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Direct Evidence of the Amino Acid Side Chain and Backbone Contributions to Protein Anharmonicity

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Abstract: Elastic incoherent neutron scattering has been used to study the temperature dependence of the mean-square displacements of nonexchangeable hydrogen atoms in powders of a series of homomeric polypeptides (polyglycine, polyalanine, polyphenylalanine and polyisoleucine) in comparison with myoglobin at the same hydration level ($h = 0.2$). The aim of the work was to measure the dynamic behavior of different amino acid residues separately and assess the contribution of each type of side chain to the anharmonic dynamics of proteins. The results provide direct experimental evidence that the first anharmonic activation, at ~150 K, is largely due to methyl group rotations entering the time window of the spectrometer used; however, contributions on the order of 10−20% from the motions of other groups (e.g., the phenolic ring and the methylene groups) are present. Our data also indicate that the dynamical transition occurring at ~230 K can be attributed, at least at the hydration level investigated, mainly to motions involving backbone fluctuations.

Introduction

At cryogenic temperatures, the mean-square displacements (MSDs) of nonexchangeable hydrogen atoms in proteins arise from a purely harmonic vibrational contribution. The MSDs can be measured by elastic incoherent neutron scattering (EINS) on protein powders and described in terms of a set of quantized Einstein harmonic oscillators (MSD $\approx \coth[(h\nu)/(2k_BT)] +$ constant, giving essentially a linear dependence on temperature for $T \geq 20$ K). With an increase in temperature, a first anharmonic activation occurs in the 100−150 K region, independent of the hydration level of the protein.[1,2] In powders hydrated above a critical threshold (typically ~0.2 g of water/g of protein), a second activation is observed at ~230 K, giving rise to a sharp MSD increase. This second onset is known as the dynamical transition and has received a lot of attention for two decades.[3−5] Several experimental and computational studies have suggested that this transition is strongly coupled with solvent dynamics:[6−9] it is suppressed in dry protein powders and enhanced as the hydration increases. Moreover, the occurrence of the dynamical transition is generally related to the onset of biological function[10] (although counterexamples have been reported in the literature[11]), indicating that this phenomenon is of utmost importance for protein activity. However, a clear picture of the molecular motions involved and the protein groups/regions contributing to them (e.g., segments of secondary structures, individual side chains, flexible domains at the protein surface, etc.) is far from being reached.

Despite a growing interest in recent years, only a little is known about the first activation. In particular, a definitive assignment of its molecular origin has not been obtained to date. Molecular dynamics (MD) simulations of proteins[1,12] indicate that the methyl group rotations are responsible for the increase in the MSD, similar to the conclusion based on neutron scattering data on polymers.[13] Results from NMR spectroscopy,[14] MD simulations,[12] and neutron scattering[1] indicate that the energy barriers for methyl rotation are temperature-independent, as expected for a localized motion, and count for

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2−4 kcal/mol, compatible with activation at ∼150 K;[15] moreover, a distribution of energy barriers has been found,[14] with different energy values being determined essentially by the heterogeneous local environment and much less by amino acid type and solvent exposure.[12,14] Although these results indicate that methyl rotation is a plausible origin of the first anharmonic activation, unambiguous proof is still missing. Even the beautiful experiment on t-RNA by Caliskan et al.[16] cannot be considered as direct proof, since the absence of anharmonic activation at 100−150 K in this sample, although consistent with the paucity of methyl groups in t-RNA, could in principle be due to "the different flexibility of polypeptide and polynucleotide backbones", as stated by the authors. It should also be noted that MSDs measured by EINS in deuterated protein powder samples contain contributions from all of the nonexchangeable hydrogen atoms. Because of the intrinsic heterogeneity in the amino acid composition of proteins (only 25−30% of nonexchangeable hydrogen atoms belong to methyl groups), the results of such experiments on proteins can only be *consistent* with the above hypothesis but not directly *prove* it.

The present study was aimed at obtaining direct experimental evidence of the molecular origin of the anharmonic activations that characterize the dynamics of proteins. For the first activation, observed in protein dynamics at 100−150 K, we sought direct evidence of the involvement of methyl group dynamics and possible contributions of different side chain groups. In regard to the second anharmonic activation, observed in protein dynamics at ∼230 K (the so-called dynamical transition), we wanted to obtain evidence of the contributions from the backbone in comparison with those from the amino acid side chains.

