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Anharmonic onsets in polypeptides revealed by neutron scattering: Experimental evidences and quantitative description of energy resolution dependence

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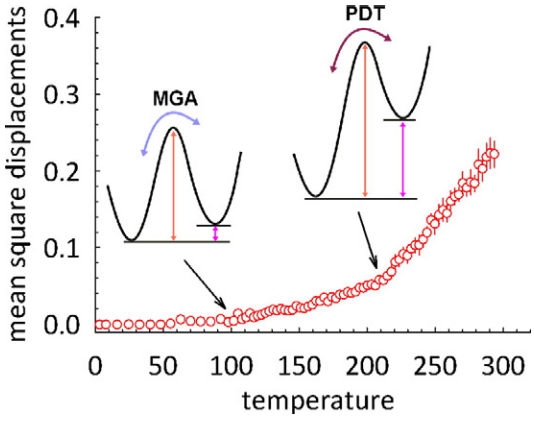
HIGHLIGHTS

- Homopeptides as model systems to catch the contributors to anharmonic dynamics
- Experimental evidence on the resolution dependence of protein anharmonic activations
- Relation between protein dynamical transition and liquid–liquid water transition

GRAPHICAL ABSTRACT



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ABSTRACT

Neutron scattering measurements on protein powders reveal two deviations from harmonic dynamics at low temperature, whose molecular origin, physical nature and biological relevance are still matter of discussion. In this study we present a new experimental and theoretical approach to evidence the resolution dependence of anharmonic onsets: the use of strategically selected homomeric polypeptides allows revealing the exact resolution dependence; a two-site energy landscape model, where resolution effects are explicitly taken into account, is able to interpret quantitatively the experimental data in terms of energy landscape parameters. The energetic description provided by this analysis, together with recent experimental evidences obtained on chemically and structurally different peptide systems, allows us to connect the protein/water energy landscape structure with the two-wells water interaction potential proposed to explain the low-density → high-density liquid–liquid transition observed in supercooled water.

1. Introduction

Neutron scattering techniques applied to biological macromolecules give information on their dynamics and energetics. Indeed, the neutron scattering function, via the time–space correlation function, is related to the shape of the energy landscape of the protein/water system. In particular, from the elastic scattering signal of D_2O-hydrated protein powders it is possible to get an estimation of the mean square displacements (MSDs) of protein non-exchangeable H atoms. The temperature dependence of MSDs is a suitable tool to reveal the occurrence of anharmonic

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dynamics, which corresponds to the capability of polypeptide chains to explore different conformational substates by thermal atomic fluctuations. Taking into account this kind of motions is essential to understand how proteins accomplish their function. A typical example is the mechanism of oxygen binding to myoglobin: in the 3D structure obtained by X-ray diffraction on a myoglobin crystal no static path for the entry of the ligand exists, so that only structural fluctuations around the equilibrium structure (which corresponds to the structure "frozen" in the crystalline state) can allow the access to the active site. This dynamical performance is due to the fluctuations of side chains and backbone occurring in the picosecond/nanosecond time scale, i.e. exactly the time scale typically accessed by neutron scattering.

The biochemical audience used to investigate in vivo biological samples or, at least, protein aqueous solutions, could argue against the biological relevance of studying freeze-dried protein powders at a low hydration level. However, the rationale of looking at such systems is related to the possibility of exploiting the neutron scattering sensitivity to the relevant time/space scale of protein motions. Indeed, the hydration level in a D_2O-hydrated protein powder is sufficiently low as to guarantee that the scattering signal is safely attributable to the incoherent signal of non-exchangeable H atoms of protein whereas the D_2O signal is negligible. At the same time, it is high enough to allow the activation of the functionally relevant large scale protein fluctuations. In this sense, hydrated protein powders are used as model systems to simplify the neutron scattering signal and to catch relevant contributions to protein dynamics which are otherwise obscured in a diluted sample.

The inspection of the MSD temperature dependence reveals two deviations from harmonic dynamics, at 100–150 K and at about 220 K [1,2]. The first deviation has been ascribed mainly to thermally activated motions of CH_3 methyl groups [1,3–6] and, for this reason, in this paper will be called "methyl groups activation" (MGA). Concerning the second deviation, it is the well known "protein dynamical transition" (PDT), which has been the matter of discussion in the biophysical community during the last twenty years, since the publication of a seminal work by W. Doster and co-workers [2]. The biological relevance of the protein large scale motions activated at the PDT temperature is well established, however a general one-to-one correspondence between PDT onset and biological activity onset, proposed for a long time, has been later questioned. Indeed, the counterexamples of residual enzymatic activity at temperatures below the PDT have been reported [7] while the PDT has been observed also in denatured protein samples [8]. Concerning the physical mechanism giving rise to the PDT, several hypotheses have been proposed to explain it:

1. a change in the thermodynamic resilience of the water–protein coupled system [9];
2. the protein response to a liquid–liquid transition from a high density to a low density form occurring in the hydration water at about 220 K [10];
3. an abrupt change of protein structural flexibility following the glass transition of hydration water at about 170 K but detectable by neutron scattering at higher temperature [11];
4. a result of the temperature dependent protein structural relaxation time crossing the time scale determined by the experimental frequency window [12–14].

