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Anharmonic activations in proteins and peptide model systems and their connection with supercooled water thermodynamics

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Summary. — Proteins, the nano-machines of living systems, are highly dynamic molecules. The time-scale of functionally relevant motions spans over a very broad range, from femtoseconds to several seconds. In particular, the pico- to nanoseconds region is characterized by side-chain and backbone anharmonic fluctuations that are responsible for many biological tasks like ligand binding, substrate recognition and enzymatic activity. Neutron scattering on hydrated protein powders reveals two main activations of anharmonic dynamics, characterized by different onset temperature and amplitude. Here we review our work on synthetic polypeptides, native proteins, and single amino acids to identify the physical origin of the two onsets —one involving water-independent local dynamics of methyl groups and, to a minor extent, of aromatic side-chains, and the other one, known as "protein dynamical transition", concerning large scale functional protein fluctuations, most likely induced by a crossover in the structure and dynamics of hydration water connected with the second critical point hypothesis.

1. – Introduction

Biological function generally occurs in the presence of water, which, for this reason, is often called the matrix of life. At the molecular level, proteins, which are the nano-machines of living matter, need to be covered by water in order to be biologically active. This so-called hydration water is generally acknowledged to enable proteins internal structural fluctuations that are so fundamental for their capacity to fulfil a specific biological function, like *e.g.* ligand binding, protein-substrate recognition and interaction in enzymatic activity, permeability regulation in protein channels, etc. The dynamical performance corresponding to structural fluctuations defines the protein flexibility and

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occurs on a time-scale ranging from picoseconds to nanoseconds. Due to the complexity of the protein structure and of the protein-water interactions, the origin of this performance is still highly debated. A way to tease apart the complex ensemble of water-protein motions is to extend experiments down to cryo-temperatures. A seminal neutron scattering experiment performed about 25 years ago by W. Doster and co-workers on hydrated myoglobin showed for the first time that: 1) neutron scattering is an ideal technique to detect protein equilibrium internal motions; 2) hydrated proteins are characterised by an onset of structural flexibility as the temperature is increased above about 200–250 K, revealed as a transition to large-amplitude anharmonic motions of hydrogen atoms on the picosecond time-scale [1]. Since then the physical origin of this transition, known as the "protein dynamical transition", is controversially debated in the biophysical community, with different scenarios proposed in the literature [2-7]. More recently, high resolution neutron scattering experiments on lysozyme powders [8] revealed that at lower temperature (100–150 K) another small anharmonic onset occurs, even in the absence of water. In analogy with data on polymers, it was suggested that this low temperature onset is linked to the presence of methyl groups in the polypeptide chain (generally about 20–30% of hydrogen atoms in proteins belong to methyl side-chains) and due to the thermal activation of their rotational motion.

When trying to unambiguously attribute a molecular origin to the anharmonic onsets revealed by neutron scattering and to understand their physical (*i.e.* dynamical and thermodynamical) nature, the main difficulties arise from the fact that: 1) neutron scattering is sensitive to all the nuclei in the protein-hydration water system and 2) its accessible dynamical window is limited by the energy resolution of a given neutron spectrometer. The use of D_2O-hydrated proteins, taking into account the scattering lengths of the different atoms present in a protein, allows to make a first fundamental assumption: most of the signal (about 90% in protein powders at low hydration) coming from a D_2O-hydrated protein can be attributed to the so-called incoherent scattering from protein hydrogen nuclei. This evidence, together with the observation that non-exchangeable hydrogen atoms are almost uniformly distributed on the protein, allows to use neutron scattering to characterize the "mean" dynamics of a protein and largely simplifies the interpretation of neutron scattering patterns; yet a safe attribution of the observed dynamics to the different molecular groups and side-chains is impossible. In the last years we introduced the use of homomeric synthetic polypeptides and amino acids mixtures as model systems for proteins to address the following questions:

1) What is the molecular origin of the two anharmonic onsets revealed by neutron scattering in protein powders?

2) Is the polypeptide chain necessary to the protein dynamical transition?

3) Is there a direct equivalence between the protein dynamical transition and the onset of biological activity?