To this end, we studied a set of relevant homomeric polypeptides having a number of residues comparable to that in proteins. This approach overcomes the problem posed by the intrinsic heterogeneity of proteins: it allows the study of amino acid chains (in view of the fact that the dynamical properties of proteins are expected to be intimately linked to their chainlike nature) but at the same time permits the measurement of the dynamic behavior of each type of residue separately, making possible an assessment of the contributions of each type of side chain to the anharmonic transitions and hence an identification of the transitions' molecular origin.

We therefore performed an EINS study of four D_2O-hydrated polypeptides with and without methyl groups in the side chain, as shown in Figure 1: polyglycine (poly-gly), polyalanine (poly-ala), polyisoleucine (poly-ile), and polyphenylalanine (poly-phe). Poly-gly has only α-carbon hydrogens: it can be taken as a "backbone-only" polypeptide. Poly-ala has a single methyl group as its side chain. Poly-ile has a more complex side chain containing both methyl and methylene hydrogens, while poly-phe has a complex side chain containing methylene hydrogens but no methyl groups at all. By a comparison of the first two samples, an indication of how the backbone (poly-gly) and methyl groups (poly-ala) contribute to the anharmonic dynamics can be achieved; the other samples reveal the contributions of more extended amino acid side chains and of hydrogens belonging to different chemical groups.

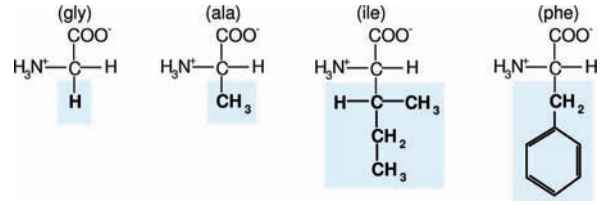

Figure 1. The four different amino acids that compose the homomeric polypeptide chains studied in this work. The different side chains are highlighted in bold print and blue shading.

Our approach is different from, but complementary to, the selective hydrogen/deuterium labeling method that has recently been applied to the study of the dynamical heterogeneity of bacteriorhodopsin within the purple membrane.[17−19]

Experimental Section

Samples. Polypeptide powders (chain length ≈ 100 residues) 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 at 45 °C 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 amino acid polymer) was determined by measuring the mass change. The myoglobin (Mb) powder sample was the same as that used in ref 20.

Neutron Scattering Measurements. EINS measurements as a function of temperature ($T = 20-300$ K) and momentum transfer ($Q = 0.2-4.9$ Å^{-1}) were performed on the thermal back-scattering spectrometer IN13 (incident wavelength λ_i, 2.23 Å; energy resolution, 8 μeV fwhm) at the Institut Laue Langevin (Grenoble, France). IN13 allows access to the space−time scale of 1−6 Å and 0.1 ns. The elastically scattered intensity $S(Q, \omega = 0)$ 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 ≈ 88%) was chosen in order to avoid corrections for multiple scattering.

Neutrons scattered incoherently by nonexchangeable hydrogen atoms of polypeptides dominate the total scattered intensity, allowing us to assume (as is usually done for protein powders) that we were probing the self-dynamics of these atoms.[4] It is worth noting that in the time−space window investigated, the hydrogen atom dynamics reflects the motions of the molecular groups to which the H atoms are bound.[21]

Secondary Structure Control and Infrared Measurements. Since the local dynamics of nonexchangeable hydrogen atoms (e.g., methyl group rotation) can depend on the local structure,[22] it was important to check the secondary structure of the investigated polypeptides and compare it with that of Mb powder. Moreover, as the dehydration process can modify the secondary structure with

(15) The expression "activation of methyl group rotations at $T \approx 150$ K" as used in this work is not meant to imply that methyl groups rotate only for $T > 150$ K but not for $T < 150$ K. It rather means that at $T \approx 150$ K, the characteristic time for methyl group rotation, τ, reaches the subnanosecond time scale and therefore enters the time window accessible with IN13 (also see eq 2 and related comments).