The above hypotheses propose physical origins for the PDT partially alternative, thus revealing that the biophysical community does not share a common picture of the phenomenon, yet. However, there is a clear experimental evidence that the PDT occurs only in the presence of a sufficient amount of hydration water: this suggests that water properties and protein–water interactions are involved in the transition. A way to discriminate among the different hypotheses listed above is to investigate whether and how the onset temperature and amplitude of the anharmonic fluctuations depend on the given time scale accessed by neutron spectrometers, which is set by the width of the instrumental energy resolution function [15,16]. The way how anharmonic activations depend on energy resolution is indeed related to the nature of the underlying energy surface of the system and then to the dynamic/thermodynamic event responsible for the observed activation: a PDT onset temperature independent of resolution is compatible with the hypotheses 1 and 2, while it would exclude 3 and 4, that assume an instrumental finite-resolution effect. In other words, changing energy resolution width is a tool to test the validity of the models proposed. In the literature there are some neutron scattering studies showing that the PDT onset temperature depends on energy resolution; however they are controversial because they are performed on systems where the solvent signal cannot be neglected but probably dominates the total scattering signal [17] or they are not sufficiently supported by clear experimental evidences [14]. Indeed, it is important to notice that experimental problems arise when determining the onset temperature: 1) the definition of MGA and PDT onset temperatures is not well established thus making their identification highly dependent on the researcher's eye (very recently, a new protocol to detect transitions in elastic neutron scattering data has been proposed [18]); 2) in hydrated systems both anharmonic activations occur and their partial overlapping can make hard to identify the onset temperature. Moreover, there are no investigations on the resolution dependence of anharmonic fluctuation amplitudes: comparing on the same scale data obtained with different spectrometers (and then with different MSD amplitudes) results in a flattening of the small amplitude data, which makes difficult to identify the activation temperatures.

In a recent Letter [19], we have presented experimental evidences on the resolution dependence of anharmonic MSDs, where the above experimental issues have been substantially overcome. We have also showed that a simple model of anharmonic free energy landscape, if properly improved to take into account explicitly the resolution effects on onset temperature and fluctuation amplitudes, is able to quantitatively and consistently explain the results in terms of the shape parameters of energy landscape. In the present work, I describe thoroughly the experimental details and the theoretical scheme of this approach, which are lacking in the Letter, with a full discussion on the relationship between energy landscape parameters and resolution effects on the detection of anharmonic activations. Moreover, I report the complete set of elastic neutron scattering data, that are used to test the model, and new experimental evidences by quasi-elastic neutron scattering, that further confirm the consistency of our analysis and results.

Our experimental strategy is aimed at overcoming the intrinsic protein heterogeneity, which makes difficult to identify the dynamical behavior of different side-chains and backbone from the overall protein scattering signal. To this purpose we used homomeric polypeptides, i.e. peptide chains with one type of side-chain but a number of residues similar to that of functional proteins. In particular, we studied poly-glycine (poly-gly) and poly-alanine (poly-ala) in the dry state (where PDT does not occur) and at a hydration level ($h = 0.2$ g D_2O/g poly-amino acid) low enough to get a signal attributable to protein non-exchangeable H atoms with a negligible D_2O contribution [20], but high enough to allow the PDT. We have previously shown [3,5] that hydrated poly-gly undergoes only the PDT (no CH_3 is present) while dry poly-ala shows only the MGA. This evidence allows us to investigate the resolution dependence of the two transitions avoiding their superposition. Since the aim of our approach is to give information on the protein dynamical behavior, the results obtained with these systems have been compared with analogous results obtained with a representative globular protein, bovine serum albumin (BSA). The energy resolution dependence is investigated by using three different spectrometers at the Institut Laue-Langevin in Grenoble (France): IN16 (energy resolution FWHM: 0.9 μeV), IN13 (8 μeV) and IN6 (70 μeV). The energy resolution range spanned by the three spectrometers approximatively corresponds to the 100 ps–10 ns time range.

2. Materials and methods

2.1. Sample preparation

Poly-gly and poly-ala powders were purchased from Sigma-Aldrich (St. Louis, MO); they were obtained by direct lyophilization from water solution (poly-ala) or filtration from acidic solution followed by several water washes (Sigma-Aldrich Technical Service, private communication). The powders were dried under vacuum and held in a D_2O atmosphere to reach the desired hydration level. This procedure was repeated several times to guarantee that most of the exchangeable H atoms had been replaced by D atoms. The final hydration level ($h = 0.2$ g of D_2O/g of poly-amino acid) was determined by measuring the mass change.

Bovine serum albumin (BSA) powder sample was purchased from Sigma-Aldrich (St. Louis, MO). A treatment analogous to that described above for polypeptides was applied to obtain a sample at $h = 0.2$ hydration level.