4) What is the thermodynamical nature of the two onsets? Do they correspond to real transitions or to kinetic effects due the finite energy resolution?

5) Is there a connection between the protein dynamical transition and the liquid-liquid crossover proposed to occur in supercooled water in the same temperature region?

In this paper, we present an overview of the main results obtained with the approach described above.

2. – Materials and methods

All the data presented here have been obtained on D_2O-hydrated powders of proteins, polypeptides and amino acids mixtures. Elastic neutron scattering experiments have been performed at the Institut Laue Langevin (ILL), Grenoble, France, using the spectrometers IN6, IN13 and IN16 with the following settings:

- IN6: incident wavelength 5.1 Å, resolution 70 μeV FWHM.
- IN13: incident wavelength 2.3 Å, resolution 8 μeV FWHM.
- IN16: incident wavelength 6.3 Å, resolution 0.9 μeV FWHM.

Raw data correction has been done using standard procedures available at ILL. The calorimetric data reported in fig. 7 have been obtained on the very same samples used for neutron scattering using a Pyris Diamond Differential Scanning Calorimetry apparatus from Perkin-Elmer.

For details on sample preparation, neutron scattering spectrometers used and data analysis, we refer the reader to refs. [9-16].

3. – Results and discussion

3˙1. *Elastic neutron scattering reveals anharmonic onsets in hydrated proteins.* – Figure 1 shows the typical temperature evolution of the mean squared displacements (msd) of hydrogen atoms in a protein powder. As described many times in the literature, like *e.g.* in ref. [17], in the limit of the momentum transfer $q \to 0$, the decay of the elastic scattering intensity can be described by a single Gaussian decay, where the decay rate is proportional to the msd of scattering nuclei. In particular we report the msd of a myoglobin powder at the hydration level $h = 0.5$ (gr D_2O)/(gr protein), which corresponds to complete coverage of the protein surface by hydration water, compared to the same protein powder in dry conditions, virtually $h = 0$ (it is actually known that a small number of water molecules remain attached to the protein even after prolonged drying under vacuum/high temperature). Data in fig. 1 show that around 150 K a first small but evident deviation from the harmonic regime occurs in both the dry sample and the hydrated one. Then, at higher temperature, between 200 and 250 K, an onset of large scale motions is revealed in the hydrated sample as a marked deviation from the msd of the dry sample, corresponding to the so-called protein dynamical transition. As already discussed in the introduction, the results shown in fig. 1 reveal that the presence of water is necessary to allow the onset of large scale fluctuations, while it does not influence at all the presence and the amplitude of the low temperature onset —yet no molecular attributions can be done on the basis of the msd of a protein.

3˙2. *Homomeric polypeptides allow to overcome the structural heterogeneity of proteins.* – In order to obtain direct experimental evidence of the molecular origin of the anharmonic onsets characterizing protein dynamics, we studied a set of relevant homomeric polypeptides having a number of residues comparable to that present in real proteins [9, 10]. This approach allows to overcome the intrinsic heterogeneity of protein sequences, since it enabled us to study amino acid chains where the measurement of the dynamic behavior of each type of residue can be isolated. This made it possible to assess the contribution of each type of side-chain to the anharmonic onsets revealed in fig. 1 and hence to identify their molecular origin.

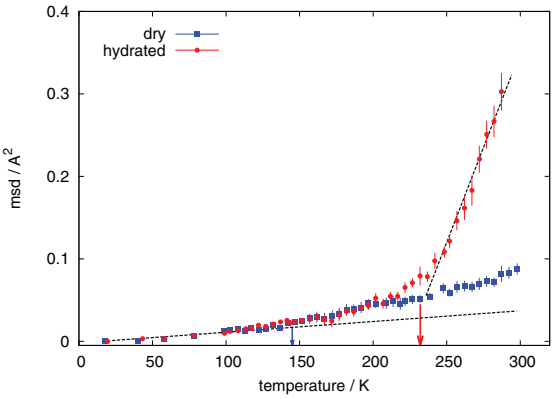

Fig. 1. – Mean squared displacements of myoglobin in a D_2O-hydrated (red circles) and a dry (blue squares) state, measured by elastic neutron scattering on the backscattering spectrometer IN13 (8 μeV resolution, ILL, Grenoble). Experimental error bars are indicated as vertical lines. Blue and red arrows indicate the temperature regions of the two main anharmonic onsets.