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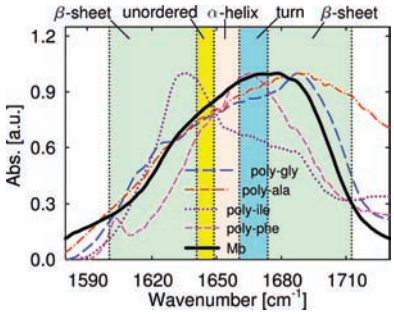

Figure 2. Amide I′ band measured by diffuse-reflectance FT-IR spectroscopy at room temperature for the studied polypeptides and a myoglobin powder sample.

respect to the solution state,[23,24] it was essential to perform measurements on the same powder samples used for the EINS measurements. To this end, the polypeptide and Mb powders used for the EINS experiments were mixed in a 1:10 proportion with KBr powder, and diffuse-reflectance FT-IR spectra in the amide I′ region near 1650 cm^{-1} were measured with a Jasco FT-IR-410 spectrometer equipped with a Pike diffuse reflectance accessory.

Data measured at room temperature are reported in Figure 2. The spectra of poly-gly, poly-ala, and poly-phe are very similar to that of Mb, whereas the spectrum of poly-ile has a marked peak at ~1630 cm^{-1} attributable to an increased β-sheet structure.[25,26] We note also that all of the spectra were considerably broader than those measured in solution,[25,26] thus indicating a relevant degree of secondary structure heterogeneity in all of the measured samples. Overall, the comparison with Mb revealed that the percentage of ordered secondary structures and the structural heterogeneity in the polypeptide powders were comparable to those in a powder of a functional protein. The structural similarity between the protein and the polypeptides, supported by the data in Figure 2, suggests that the polypeptide powders investigated in this work can be considered (at least at the level of secondary structure) as good model systems for protein powders.

Results and Discussion

Poly-Gly, Poly-Ala, and Myoglobin. Figure 3 shows the temperature dependence of the elastic intensity (binned over the total Q range explored) for poly-gly and poly-ala compared with that for Mb. This simple procedure detects relevant transitions as sharp changes in the elastic scattering decay[13] and has the advantage of being model-independent. As expected from the temperature dependence of MSDs investigated in previous works,[20,27] Mb undergoes two transitions, one at ~150 K and the other at ~230 K. The curves for poly-gly and poly-ala provide direct experimental evidence of the involvement of methyl groups in the transition at ~150 K; in fact, this activation is present for poly-ala, which has only methyl groups as its side chains, and it is absent for poly-gly, which does not have side chains at all. Moreover, poly-gly shows only one transition, at 220−230 K, indicating that backbone dynamics (probed by the hydrogen atoms in poly-gly) is involved in the dynamical transition at ~230 K.

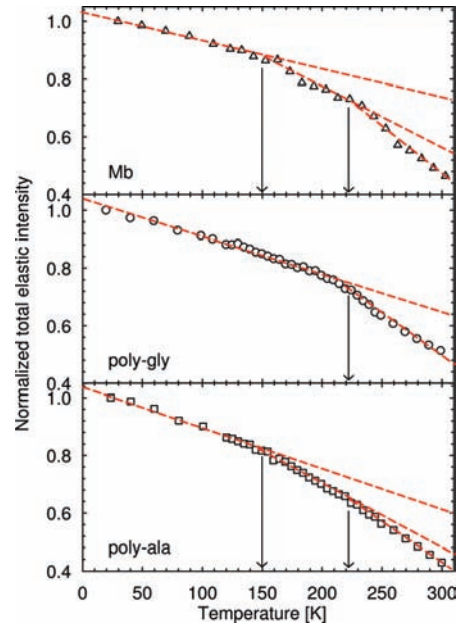

Figure 3. Elastically scattered intensity binned over the explored Q range as a function of temperature for hydrated powders ($h = 0.2$) of myoglobin, polyglycine and polyalanine. The arrows mark the temperatures at which breaks in the temperature dependence are observed.