2.2. Neutron scattering measurements: data collection and analysis

Elastic neutron scattering measurements as a function of temperature T and momentum transfer Q were performed at the Institut Laue Langevin (Grenoble, France) in the back-scattering spectrometers IN13 ($\lambda_i = 2.23$ Å; energy resolution $\Delta E = 8$ μeV FWHM) and IN16 (incident wavelength $\lambda_i = 6.27$ Å; energy resolution $\Delta E = 0.9$ μeV FWHM) and in the time-focusing time-of-flight spectrometer IN6 (incident wavelength $\lambda_i = 5.12$ Å; energy resolution $\Delta E = 70$ μeV FWHM). The elastically scattered intensity $S(Q,T,E = 0)$ was corrected for the contribution of the empty cell and normalized to the lowest temperature data in order to compensate for spurious background and detector efficiency.

Flat aluminum sample holders were used, and a sample thickness of 0.3 mm (transmission ~ 90%) was chosen in order to avoid corrections for multiple scattering. Neutrons scattered incoherently by non-exchangeable hydrogen atoms of D_2O-hydrated polypeptides dominate the total scattered intensity, allowing us to assume that we are probing the self-dynamics of these atoms.

To calculate the mean square displacements (MSDs) from the elastic scattered intensity $S(Q,T,E = 0)$, according to the Gaussian approximation, we used the following definition:

$$-\ln\left[\frac{S(Q,T,E=0)}{S(Q,T_m,E=0)}\right] = \frac{\left\langle u^2\right\rangle(T) - \left\langle u^2\right\rangle(T_m)}{6}Q^2 = \frac{\left\langle \Delta u^2\right\rangle(T)}{6}Q^2 \qquad (1)$$

where T_m is the lowest temperature and, assuming that the sample is isotropic, $\frac{\langle \Delta u^2\rangle(T)}{6} = \frac{\langle \Delta x^2\rangle(T)}{2}$, that is our definition of MSD. In practice, MSD is determined by fitting the Q^2 dependence of $-\ln\left[\frac{S(Q,T,E=0)}{S(Q,T_m,E=0)}\right]$ with a straight line over a suitable Q^2 range and checking a posteriori that the condition $Q^2\langle \Delta u^2\rangle \leq 2$, necessary to verify the validity of Gaussian approximation, is obeyed. The temperature dependence of the complete set of MSD of poly-gly and poly-ala measured at IN16, IN13 and IN6 is reported in Fig. 1.

In order to determine the anharmonic onset temperatures, we used the following strategies for MGA and PDT:

- MGA onset temperature: we calculated the reduced χ^2 fitting the MSD vs. T with a linear dependence (expected for harmonic dynamics) from the lowest temperature up to a given temperature T_f. A sharp increase of this quantity indicates the harmonic-to-anharmonic transition temperature.
- PDT onset temperature: the quantity $\Delta MSD = MSD_{hydrated} - MSD_{dry}$ was first obtained by subtracting from MSD relative to hydrated poly-gly the MSD relative to the corresponding dry sample [21], where PDT does not occur. MSD values relative to dry and hydrated samples are identical up to the PDT onset (see Fig. 1). ΔMSD was then normalized to the room temperature value. In order to minimize systematic errors arising from normalization to a single experimental value, the "room temperature value" is obtained by extrapolation from the MSD temperature dependence in the entire temperature region above the PDT. It is clear that a deviation from zero of the normalized ΔMSD reveals the PDT onset temperatures in hydrated poly-gly. Moreover, after normalization to room temperature, the comparison of data obtained with different spectrometers is no more dependent on the MSD amplitude.

2.3. Neutron scattering measurements: theoretical model and analysis

A quantitative description of the MSD temperature dependence can be obtained with a model of anharmonicity already used by W. Doster and co-workers to describe anharmonic activations in proteins [2]. In this model, the appearance of anharmonic dynamics is described as a jumping motion of protein hydrogen atoms between two sites, separated by a distance *d*, corresponding to two minima in the energy landscape, each characterized by a different equilibrium free energy (see Fig. 2A). The scattering function S(E,Q,T) can be derived with a semi-classical treatment, as reported in [22]. The main parameters describing this model are the free energy difference ΔG between the two sites at distance *d*, the activation barrier ΔG^* is for the jumping from the site at lower energy to the other (the motion in the opposite direction, with activation energy ΔG^* can be also taken into account, as shown in Ref. [19]). For the sake of simplicity, here we assume that free energies are independent of temperature. The scattering function obtained with this model is:

$$S(E,Q,T) = e^{-Q^2u_v^2}\left(S_0\delta(E) + S_1\frac{\frac{\tau_0}{\pi}\frac{e^{\frac{\Delta G^*}{RT}}}{1+e^{\frac{\Delta G}{RT}}}}{1+\frac{(E\tau_0)^2}{\hbar^2}\frac{e^{\frac{2\Delta G^*}{RT}}}{\left(1+e^{\frac{\Delta G}{RT}}\right)^2}}\right) \qquad (2)$$

where:

$$S_0 = 1 - 2\frac{e^{-\frac{\Delta G}{RT}}}{\left(1+e^{-\frac{\Delta G}{RT}}\right)^2}\left(1-\frac{\sin(Qd)}{Qd}\right) \qquad (3)$$

is the so called Elastic Incoherent Structure Factor (EISF) and $S_1 = 1 - S_0$. u_v^2 is the temperature dependent mean harmonic vibrational term.