Among the 20 amino acids forming native proteins, we selected six different ones to form homomeric polypeptides of about 100 residues/chain, which we list in the following together with the rationale of the choice:

- poly-glycine (poly-gly): only α-carbon hydrogens, "pure backbone" contribution;
- poly-alanine (poly-ala): side-chain composed of a single methyl group, methyl groups contribution to anharmonic dynamics;
- poly-isoleucine (poly-ile): more complex methyl-group–containing side-chain;
- poly-lysine (poly-lys): four methylene groups and a charged amino terminus, essentially methylene contribution;
- poly-phenylalanine (poly-phe): benzyl side-chain not containing methyl groups, contribution of aromatic side-chains to anharmonicity;
- poly-proline (poly-pro): methylene side-chain with the terminus bound to the N atom of the backbone.

In all cases the presence of secondary structure elements similar to those of a protein (myoglobin) powder was checked with FTIR spectroscopy [9], in order to confirm that our homomeric polypeptides could indeed serve as good model systems.

The msd relative to D_2O-hydrated ($h = 0.2$) powders of the six samples listed above are shown in fig. 2. Data clearly reveal that poly-gly, poly-lys and poly-pro have a linear behaviour up to the protein dynamical transition temperature, while poly-ala and poly-ile deviate from linear dependence much earlier, at about 150 K, as observed also in Mb (see fig. 1). In the case of poly-phe, close inspection of the data reveals a slight departure from linearity occurring at about 170 K. These results give direct experimental evidence that a) methyl groups largely contribute to the low temperature anharmonic onset (with a small contribution from phenyl ring motions) and b) protein backbone is involved in the protein dynamical transition (it is observed in poly-gly, where no side-chains are

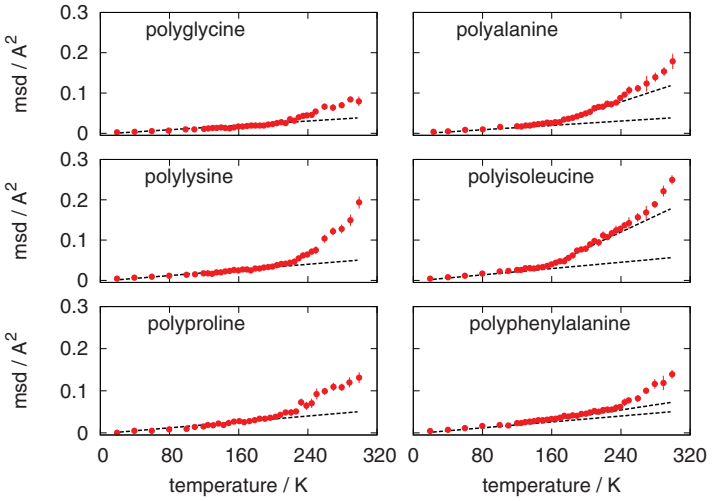

Fig. 2. – Mean squared displacements of non-exchangeable H atoms as a function of temperature. Dashed lines are linear fits in the suitable temperature regions.

present), with the presence of extended side-chains enhancing significantly the amplitude of measured msd (poly-lys and poly-pro).

3˙3. *Hydration dependence reveals different water-backbone and water–side-chains interactions.* – The results presented in the preceding section show that the protein dynamical transition occurs even in the absence of side-chains (poly-gly) and that adding a single methyl group as side-chain (poly-ala) is enough to qualitatively reproduce the anharmonic onsets characterizing the dynamical behavior of a real protein. Poly-gly and poly-ala then represent two minimal model systems to study the mean dynamics in the absence and in the presence of side-chains, respectively. We used this opportunity to study the effect of the hydration level, directly related to the average number of water molecules on the surface of proteins, on the dynamics of backbone and side-chains [10]. In fig. 3 we show the msd of poly-gly and poly-ala as a function of the hydration level h in the range from $h = 0$ to $h = 0.35$. In globular proteins, this hydration range largely affects the size of internal motions. Data in fig. 3 reveal that:

- hydration water triggers the anharmonic dynamics associated to the protein dynamical transition in protein backbone (poly-gly); however, increasing the hydration level does not enhance the amplitude of protein fluctuations;
- as already mentioned, the low temperature onset, largely due to methyl groups, does not need hydration water to occur (dry poly-ala) and is independent of the presence of hydration water; however the size of fluctuations related to the protein dynamical transition is strongly affected by the hydration level (hydrated poly-ala).