To calculate the MSDs,[28] the elastic scattered intensity $S(Q, T, \omega = 0)$ was analyzed in the framework of the Gaussian approximation, according to the following definition:[4,30]

$$-\ln\left[\frac{S(Q,T,\omega=0)}{S(Q,T=20\text{ K},\omega=0)}\right] = \frac{\langle u^2\rangle}{6}Q^2 \qquad (1)$$

where, assuming an isotropic sample, $\langle u^2\rangle/6 = \langle x^2\rangle/2$. In practice, $\langle x^2\rangle/2$ is determined by fitting the Q^2 dependence of $\ln[S(Q, T, \omega = 0)/S(Q, T = 20\text{ K}, \omega = 0)]$ with a straight line over a suitable Q^2 range (see the captions of Figures 4 and 7) and verifying a posteriori that the condition $Q^2\langle u^2\rangle \leq 2$ is obeyed.[4] The resulting curves reported in Figure 4 fully confirm the conclusions drawn from the data in Figure 3, namely, that poly-ala and Mb (which contain methyl groups) clearly exhibit anharmonic activations at both ~150 and ~230 K while poly-gly (which has no methyl groups) shows only one activation, at ~230 K.

The reduced χ^2 values obtained by fitting the MSD-versus-T data with a linear dependence from 20 K up to a given temperature T_f are shown in Figure 5 and provide a quantitative estimate of the harmonic-to-anharmonic transition temperature. While poly-gly shows linear behavior up to the dynamical transition ($T \approx 215$ K for poly-gly), poly-ala and Mb deviate from linear dependence much earlier ($T \approx 150$ K for Mb, in agreement with previous results in the literature,[12,20,30] and $T \approx 160$ K for poly-ala). The shift in the activation temperature with respect to that previously reported for lysozyme[1] ($T \sim 100$ K) may be due to the different energy resolution of the instruments used or to differences in the activation enthalpies and entropies for methyl group rotation among the various samples investigated.

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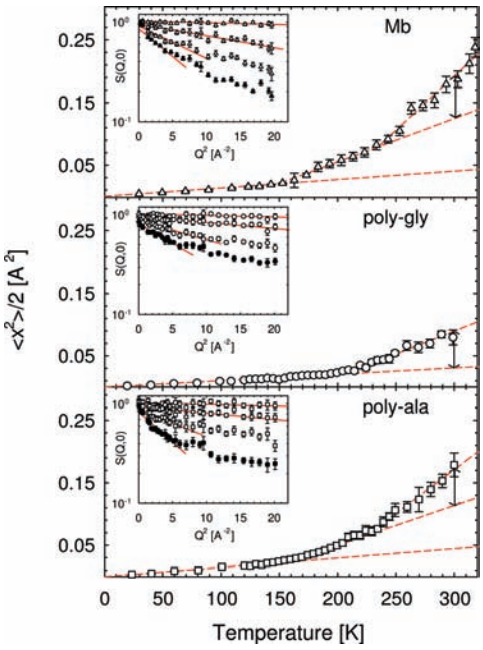

Figure 4. Total MSDs as a function of temperature obtained by analyzing the elastically scattered intensity $S(Q, T, \omega = 0)$ using the Gaussian approximation. A small offset ($\sim 10^{-3}$ Å^2) was added to take into account zero-point vibrations. Dashed lines are linear fits in the suitable temperature regions. The double-headed arrows indicate the contributions of the dynamical transitions to the total MSDs at 300 K. Insets: $S(Q, T, \omega = 0)$ vs Q^2 and corresponding fits using the Gaussian approximation for selected temperatures (typical Q ranges over which the linear fits were performed are 0−4.0, 0−3.0, and 0−2.0 Å^{-1} at $T = 100$, 200, and 300 K, respectively). Solid symbols show the 300 K data.

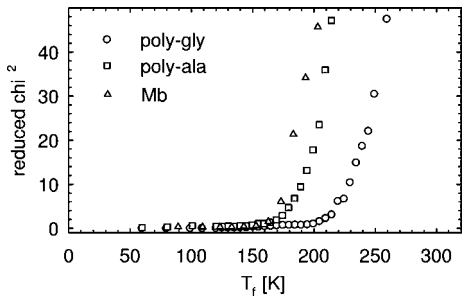

Figure 5. Reduced χ^2 values obtained from fitting the MSD curves reported in Figure 4 with a linear function up to a given temperature T_f, as a function of T_f.