To take into account the finite resolution of the spectrometers used, the scattering function must be convoluted with the energy resolution function R(E;ΔE), typical of a spectrometer with ΔE resolution:

$$S_{model}(E,Q,T) = S(E,Q,T)\otimes R(E;\Delta E) \qquad (4)$$

R(E;ΔE) can be obtained measuring the neutron scattered intensity from the sample at low temperature, where the signal is totally elastic; the symbol ⊗ is the convolution operator in the energy domain. The theoretical MSD_{model} can be obtained, according to the two-site model, using the scattering function defined in Eq. (4). The same procedure described above to obtain the MSD from the experimental data has been applied to calculate the MSD_{model}: it is defined as the slope of $-\ln[S(E = 0, Q, T)]$ vs. Q^2 in the low Q region accessed by each spectrometer used in this work. In order to demonstrate the consistency of our analysis, we show in Fig. 3 a comparison between $S(E = 0,Q,T)$ theoretical curves and experimental data at a representative temperature (analogous results are obtained at all the temperatures explored), in the entire Q range accessed by IN13. At IN13, indeed, the Q range is larger than in the other two spectrometers and, as a consequence, the comparison is more significant. Fig. 3 shows that a good agreement between experimental data and theoretical curves is obtained in the entire Q-range.

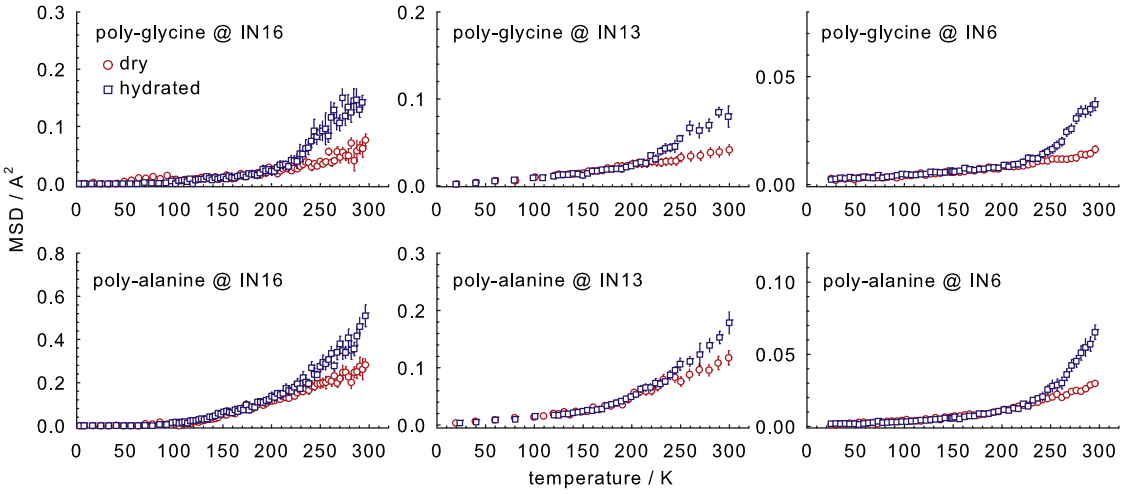

Fig. 1. MSD temperature dependence for dry and D_2O-hydrated poly-gly (top) and poly-ala (bottom) measured at IN16, IN13 and IN6.

As shown in Fig. 4, the elastic intensity counted in the "elastic channel" contains also the quasi-elastic signal falling under the resolution function. This implies that a decay with temperature of the elastic signal can be due to the decrease of the elastic peak and/or to the broadening of quasielastic peak. The first contribution does not depend on ΔE, the width of the resolution function, while the second does. As shown in Fig. 5, in the two-site model modified to consider the effects of energy resolution, the main parameter describing how the anharmonic activation onset temperature may change or not with the resolution is the ratio $\rho = \Delta G^*/\Delta G$. Indeed:

1. for $\rho \gg 1$, the appearance of an activation in the elastic intensity decay is mainly due to the (temperature dependent) broadening of quasielastic width compared to the (temperature independent) resolution width; in this case (see Fig. 5) the onset temperature markedly depends on resolution function.
2. for $\rho \sim 1$, the anharmonic activation corresponds to a pronounced depletion of the elastic peak or, in other words, to the thermal population of the high energy site; in this case (see Fig. 5) the onset temperature does not depend on resolution function.

While this model can easily explain the resolution dependence/independence of onset temperature, it cannot take into account the dependence of MSD amplitude on resolution function, which is commonly observed (see Fig. 1). This is mainly due to the simplistic assumption of an energy landscape made by only two energy wells. It is well known, indeed, that proteins are characterized by a complex multi-minima anharmonic landscape that proteins explore via a Brownian-like motion within the conformational space [23]. Here we propose a euristic way for taking into account this aspect (see Fig. 2B): we define the distance d between the two sites as the average length scale explored by protein H atoms in the time scale $\tau = h/\Delta E$ imposed by the resolution ΔE of the neutron spectrometer. We introduce the relation $d = \phi\tau^{\lambda}$; the parameter ϕ is related to the diffusion coefficient describing the diffusive behavior of H atoms, while $\lambda \leq 0.5$ introduces the possibility of a sub-diffusive character, which is generally observed in polymeric systems [24]. The two-site model described above is a good approximation of the real physical system if the MSD obtained from experimental S(E = 0,Q,T) and relative to the three different spectrometers can be reproduced by the MSD_{model} with the same parameters but ΔE.