The results reported in fig. 3 show that the effect of water in promoting and sustaining anharmonic dynamics is different depending on the class of motions involved and depends on the structural complexity of peptide chains. Both the onset temperature and size of the local dynamics of methyl side-chains is independent of the presence and amount of hydration water. Concerning the anharmonic fluctuations involved in the dynamical

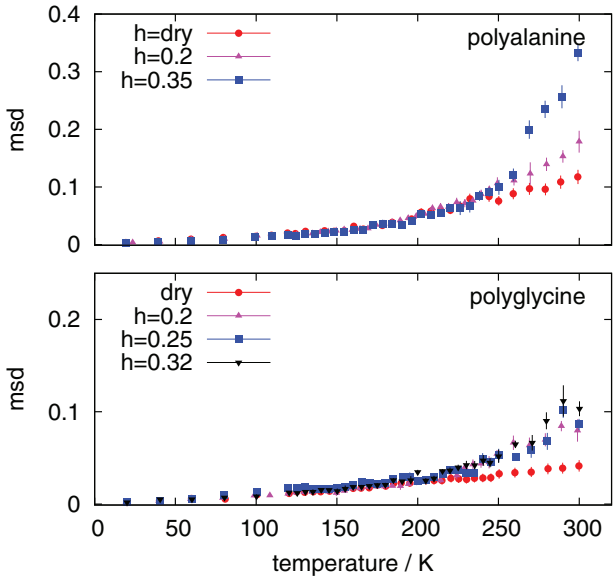

Fig. 3. – Comparison of mean squared displacements in polyalanine (upper panel) and polyglycine (lower panel) at different hydration levels.

transition, both poly-gly and poly-ala data confirm that there is a minimum hydration level ($h \geq 0.2$) which is needed to activate protein large scale fluctuations. When only the backbone is involved, probably due to the limited conformational freedom determined by the geometrical constraints of the main chain, the size of these fluctuations does not increase by increasing the hydration above this threshold; on the contrary, the presence of side-chains increases the number of conformational degrees of freedom and this in turn determines a strong hydration dependence.

3˙4. *Resolution-dependent neutron scattering reveals the physical nature of anharmonic onsets.* – The use of a single resolution-limited neutron spectrometer makes it impossible to discriminate between a real thermodynamic transition and a thermally activated motion whose temperature-dependent relaxation time crosses the instrumental time-scale. The way to clarify this question is to explore the resolution dependence of the onset temperature and amplitude of the detected anharmonic msd [11, 12]. Indeed, gaining information on the resolution dependence allows to describe the topology of the protein energy landscape, that determines the equilibrium protein dynamics observed by neutron scattering. Previous neutron scattering studies [18, 19] on the resolution dependence of the protein dynamical transition temperature are controversial in that they concern samples where the solvent signal is non-negligible [20] or they are poorly supported by clear experimental evidences. In fact, experimental problems come from the presence in proteins of multiple anharmonic activations, often partially overlapping, and from the ambiguity in defining the onset temperatures whose identification thus heavily depends on the researcher's eye. One therefore needs: a) systems in which the anharmonic activations are clearly disentangled and b) a clear operative definition of the onset temperatures.