To obtain an estimate of the activation enthalpies for methyl group rotation in our samples (Mb and poly-ala), we followed the procedure adopted by Roh et al.,[12,13] i.e., we fitted the temperature dependence of $S(Q, T, \omega = 0)$ over the temperature range 20−225 K in terms of the following expression:

$$S(Q,T,\omega=0) = e^{-Q^2\langle x^2\rangle/2}\left[k_1(Q) + k_2(Q)\int_{-\infty}^{+\infty}\rho(\omega-\omega')\times \int_0^{+\infty} g(H)\frac{1}{\pi}\frac{2\tau}{1+(2\omega'\tau)^2}\,dH\,d\omega'\right|_{\omega=0}\Bigg] \quad (2)$$

where $\langle x^2\rangle/2$ at temperature T was obtained by extrapolating the harmonic trend observed for $T < 150$ K (see the data reported in Figure 4); $\tau = {}^2/_3\tau_0 e^{H/RT}$ is the characteristic time for methyl group rotation having an Arrhenius temperature dependence with activation enthalpy H; $\rho(\omega - \omega')$ is the resolution function of the spectrometer, which was measured in a separate vanadium

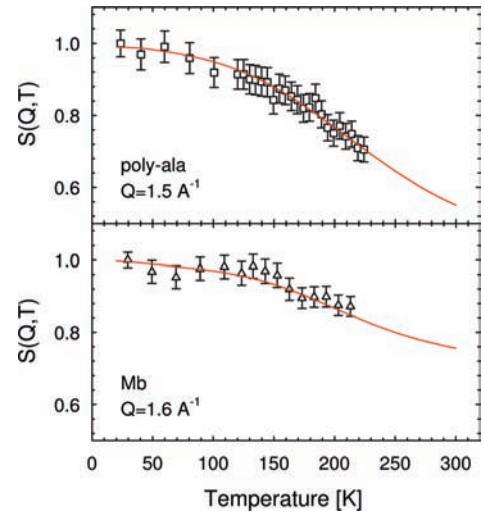

Figure 6. Temperature dependence of the elastically scattered intensity $S(Q, T, \omega = 0)$ normalized to $S(Q, T = 20$ K, $\omega = 0)$ for (top) poly-ala and (bottom) Mb powders; the red curves are fits to eq 2.

experiment and modeled as a Gaussian with 8 μeV fwhm; $g(H)$ is the distribution of activation enthalpies; and $k_1(Q)$ and $k_2(Q)$ are given by

$$k_1(Q) = 1 - 2p_m\left[\frac{1}{3} + j_0(Qr\sqrt{3})\right] \quad (3)$$

$$k_2(Q) = \frac{2}{3}p_m\left[1 - j_0(Qr\sqrt{3})\right] \quad (4)$$

in which p_m is the fraction of hydrogen atoms involved in methyl group rotation (0.29 for Mb and 0.75 for poly-ala), $j_0(x)$ is the zeroth-order spherical Bessel function, and $r = 1.2$ Å is the radius of the methyl group rotation. As in refs 1 and 2, we assumed a Gaussian distribution of activation enthalpies:

$$g(H) = \frac{1}{\sqrt{2\pi}\Delta_H}e^{-(H_0-H)^2/2\Delta_H^2} \quad (5)$$

The fitting parameters are H_0, Δ_H, and τ_0. Results are shown in Figure 6 for selected Q values; we obtained $H_0 = 3.6$ kcal/mol, $\Delta_H = 0.8$ kcal/mol, and $\tau_0 = 1.6 \times 10^{-13}$ s for Mb and $H_0 = 3.8$ kcal/mol, $\Delta_H = 1.2$ kcal/mol, and $\tau_0 = 1.5 \times 10^{-13}$ s for poly-ala, in good agreement with literature data for methyl rotation.[1,12−14]

Poly-Ile and Poly-Phe. Although the data in Figures 3−5 clearly show that methyl group rotations are involved in the first anharmonic activation observed at lower temperatures in protein dynamics, they do not exclude the possibility that other functional groups in more extended amino acid side chains make a sizeable contribution to this effect. To investigate this point, we studied poly-ile and poly-phe; in fact, isoleucine has a complex side chain that in addition to two methyl groups also contains a methylene group, while phenylalanine has an extended side chain that contains a methylene group and a phenyl group but no methyl groups (see Figure 1).

