protein stability through hydration dynamics of two homologous proteins: one from the non-thermophilic *Escherichia coli* and one from the hyperthermophile *Sulfolobus solfataricus*. Surprisingly, they found that the surface water dynamics of both proteins were unaffected by their different amino acid compositions, because the main perturbation stems from their similar geometries. When the proteins were folded, interfacial water dynamics also responded in similar ways to temperature fluctuations. Therefore, the researchers concluded that hydration dynamics around thermophilic proteins is essentially no different from that near their homologues which function at ambient temperature. However, questions remain: “Water dynamics tells us only partial information concerning the kinetic processes at the molecular interface,” warns Sterpone. “What we really need to know is how the chemical composition affects thermodynamic quantities, such as the entropy of the hydration layer. Moreover, inside proteins,
and originally developed by Derreumaux at the LBT. A MULTI-SCALE SIMULATION TOOLKIT

Along a different line of enquiry, Sterpone, Melchionna and PhD student Maria Kalimeri addressed the common belief that rigidity of the protein matrix accounts for enhanced thermal resistance in X-proteins, undertaking an analysis of intrinsic conformational flexibility. Using the same proteins, from *E. coli* and *S. solfataricus*, they applied computer simulations at microsecond timescales to assess changes in topology and the role of internal fluctuations at ambient temperatures.

The researchers found that, while the magnitude of fluctuations was comparable between the proteins, in the X-protein the distribution of regions of flexibility and rigidity in amino acid composition was more regular, which had the effect of confining and so limiting the effects of mechanical excitation. The thermally excited motion of an extended flexible region in the *E. coli* protein, on the other hand, eased unfolding and so made it substantially less resistant to thermal stress: “A small molecular change can result in a very different stability. It is safer to confine flexibility than to localise it in a very large and very flexible fragment of the protein,” elucidates Sterpone, who sees the next step as being to identify those parts of a protein structure where it is safe to site or confine flexibility. He has already located weak spots in some model proteins where molecular adjustment would increase their stability.

A MULTI-SCALE SIMULATION TOOLKIT

Over the course of THERMOS, Sterpone has developed a new methodology to simplify the computational representation of the molecular dynamics in protein interactions based on the coarse-grained optimised potential for efficient protein structure prediction (OPEP) model for protein folding and aggregation, originally developed by Derreumaux at the LBT.

The method extends the OPEP force field, and improves the description of ion pairs without the need for explicitly declaring electrostatic terms. Results improve upon OPEP by refining the packing of charged amino acids, influencing the representation of the stability of secondary structure motifs and the population of intermediate states during temperature folding/unfolding, and also improves the aggregation propensity of peptides. It therefore delivers a more realistic picture of a wide variety of situations where salt bridges are key interactions, as in X-proteins.

Also within THERMOS, Sterpone, Derreumaux and Melchionna have developed a multi-scale framework which allows modelling and tracking of protein behaviour and functions over time and space according to the characteristics of particular biosystems. The framework couples Sterpone’s refined version of the OPEP force field with an algorithm that reproduces the hydrodynamic behaviour of a fluid. This multi-scale method opens a route to the investigation of processes like protein diffusion, stability and misfolding in a crowded environment, as occurs within a living cell. The framework is thus capable of simulating protein behaviour for key applications, such as the stability of antibodies for medical purposes, or assessing the effects of protein dysfunction in diseases such as Alzheimer’s.

To reveal the microscopic basis for protein thermal stability by taking a systematic multi-scale and multi-method computational approach to considering the behaviour of thermophiles. Investigations focus on three key aspects:

- The role of hydration
- The effect of protein rigidity/flexibility on stability
- The molecular mechanism sustaining protein activity at high temperature

KEY COLLABORATORS

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