Onsets of Anharmonicity in Protein Dynamics

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Two onsets of anharmonicity are observed in the dynamics of the protein lysozyme. One at $T \sim 100$ K appears in all samples regardless of hydration level and is consistent with methyl group rotation. The second, the well-known dynamical transition at $T \sim 200$–230 K, is only observed at a hydration level h greater than ~ 0.2 and is ascribed to the activation of an additional relaxation process. Its variation with hydration correlates well with variations of catalytic activity suggesting that the relaxation process is directly related to the activation of modes required for protein function.

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It has previously been shown that the onset of anharmonic motion observed in many hydrated proteins at a dynamic transition temperature $T_D \sim 200$–230 K correlates with the onset of measurable biochemical activities [1–5], although exceptions have been reported [6,7]. This onset of anharmonicity is usually observed as a sharp increase of the mean squared atomic displacement $\langle x^2 \rangle$ with temperature. The biochemical activity of proteins also depends on hydration. In lysozyme, enzymatic activity remains very low up to a hydration (h) of ~ 0.2 (h is measured in g of water per g of protein) and then increases sharply with an increase in h from 0.2 to 0.5. [8]. Various experiments and computer simulations have demonstrated the strong influence of hydration on protein dynamics [1–5,9–11]. Thus, the detailed analysis of the dependence of internal protein motions on hydration can provide another methodology to study the relationship between protein dynamics and activity.

In this Letter we emphasize the existence of two temperature regions where the onset of anharmonicity in the dynamics occurs: one at low temperature that appears in all samples and is consistent with methyl group rotation, and the well-known dynamical transition at $T_D \sim 200$–230 K that appears only in samples with $h > 0.2$. After correction for methyl group contributions, variations of the neutron spectra with hydration show a good correlation with variations of catalytic activity. These results suggest that the relaxation process that is activated above T_D and $h > 0.2$ might be directly related to the onset of biochemical activity of proteins.

Hen-egg white lysozyme (Sigma-Aldrich) was dialyzed to remove salts and lyophilized. Then it was dissolved in D_2O, to exchange all exchangeable H atoms, and lyophilized. Isopiestic equilibration of the deuterium-exchanged protein with saturated solutions of LiCl, NaCl, and K_2SO_4 in D_2O resulted in hydration levels of $h \sim 0.05$, 0.18, and 0.29, respectively. Samples with $h \sim 0.45$, 0.51, and 0.80 were prepared by adding D_2O to the $0.29h$ sample and equilibrating the powders for at least 12 h. The hydration levels were determined from the observed mass change on drying by thermogravimetric analysis. Neutron scattering spectra were measured using the High Flux Backscattering Spectrometer (energy range ± 36 μeV, resolution $\delta E \sim 1$ μeV, wave vector range $0.25\ \text{Å}^{-1} < Q < 1.75\ \text{Å}^{-1}$) at NIST. Elastic scans were performed upon cooling from 300 K down to 10 K at a 0.7 K/min cooling rate. Total neutron scattering from the samples was $\sim 10\%$. Scattering of neutrons by nonexchangeable hydrogen atoms of lysozyme dominates the spectra and provides information on protein motion. The data were treated using DAVE software provided by NIST and normalized by the mass of lysozyme in each sample. The mean squared atomic displacement was calculated assuming a Gaussian approximation, $\langle x^2(T) \rangle = -3Q^{-2} \ln[I_{el}(Q,T)/I_{\text{el}}(Q, 10\ \text{K})]$, in the low-$Q$ range $0.35\ \text{Å}^{-1} < Q < 1.00\ \text{Å}^{-1}$. Here $I_{\text{el}}(Q,T)$ is the elastic intensity measured at a particular Q and T. Molecular dynamics simulation of lysozyme (6LYZ) was carried out in the NVE ensemble using the program NAMD [12] utilizing the CHARMM-27 protein force-field. The TIP3 water model [13] was used along with periodic boundary conditions with two lysozyme molecules per box. Systems were equilibrated for 1 ns followed by a further 10 ns that was used for analysis. Effective rotational correlation times were calculated using the Lipari-Szabo formalism [14]. Average $\langle x^2 \rangle$ of nonexchangeable protons was calculated in the same manner as the neutron results described above: from $I_{\text{el}}(Q,T)$ simulated using an identical resolution function and Q range. This method is known to provide quantitative amplitudes in agreement with experiment [15].