3. Results and discussion

The reduced χ^2 values relative to dry poly-ala as a function of T_f and obtained with the three spectrometers (Fig. 6A) show that deviations from the harmonic trend occur at different temperatures: ~100 K at IN16, ~150 K at IN13, and ~180 K at IN6. The ΔMSD values obtained for hydrated poly-gly as a function of temperature are reported in Fig. 6B and show that inside a region of ~20 K, i.e. 215 ± 10 K there is no evidence of any PDT onset temperature dependence on the energy resolution. From the results in Fig. 6, we conclude that, within the resolution range here explored, the MGA onset temperature markedly depends on energy resolution while the one of PDT does not. The same conclusions seem to hold also for a functional protein, as suggested in Fig. 7 by the temperature dependence of MSDs measured on a sample of D_2O-hydrated (h = 0.2) BSA at IN16, IN13 and IN6.

In Fig. 7 we report the MSDs of dry poly-ala (panel A) and of hydrated poly-gly (panel B), together with the fittings in terms of the

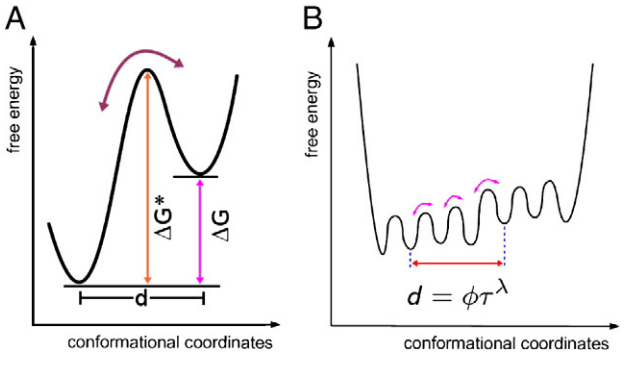

Fig. 2. Structure of the two-site energy model (panel A) and schematic representation of the diffusive-like motion through the multiminima protein energy landscape (panel B).

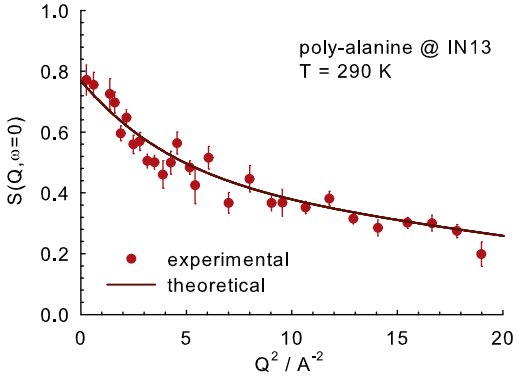

Fig. 3. Example of the consistency between S(E = 0,Q,T) theoretical curves and experimental data for the dry poly-alanine sample measured at the IN13 spectrometer (T = 290 K).

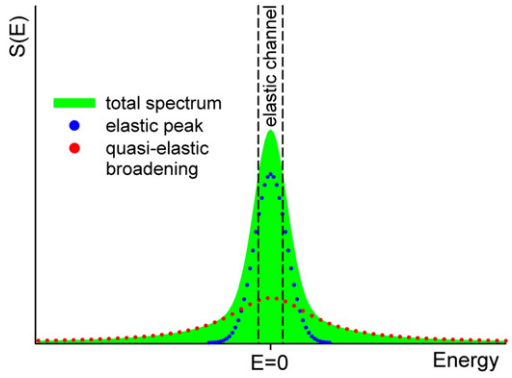

Fig. 4. Typical shape of an experimental neutron scattering spectrum around the elastic channel.

model described in the previous paragraph, while the obtained parameters are reported in Table 1. As already mentioned, in dry poly-ala and hydrated poly-gly only the MGA and the PDT are present, respectively. Fig. 7 shows that the two-site model, modified as described above, is able to reproduce the entire temperature evolution of MSD, from cryogenic to room temperatures, including the resolution dependence of anharmonic transitions onset temperatures and MSD amplitudes. In Fig. 8 we show the quasielastic spectra measured at IN16 on dry poly-ala (upper panel) and wet poly-gly (lower panel) at 5 K and 300 K, summed up over the explored Q range to improve the signal to noise ratio. It is evident from the raw data that the spectrum relative to dry poly-ala at room temperature is characterized by both a decrease of elastic peak and a change in the shape of quasielastic tails if compared to the low temperature spectrum (as suggested by the slope of tails indicated with dashed lines in the plot); in the case of wet poly-gly only a decrease of the elastic peak is observed (and a consequent increase of quasielastic intensity), while the shape of quasielastic tails is only barely affected by the increased temperature. These experimental evidences from quasielastic data are in line with the results presented above and obtained with our analysis of the elastic data: the MGA onset is a finite resolution effect while the PDT is due to a decrease of the EISF and then it is independent of the resolution width (cfr. the discussion relative to Fig. 4 in Section 2).