We think that our homomeric polypeptides at various hydrations are suitable systems fulfilling the above two conditions. In fact, dry poly-ala shows only the low temperature

onset, while hydrated poly-gly undergoes only the protein dynamical transition, thus giving us the possibility of studying the resolution dependence of the two transitions, separately, avoiding their superposition. We also introduced a clear operative definition of the onset temperatures. To identify the onset temperature of methyl groups anharmonic dynamics in dry poly-ala, we first subtracted from the measured msd the harmonic contribution estimated by extrapolation from the linear low temperature trend; the so obtained msd were normalized to their room temperature value. We used an analogous procedure to define the onset temperature of protein dynamical transition: we first subtracted from the msd relative to the poly-gly hydrated sample those relative to the dry poly-gly sample, where the transition does not occur (note that msd of dry and hydrated samples are identical up to the protein dynamical transition onset); the so obtained msd were normalized to their room temperature value. It is straightforward to see that any deviation from zero of the normalized msd reveals the methyl groups onset temperature in dry poly-ala and the protein dynamical transition temperature in hydrated poly-gly; moreover, comparison of data obtained with different spectrometers is no more influenced by the resolution dependence of msd amplitude.

The results of the procedure described above applied on data measured on three different spectrometers (IN6, IN13 and IN16 at ILL, spanning over two orders of magnitude in energy/time-scale) are shown in fig. 4. Results clearly reveal that deviations from the harmonic trend in dry poly-ala (upper panel) occur at different temperatures: 110 K at IN16, 150 K at IN13, 180 K at IN6, while there is no evidence (bottom panel) of a dependence of the protein dynamical transition temperature on the energy resolution. In order to confirm the validity of homomeric polypeptides as model systems for proteins, we verified that the same conclusions hold also for a real protein, as suggested in fig. 5 by the temperature dependence of the msd of a D_2O-hydrated native globular protein,

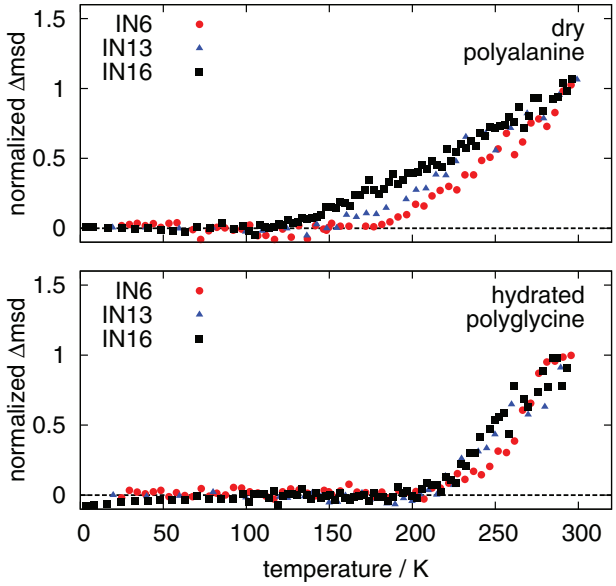

Fig. 4. – Upper panel: normalized Δmsd (dry-harmonic) in dry polyalanine. Lower panel: normalized Δmsd (hydrated-dry) in hydrated polyglycine.