Figure 1 presents the quasielastic scattering spectra (QES) of different samples summed up over all detectors in the Q range $0.5\ \text{Å}^{-1}$–$1.75\ \text{Å}^{-1}$ (consistent with [16], analysis of the QES width reveals no significant dependence on Q in this range). Surprisingly, even the spectrum of the dry ($h \sim 0.05$) sample shows a significant QES

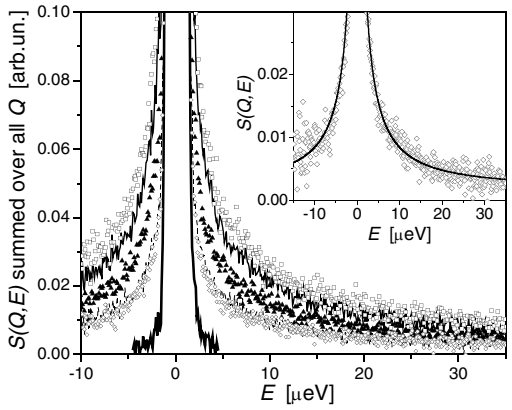

FIG. 1. $S(Q, \omega)$ summed over all Q and normalized by mass of lysozyme in each sample at $T = 295$ K. (h: ○, 0.05; dashed line, 0.18; ▲, 0.29; solid line, 0.51; □, 0.8). A vanadium spectrum (thick solid line) provides the resolution function. Inset shows the spectrum of the dry sample (symbols) and the fit using Eq. (3) (solid line).

contribution which remains essentially unchanged between $h \sim 0.05$ and $h \sim 0.18$. $\langle x^2 \rangle$ shows an onset of anharmonicity at $T \sim 200$–220 K [Fig. 2(a)] associated with the dynamical transition. However, it appears only in the samples with $h > 0.18$. An unexpected result is the existence of a low-temperature onset of anharmonicity at $T \sim 100$ K that appears in all the samples studied [inset of Fig. 2(a)]. A low-temperature onset of anharmonicity appears in neutron scattering data for a number of proteins [2,10,11,17], but its microscopic nature was not discussed. The authors of [17] ascribed the observed increase in $\langle x^2 \rangle$ at $T \sim 100$ K to quantum effects for zero-point vibrations with characteristic energy $\nu \sim 210$ cm^{-1}. However, there are no strong vibrational modes at this frequency range. The low-frequency mode, the so-called boson peak at $\nu \sim 20$–30 cm^{-1}, dominates neutron scattering spectra of proteins and the corresponding quantum effects are expected at much lower temperatures. An onset of anharmonicity at $T \sim 100$ K has been recently indicated in molecular dynamics simulations [18]. Although the authors did not discuss its mechanism, they emphasized that quantum effects play no role at $T \sim 100$ K and cannot be a cause for this anharmonicity. Moreover, the spectrum of the dry sample (Fig. 1) shows a strong QES contribution clearly indicating presence of a relaxation-like motion.

Methyl group rotation usually appears in the neutron scattering spectra of polymers in this temperature range [19]. It is reasonable to assume that methyl group rotations can make a significant contribution to the lysozyme spectra because ~26% of all nonexchangeable H atoms in lysozyme are on methyl groups. The dynamic structure factor