MSDs for poly-ile and poly-phe are reported the top and middle panels, respectively, in Figure 7; the bottom panel reports the temperature dependence of the reduced χ^2 values. The general trend of the data confirms the conclusions already drawn from the experiments on poly-gly and poly-ala: polypeptides with side chains containing methyl groups exhibit two clear

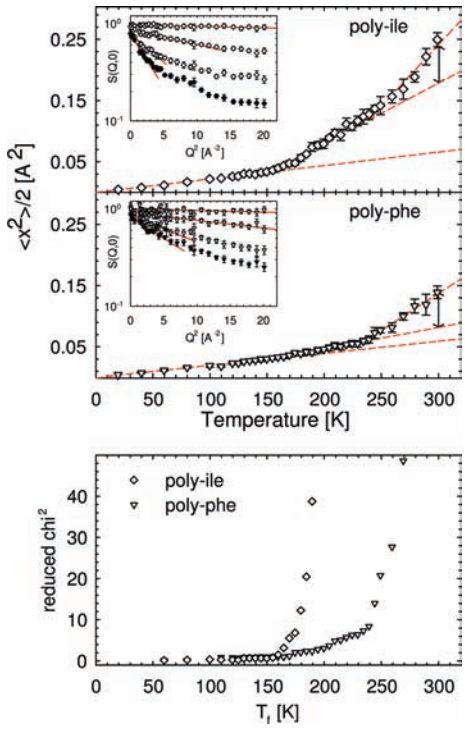

Figure 7. Same as Figure 4 (top and middle panels) and Figure 5 (bottom panel) for poly-ile and poly-phe. Insets: $S(Q, T, \omega = 0)$-vs-Q^2 plots and fits using Gaussian approximation for selected temperatures (typical Q ranges over which linear fits were performed are 0−4.0, 0−3.0, and 0−2.0 $Å^{-1}$ at $T = 100$, 200, and 300 K, respectively).

anharmonic activations, the first one at ∼150 K and the second at ∼230 K, while polypeptides with side chains that do not contain methyl groups exhibit only one clear anharmonic activation, at ∼230 K. However, closer inspection of the data reveals that poly-phe also shows a slight departure of MSD values from linearity at ∼150 K (Figure 7, middle panel), and the effect is more clearly evidenced in the lower panel of Figure 7, where an increase in the reduced χ^2 values starting at ∼150 K is evident. Moreover, for poly-ile, the extent of the first anharmonic activation, as estimated from the slope of the MSD-versus-T plot over the temperature range 150 K < T < 220 K, is larger than that for poly-ala. This indicates that fluctuations of side-chain groups other than methyl groups (e.g., librations of the phenyl ring and the methylene hydrogens) take place as early as ∼150 K; however, since they occur on a space scale smaller than methyl group rotations, they contribute less to the total MSD. To better quantify the extent of the motions activated at ∼150 K with respect to harmonic vibrations, we calculated the ratio α of the MSD slopes in the temperature regions just above (150 K < T < 220 K) and below (20 K < T < 100 K) the first anharmonic activation (see Figures 4 and 7). The α values scale in the order poly-ile (4.52 ± 0.08) > poly-ala (4.31 ± 0.07) > poly-phe (1.78 ± 0.04) > poly-gly (1.0). This confirms that although methyl group rotation gives the predominant contribution to the first anharmonic activation, contributions from other groups are present and can be estimated to be on the order of 10−20%. To assess the relative contributions of different side chains and functional groups, a thorough study of different polypeptides (e.g., polylysine, whose side chains contain only methylene groups) would be in order. Alternatively, specific hydrogen labeling[17−19] of one amino acid type at a time in an otherwise deuterated protein would provide information on amino acid dynamics in a protein instead of in a model system.

Table 1. Contributions of the Dynamical Transition to the Total MSD at 300 K and Resilience Parameters Obtained in the Low- and High-Temperature Regimes (See the Text)

	$\langle x^2\rangle_{DT}/2$ at 300 K [Å²]	k [N/m]	k' [N/m]	α
poly-gly	0.060 ± 0.004	2.4 ± 0.2	0.35 ± 0.03	1
poly-ala	0.058 ± 0.005	1.5 ± 0.1	0.16 ± 0.02	4.31 ± 0.07
poly-ile	0.062 ± 0.006	1.0 ± 0.1	0.10 ± 0.01	4.52 ± 0.08
poly-phe	0.056 ± 0.004	1.2 ± 0.1	0.19 ± 0.02	1.78 ± 0.04
Mb	0.068 ± 0.007	1.8 ± 0.2	0.13 ± 0.01	5.48 ± 0.08