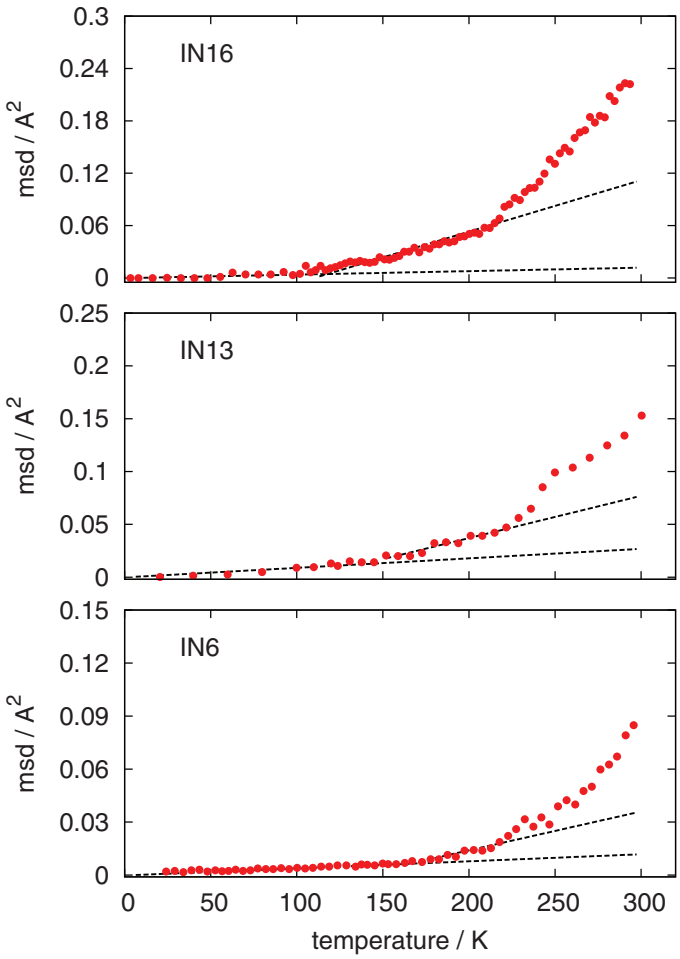

Fig. 5. – Mean squared displacements of D_2O-hydrated bovine serum albumin measured at IN16 (upper panel), IN13 (middle panel), and IN6 (lower panel).

i.e. bovine serum albumin, measured with the same energy resolutions at IN16, IN13, and IN6 [11].

A quantitative analysis of the data reported in figs. 4 and 5 [11, 12] revealed that both anharmonic onsets can be interpreted with similar de-trapping mechanisms, but with large differences in the parameters of the energy landscape. In particular, the protein dynamical transition reflects a transition between two main states with a large equilibrium free energy difference and a mean energy barrier of the order of 20 kJ/mol. Interestingly, the protein dynamical transition shares the same temperature region with the so-called water liquid-liquid crossover, proposed to occur in supercooled water, like *e.g.* protein hydration water [3, 21]. Moreover, a simplified model proposed to explain the polymorphism induced by the liquid-liquid crossover considers the existence of a two-wells interaction potential [22], analogous to that used to explain our data, with an energy barrier set by the energy necessary for hydrogen bond breaking, estimated to be about 20 kJ/mol [21], then compatible with our estimation for the protein dynamical transition.

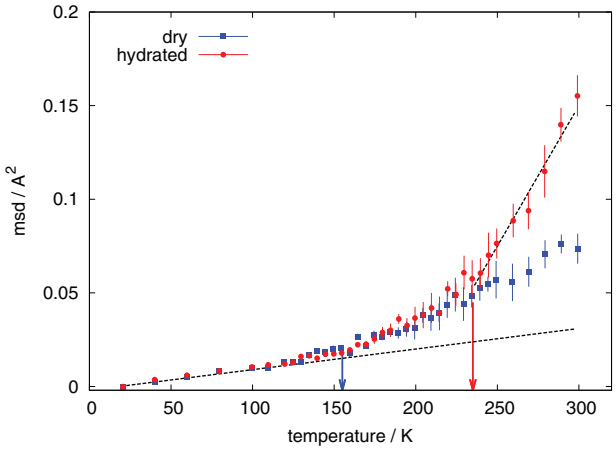

Fig. 6. – Mean squared displacements of the amino acid mixture in a D_2O-hydrated (red circles) and a dry (blue squares) state, measured on the backscattering spectrometer IN13 ($8\,\mu$eV resolution, ILL, Grenoble). Experimental error bars are indicated as vertical lines. Blue and red arrows indicate the temperature regions of the two main anharmonic onsets.

The analogy in the onset temperature and in the shape parameters of the energy profile is suggestive of a direct connection between the structural relaxation occurring in the protein and the dynamical/structural crossover of the water matrix (see subsect. **3**˙6 below).

3˙5. *The protein dynamical transition does not require the polypeptide chain.* – It is evident from the data presented in the previous sections that both backbone and side-chains contribute to the protein dynamical transition. However, while the existence of a transition in poly-gly demonstrates that its occurrence does not require the presence of side-chains, a question remained unaddressed is whether or not the dynamical transition can be observed even in the absence of backbone and, if so, what are the backbone and side-chains relative contributions to protein dynamics. Moreover, the fact that we observed the dynamical transition in non-biological polypeptides questions its relevance and direct connection to the onset of biological function. We addressed these open questions by studying with elastic neutron scattering the dynamics of a mixture of amino acids powders, with a molecular composition chosen so as to reproduce the stoichiometry of the real protein myoglobin, in both dry and hydrated ($h = 0.2$) conditions [13].