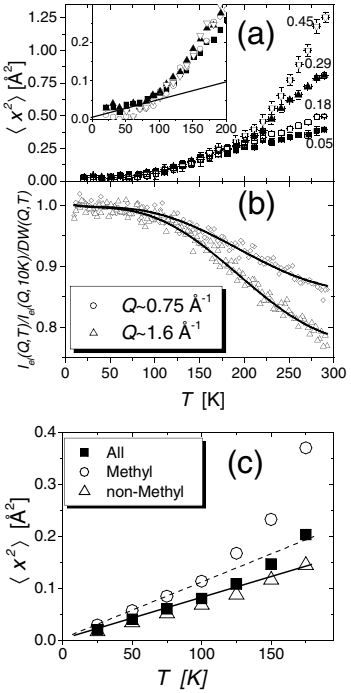

FIG. 2. (a) $\langle x^2 \rangle$ vs T. (h: ■, 0.05; ○, 0.18; ▲, 0.29; ▽, 0.45). Inset shows low-temperature onset of anharmonicity at $T \sim 100$–120 K with a line to guide the eyes. Analysis of samples with $h > 0.45$ was not possible due to the crystallization of water. (b) Temperature dependence of $I_{\rm el}(Q, T)$ in dry sample normalized to $I_{\rm el}(Q, 10\ {\rm K})$ and corrected for the Debye-Waller factor (symbols) and its fit using Eq. (2) (solid lines). (c) Simulated $\langle x^2 \rangle$ for all nonexchangeable protons, methyl protons, and nonmethyl protons in dry lysozyme. Harmonic behavior is shown as a linear extrapolation from low-temperature data for all protons (solid line) and for methyl protons (dashed line). Similar results were obtained for the wet sample.

$S(Q, \omega)$ for methyl group rotation can be approximated by a sum of Lorentzians $L_i(\omega, \tau_i)$ [19]:

$$S_{\rm met}(Q, \omega) = \frac{1}{3}[1 + 2j_0(QR\sqrt{3})]\delta(\omega) + \frac{2}{3}[1 - j_0(QR\sqrt{3})]\sum_i L_i(\omega, \tau_i). \quad (1)$$

Here $J_0(x)$ is the spherical Bessel function, R is the radius of the methyl group rotation, and τ_i is the relaxation time, $\tau_i = \tau_0 \exp(E_i/kT)$, with $\tau_0 \sim 1.8 \times 10^{-13}$ sec [19]. The activation energy for methyl group rotation, E_i, depends on the type of amino acid residue and its environment. The distribution of energy barriers $g(E_i)$ can be estimated from analysis of the temperature variation of the elastic intensity [19]:

$$I_{\rm el}(Q, T, \omega \sim 0) = DW(Q, T)[1 - p_m + p_m \int_{-\infty}^{\infty} S_{\rm met}(Q, \omega')R(\omega - \omega')d\omega$$
$$\propto DW(Q, T)\left[{\rm const}(Q) + \int_{-\infty}^{\infty} R(\omega - \omega') \int_0^{\infty} g(E_i) \frac{\tau_i}{1 + \omega'^2\tau_i^2} dE_i d\omega' |_{\omega=0} \right]. \quad (2)$$

Here $DW(Q, T)$ is the Debye-Waller factor, $DW(Q, T) = \exp(-Q^2\langle x_{\rm vib}{}^2 \rangle/3)$, $\langle x_{\rm vib}{}^2 \rangle$ is the vibrational mean square

displacement, p_m is the fraction of hydrogen atoms involved in the methyl group rotation. $R(\omega)$ is the spectrometer resolution function approximated by a Gaussian function with full width at half maximum ~ 1.2 μeV. $DW(Q, T)$ has been estimated from the slope of $\langle x^2 \rangle$ vs temperature at $T < 100$ K [inset of Fig. 2(a)]. Temperature variations of $I_{\mathrm{el}}(Q, T)/DW(Q, T)$ in the dry sample can be well described assuming a Gaussian distribution, $g(E_i) \propto \exp[-(E_0 - E_i)^2/2\Delta E^2]$, with $E_0 \sim 16.6$ kJ/mol and $\Delta E \sim 5.8$ kJ/mol [Fig. 2(b)]. These values of E_i agree well with NMR data for methyl group rotation in lysozyme [20,21].

Knowledge of $g(E_i)$ allows calculation of the methyl group relaxation spectrum:

$$\sum_Q S(Q, \omega) \propto \mathrm{const} + S_{\mathrm{met}}(\omega) = \mathrm{const} + \int_{-\infty}^{\infty} R(\omega - \omega') \int_0^{\infty} g(E_i) \times \frac{\tau_i}{1 + \omega'^2 \tau_i^2} dE_i d\omega', \quad (3)$$