Further Comparisons among the Various Amino Acid Polymers Investigated. The data reported here also give interesting suggestions on how the backbone and different amino acid side chains contribute to protein flexibility. In the harmonic regime at T < 150 K, the slope of the MSD-versus-T plot is inversely proportional to the elastic constant k of the oscillators, where k is also determined by the environment surrounding the hydrogen atoms. This idea has been phenomenologically generalized[5] to the high-temperature regime above the dynamical transition by introducing the resilience, k'. In Table 1, k and k' values obtained for the investigated samples are reported. Interestingly, k for poly-gly is about twice as large as those for the other polypeptides, indicating that the harmonic dynamics of the backbone is more rigid than that of the side chains. Moreover, k clearly depends on the dimension of the side groups (see Figure 1), with poly-gly the most rigid system and poly-ile the softest one. Approximately the same trend is found for k', although in this case the values for poly-ala and poly-phe are comparable. Possible explanations for the slight differences in the k and k' trends could be (a) a different (i.e., ordered/unordered) structure of the polypeptides, as suggested by data in Figure 2, or (b) a different distribution of water around the side chains, as k' is related to the protein−solvent coupling. This analysis supports the expected picture of proteins as composed of a *hard* backbone and *soft* side chains.[31] Moreover, the different degree of softness of the amino acids is an intriguing result that could help in understanding the selection strategy of the amino acid sequence in proteins.

We stress also that poly-gly exhibits only the activation at ∼230 K; as it has no side chains at all, this implies that the picosecond-timescale backbone fluctuations (e.g., the ones detected by NMR spectroscopy on the picosecond time scale[32]) are involved in the dynamical transition. The MSDs associated with the onset of the dynamical transition, which we call $\langle x^2\rangle_{DT}/2$, were obtained by subtracting the linear trend observed for 150 K < T < 220 K from the total MSD. The values of $\langle x^2\rangle_{DT}/2$ obtained at 300 K for the various samples investigated are reported in Table 1 and shown as double-headed arrows in Figures 4 and 7; values almost identical to the pure backbone component (poly-gly) were obtained, strongly indicating that backbone dynamics is actually the main contribution (at least at the explored hydration level) to the dynamical transition.

Finally, interesting hints for future work arise from the whole Q dependence of $S(Q, \omega = 0)$. In fact, it has been

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proposed that the non-Gaussian decay of $S(Q, \omega = 0)$ in protein powders can be fitted by a model of structural heterogeneity (e.g., methyl and non-methyl H atoms giving a sum of two different Gaussian decays[20,27,30,33]). However, alternative models of homogeneous anharmonicity (e.g., a two-well potential model[3]) reproduce the decay with comparable statistical parameters.[20] The polypeptides investigated here are good model systems for testing what is the predominant contribution: indeed, poly-gly is in principle a homogeneous system, while poly-ala contains a simplified heterogeneity (75% methyl hydrogens, 25% backbone hydrogens). Our data reveal that $S(Q, \omega = 0)$ at room temperature is non-Gaussian even in poly-gly (see the inset in the middle panel of Figure 4). Furthermore, a bimodal distribution with fixed weights[30,33] was unable to fit the data for poly-ala (fit not shown). This is evidence that the heterogeneous model alone does not well describe the dynamics of our system, suggesting that the relevant features of the Q dependence are related to the activation of anharmonicity. This result clearly implies the need to develop more sophisticated models in which both anharmonicity and heterogeneity are taken into account; data on polypeptides could be helpful in testing their validity.

Conclusions

The results presented in this work are the first unambiguous and model-independent experimental evidence for different contributions to anharmonic activations in proteins. The first anharmonic activation, at ~150 K, is largely due to the activation of methyl group rotations, although in view of the poly-phe and poly-ile results, contributions on the order of 10−20% from motions of other groups (e.g., the phenyl ring and the methylene groups) may be present. On the other hand, data on a "pure backbone" polypeptide (poly-gly) does not show the first activation, thus confirming that the first anharmonic transition is due to side-chain dynamics. Our data also indicate that the second dynamical transition, which occurs at ~230 K, can be attributed, at least at the hydration level investigated here, to motions involving backbone fluctuations. Finally, a simple analysis shows that each type of amino acid contributes differently to the protein dynamics and flexibility, in agreement with recent results on bacteriorhodopsin.[19]

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