The msd of the amino acids mixture are shown in fig. 6, obtained by elastic neutron scattering experiments performed at IN13 with a resolution of $8\,\mu$eV. If compared with data of myoglobin in fig. 1, data in fig. 6 show that the dependence on temperature and hydration is qualitatively the same in the two different systems, with methyl groups onset and protein dynamical transition occurring in the mixture at the same temperatures as for the protein. As far as the msd amplitudes are concerned, a direct comparison between the two samples at the same hydration levels is reported in ref. [13] and shows that msd relative to myoglobin are larger than those of the mixture in the anharmonic region with a difference of $0.04\,\text{Å}^2$ at room temperature, which corresponds to the anharmonic contribution to msd measured in hydrated poly-gly at room temperature (see fig. 2 in ref. [13]). On the basis of the results obtained on the amino acids mixture, we

can conclude that the protein dynamical transition is relevant for the functional protein dynamics but that there is no direct equivalence between occurrence of the transition and protein biologically active state. Moreover, the fact that the same dynamical transition temperature is observed in structurally different *hydrated* systems such as poly-gly (pure backbone contribution), amino acid mixture (pure side-chains contribution), and myoglobin (backbone and side-chains contributions) strongly suggests that the origin of the transition must be searched not in the details of the polypeptide chain dynamics but in the thermodynamics and dynamics of hydration water.

3˙6. *The protein dynamical transition vs. the hydration water liquid-liquid crossover*. – In order to find experimental evidence of the connection between the protein dynamical transition and hydration water thermodynamics, we compared our data obtained on hydrated myoglobin using elastic neutron scattering and differential scanning calorimetry (DSC) [14, 15] with data on the dynamics of hydration water of lysozyme obtained by S.-H. Chen and co-workers [3] using quasi-elastic neutron scattering (fig. 7). In the case of myoglobin we investigated two different hydration levels: $h = 0.3$, which corresponds approximately to a single water layer on the protein surface and $h = 0.5$, where a fraction of hydration water does not interact directly with the protein surface. In fig. 7 (upper panel) we report the msd obtained with elastic neutron scattering, while the middle panel shows the DSC results obtained in the temperature range 100–300 K. The thermodynamic scenario obtained from DSC data reveals first that no transitions occur in the dry sample, indicating that the features observed in the hydrated samples originate from hydration water. In particular, hydration water undergoes a glass transition at about 170 K. Around this temperature glassy water becomes liquid, then at about 230 K it shows a first-order-like transition toward another liquid state. An analogous observation has been made on supercooled water confined in Vycor porous matrices, where neutron diffraction data identified a similar transition as a change in water density. An endothermic peak appears at higher temperature (about 280 K) in the sample at $h = 0.5$, revealing the melting of D_2O icy molecules out of the first hydration shell.

The lower panel of fig. 7 reports the temperature dependence of the average translational relaxation times of the hydration water of lysozyme ($h = 0.3$) obtained by S.-H. Chen and co-workers using quasi-elastic neutron scattering [3]. In these data a clear dynamic crossover occurring at about 220 K is evident, from a super-Arrhenius (fragile) behaviour at high temperatures to an Arrhenius (strong) behaviour at low temperatures. This Fragile-Strong Crossover (FSC) in confined supercooled water is attributed to the crossing of the so-called Widom line, originating from the existence of the second critical point of water [23, 24].

Comparison of the various data reported in fig. 7 provides a clear description of the protein dynamical transition. Protein hydration water, after the glass transition at about 170 K, experiences a liquid-liquid crossover at about 230 K, that we can identify with the liquid-liquid crossover observed in various supercooled water conditions [21, 25, 26], and in lysozyme hydration water [3]. As already reported, this crossover is related to a change from a low temperature locally fully tetrahedrally H-bonded strong water to a fragile water, characterized by structural and dynamical properties similar to supercooled bulk water at higher temperature. This change makes more fluid water at temperature above about 230 K. Water molecules in the protein hydration shell, tightly coupled with protein molecular groups on protein surface via hydrogen bonds and electrostatic interactions, induce then an increase of protein atomic fluctuations, revealed by elastic neutron scattering as the protein dynamical transition.