Interesting information on the mobility of H atoms can be obtained from the parameters reported in Table 1: 1) the parameters ϕ and λ, which define the dependence of the jump distance d on the experimentally accessible time τ, are different in the different samples; this implies that different structures and hydration conditions influence the diffusive properties of H atoms; 2) the values of $\lambda < 0.5$ indicate a subdiffusive character, compatible with that observed in polymers [25]; 3) these results reveal that the dependence of PDT-related fluctuation amplitudes on the hydration level, usually observed in proteins and polypeptides [21,5], can be explained with a change in the diffusive properties as a function of the water content on the polypeptide surface.

In more general cases like hydrated homomeric polypeptides containing methyl groups (e.g. poly-ala) and hydrated proteins (e.g. BSA), methyl group rotations and PDT-related fluctuations are superimposed or, in other words, both MGA and PDT are observed by neutron scattering. Therefore, the structure factor relative to these systems can be obtained from the convolution of the $S^{MGA}_{model}(E = 0, Q, T)$ and $S^{PDT}_{model}(E = 0, Q, T)$ calculated using the parameters obtained for dry poly-ala and hydrated poly-gly, respectively. Results are reported in Fig. 7C (hydrated poly-ala) and Fig. 7D, E, and F (BSA). Note that for BSA slightly different diffusion parameters are obtained ($\lambda = 0.37$ for MGA and $\lambda = 0.21$ for PDT), as expected in line with the above considerations. The very good agreement between experimental data and lines calculated with the model is a positive test to validate the consistency of our analysis. Moreover, our results confirm that homomeric polypeptides represent valuable model systems to get information on the dynamical behavior of peptide systems including real proteins.

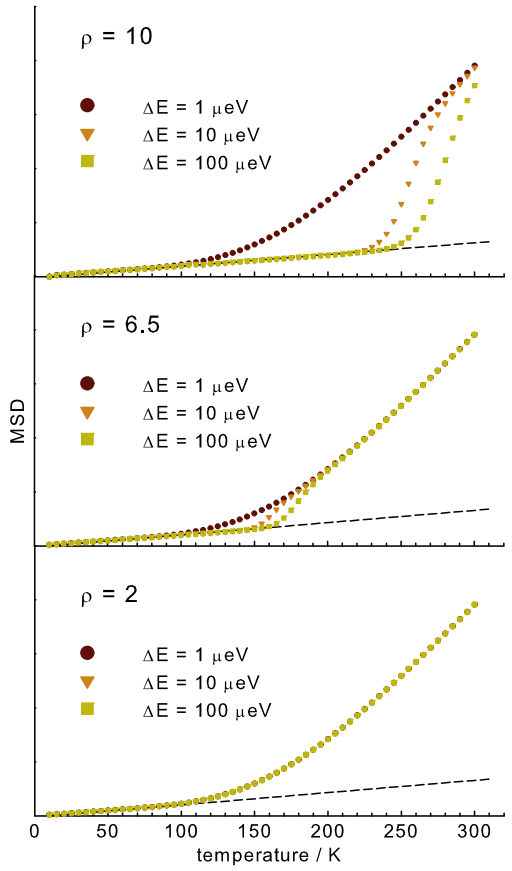

Fig. 5. Different energy resolution dependences of the anharmonic onset temperature as a function of the ρ parameter. From top to bottom: calculated MSD at three ΔE values for $\rho = 10$, $\rho = 6.5$, and $\rho = 2$.

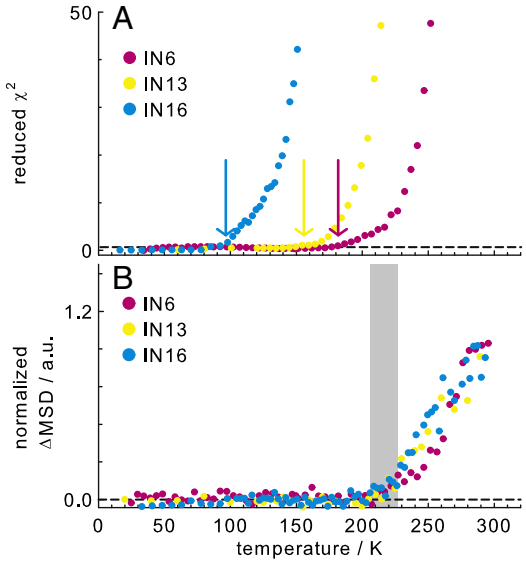

Fig. 6. Panel A: reduced χ^2 obtained fitting MSD vs. T curves, relative to dry poly-ala, with a linear function up to T_f; the arrows indicate MGA temperatures. Panel B: normalized MSD differences (hydrated–dry) relative to poly-gly; gray area indicates the PDT temperature region.