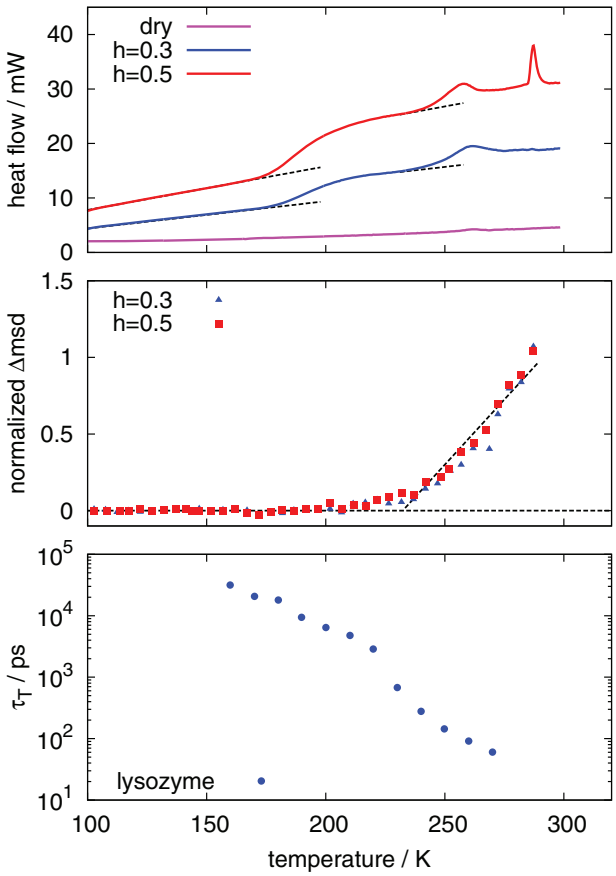

Fig. 7. – Experimental data obtained on D_2O-hydrated protein powders. From top to bottom: differential scanning calorimetry up-scans on myoglobin; mean square displacements of non-exchangeable H atoms of myoglobin obtained by elastic neutron scattering; temperature dependence of the average translational relaxation times of lysozyme hydration water, taken from ref. [3].

4. – Conclusions

From the reported results the following scenario emerges: The equilibrium dynamics of proteins (*e.g.* the equilibrium mean squared displacements measured by elastic neutron scattering) is fully harmonic at low —cryogenic— temperatures. By increasing the temperature, two anharmonic onsets are possible: the first one is observed in the temperature range 110–180 K (depending on instrumental resolution), is independent of hydration, and is essentially due to the activation of methyl groups rotations; the second one, named protein dynamical transition, is observed only in hydrated samples (typically $h > 0.2$) at temperatures around 230 K, is independent of the instrumental resolution, and can be attributed to anharmonic motions involving —in proteins— both the backbone and the amino acids side-chains. Surprisingly, the dynamical transition does not need the presence of a backbone and can be observed also in an amino acid mixture lacking of peptide bonds.

The use of homomeric polypeptides as protein model systems enables not only to assign the molecular origin of the two anharmonic onsets but also to characterize their

energetics. In fact, both onsets can be interpreted with similar de-trapping mechanisms, but with large energetic differences. The low temperature onset is due to a *relaxation* process between states energetically close (*i.e.*, of a low energy landscape tier), and without any coupling with the surrounding water matrix; it is a thermally activated motion between sites with small equilibrium energy differences and with energy barriers and jump distances characteristic of methyl groups rotations. On the other hand, the protein dynamical transition reflects a *transition* between states with a large equilibrium free energy difference (*i.e.*, of a higher energy landscape tier) and sustained by the presence of hydration water.

Comparison of elastic neutron scattering data on the protein dynamical transition with calorimetric data and with dynamic data on deeply cooled confined water (including protein hydration water) enables to suggest that the low-density–to-high-density crossover occurring in the protein hydration water at about 230 K involves changes in the H-bond network dynamics that are coupled to protein internal motions, thus inducing the increased backbone and side-chains fluctuations revealed by elastic neutron scattering as the dynamical transition.

* * *

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