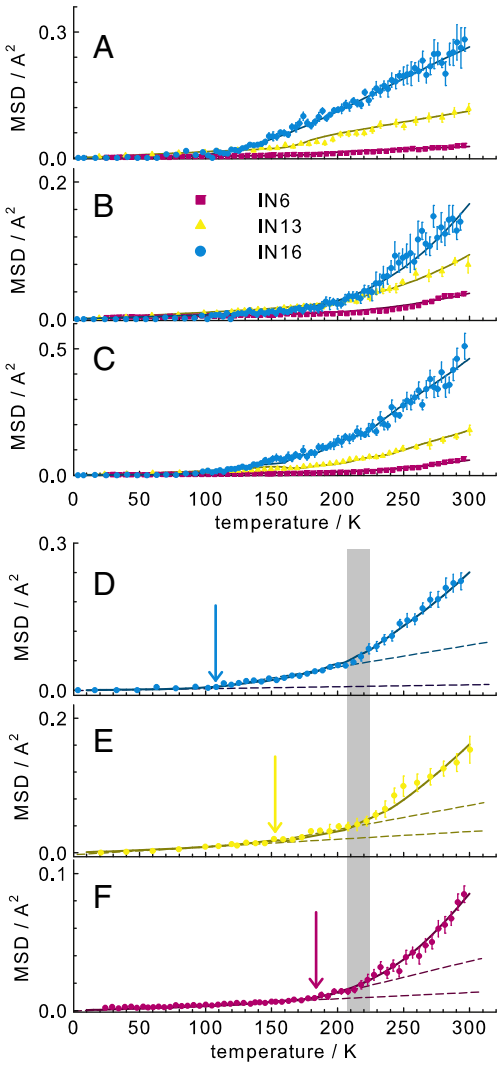

Fig. 7. Symbols: MSDs of dry poly-ala (A), hydrated poly-gly (B), hydrated poly-ala (C) and of hydrated BSA at IN16 (D), IN13 (E) and IN6 (F); lines: MSD_{model} calculated with the parameters reported in Table 1. Arrows and gray area in panels D, E and F indicate the MGA and PDT temperatures, respectively.

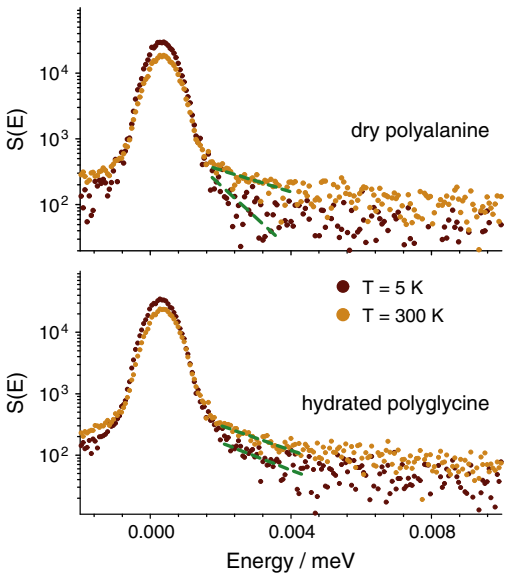

Fig. 8. Quasielastic spectra measured at IN16 and binned over the entire Q range, relative to dry poly-ala (upper panel) and hydrated poly-gly (lower panel) at T = 5 K and T = 300 K.

Looking at the energetic description obtained with the present analysis, our experimental results and their analysis reveal that both anharmonic onsets observed in proteins and polypeptides can be qualitatively described with similar de-trapping mechanisms, but with large quantitative differences. The MGA involves a thermally activated motion between states energetically close (i.e. of a low energy landscape tier, in terms of the H. Frauenfelder's terminology [26]) and without any coupling with the surrounding water matrix; the parameters obtained are in agreement with the current structural interpretation [1,3,4] of a *relaxation* between sites with small equilibrium energy differences and with jump distances of the order of 1 Å, as expected for methyl group rotations. The PDT is associated to a *transition* between states with a large equilibrium free energy difference (i.e. of a higher energy landscape tier), and allowed by the presence of hydration water. Energy barriers are of the order of 20 kJ/mol and jump distances of few Angstrom, as expected for side chain motions.

The most important question still unanswered is on the physical origin of the PDT: what is exactly the mechanism responsible for the water driven effect giving rise to the PDT? A relevant clue comes up from the experimental observation (see Fig. 9) that several hydrated peptide systems, different from both a chemical and a structural point of view, like native and denatured proteins [8], homomeric polypeptides (also for poly-gly that accounts for the pure backbone contribution), amyloid fibrils [27] and amino acid mixtures [28], share essentially the same PDT onset temperature of ~220 K. This implies that the relevant features of the protein/water energy landscape responsible for the PDT (in particular equilibrium and activation energies, which, as we showed in this work, set the PDT onset temperature) are determined by hydration water properties. As already discussed in Ref. [28], if water drives the onset of the PDT, the structural differences among different peptide systems can result in different amplitudes of the PDT related fluctuations. Another clue may be gained by noting that the activation energy here estimated for the PDT (20 kJ/mol) coincides with the value estimated for the breaking of an H-bond which initiates the transition from a low density (LDL) to a high density (HDL) liquid form, proposed for supercooled water at ~220 K (at atmospheric pressure) [29]. As proposed for supercooled water confined in solid matrices [29], it is reasonable to infer that protein hydration water at temperatures below the PDT corresponds to the LDL, where an open, locally ice-like H-bond network is fully developed, while hydration water above the PDT temperature corresponds to the HDL, where the locally tetrahedrally coordinated H-bond network is not fully developed. The energetic cost for such a transition is exactly the breaking of an H-bond. Interestingly, the two-site structure of the energy surface here adopted shares analogies with the model of interaction water potential, proposed by H. Stanley and co-workers to explain the LDL → HDL transition. In this model, at temperatures <220 K water is a one-phase low density liquid condensed into the narrow well of the potential, while at about 220 K water starts to occupy the inner well corresponding to the high density phase [30]. We believe that our results on the description of the protein/water landscape structure responsible for the PDT allow to make a connection with this two sub-well model. In this scenario, as proposed by S.H. Chen and

Table 1
Parameters obtained from the analysis of dry poly-A (MGA) and hydrated poly-G (PDT) data (see text and Fig. 7). Energies are reported in kJ/mol, τ_0 in 10^{-20} s, ϕ in Å/s$^{2\lambda}$, and d in Å. τ_0 values of the order of 10^{-20} s are unphysical and can be explained with a deviation from Arrhenius behavior at high temperatures, as discussed in [32].

	ΔG	τ_0	ΔG*	ϕ	λ	d IN16	d IN13	d IN6
MGA	5.0	2.1	44	$2.0\cdot10^3$	0.35	2.9	1.4	0.7
PDT	14	2.1	21	$5.0\cdot10^2$	0.23	7.5	4.7	2.9

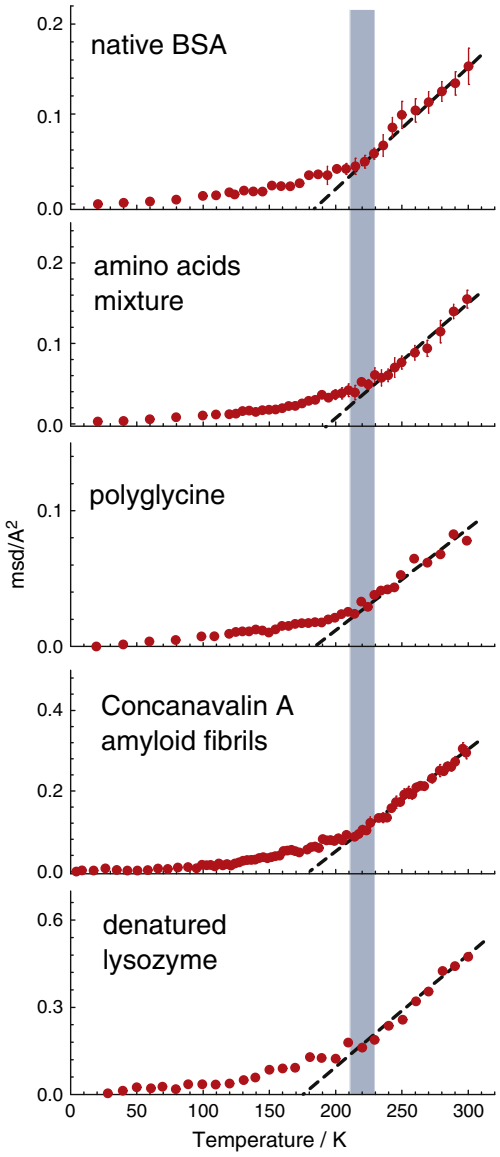

Fig. 9. MSD as a function of temperature for different hydrated peptide systems. From top to bottom: native Bovine Serum Albumin (BSA), amino acid mixture [28], polyglycine, Concanavalin A amyloid fibrils [27] and denatured lysozyme [8]. The gray area indicates the coincidence of PDT temperature region among the different samples.

coworkers [31], the LDL → HDL transition makes more "fluid" the protein hydration water thus inducing a protein structural relaxation through backbone and side chain fluctuations, revealed as the PDT.

4. Conclusions

The principal results of the present work are as follows:

1. Thanks to the novel approach of using homomeric polypeptides we obtain a clear experimental evidence on the resolution dependence of MGA and PDT.
2. The data show that MGA depends upon resolution while PDT onset temperature does not; therefore, PDT cannot be considered as a mere resolution effect.
3. This evidence helps to discriminate among different hypotheses previously put forward to explain the origin of PDT.
4. We show that a "simple" two-site model of anharmonicity, in which the effects of resolution are explicitly taken into account, is able to reproduce quantitatively the observed temperature dependence of MSD, i.e. both onset temperatures and amplitudes. This fact, together with the use of homomeric polypeptides, enables to obtain the activation barriers and equilibrium free energy differences involved in MGA and PDT. The same energy values are also found for the real protein BSA.
5. Based on the above energetics, together with experimental evidences in the recent literature, we propose that PDT is related to a real transition between states with different free energies linked to the LDL → HDL transition of the hydration water.

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