where const accounts for contributions from faster processes. Equation (3) provides a good description of the QES spectrum of the dry sample at $T = 295$ K (inset of Fig. 1). The spectrum represented by Eq. (3) has been used for analysis of the elastic incoherent structure factor ($EISF$) (for definitions see, e.g., [19]) at each Q in the range 0.6 Å$^{-1} < Q < 1.7$ Å^{-1} (lower Q were excluded due to possible multiple scattering contributions). The $EISF(Q)$ obtained for the dry sample was analyzed with a three-site jump model [19,22]:

$$EISF(Q) = 1 - p_m + \frac{p_m}{3}[1 + 2j_0(QR\sqrt{3})]. \quad (4)$$

The fit of the data in Fig. 3(a) gives estimates of the radius $R \sim 1.3 \pm 0.2$ Å and the fraction of hydrogen atoms involved in rotation, $p_m \sim 0.25 \pm 0.03$. These values agree well with the methyl group radius ($R = 1.1$ Å) and the number of methyl protons in lysozyme ($p_m = 0.26$). Thus the temperature, frequency, and Q dependencies of the dynamic structure factor of dry ($h \sim 0.05$) lysozyme can be well described by methyl group rotation. These results suggest that the onset of methyl group rotation may be the source of the low-temperature anharmonicity. This agrees well with earlier NMR analysis [20,23] that estimated methyl group reorientation to be ~73% to 84% of total proton relaxation in dry lysozyme. The recent analysis of neutron scattering data in dry myoglobin [24] also ascribes ~80% of the observed relaxation to methyl group rotation. Analysis of simulated $\langle x^2 \rangle$ [Fig. 2(c)] indeed shows that ~70% of anharmonicity at $T < 200$ K comes from methyl groups which activate at $T \sim 100$ K in both dry and wet samples.

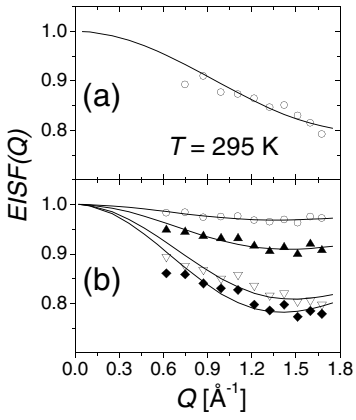

FIG. 3. (a) $EISF(Q)$ of dry sample (symbols) and the fit using Eq. (4) (solid line). (b) $EISF(Q)$ of hydrated samples after correction for $EISF(Q)$ of dry sample (h: ○, 0.18; ▲, 0.29; ▽, 0.51; ◆, 0.80). The solid lines are results of the fit using the two-site jump model.

Analysis of molecular dynamics simulations also helps explain the origin of the broad distribution of activation energies. The average rotational correlation time of methyl groups in simulations was $\tau \sim 110$ ps at $T = 295$ K. It corresponds to an average activation energy barrier ~16 kJ/mol, consistent with the experimental value of E_0. Simulations show a broad distribution of τ in agreement with the broad distribution of energy barriers found by experiment. The broad distribution of rotational correlation times appears to have two origins, the amino acid type (e.g., methionine methyl groups have $\tau \sim 20$–30 ps) and the position of the methyl group in the protein (e.g., alanine methyl groups have τ between 30 and 200 ps). Simulations of wet lysozyme ($h \sim 0.43$) at $T = 295$ K show that methyl rotations are shifted to lower effective rotational correlation times with an average $\tau \sim 75$ ps.

Now we turn to the analysis of the additional "slow" process that is activated in our frequency window at T above $T_D \sim 200$–230 K and $h > 0.2$. We fit the spectra of all the samples using a standard quasielastic-neutron-scattering procedure in DAVE, which approximates the spectra by an elastic line, $EISF(Q)$, and a Lorentzian for the quasielastic spectrum. Although a single Lorentzian does not accurately describe relaxation spectra of proteins and DNA [2,9], this approximation can provide some qualitative information. The $EISF(Q)$ for each sample has been corrected for $EISF(Q)$) of the dry sample obtained in the same way assuming that the slow process and methyl group rotations are additive and that the latter can be estimated from the QES spectra of the dry sample. The limited Q range of the resulting $EISF(Q)$ [Fig. 3(b)] does not allow us to distinguish various models (e.g., 2-, 3- site jump or diffusion in a sphere), but provides an estimate of a jump length ~3.0 Å. This is much larger than that characteristic of methyl groups. This jump distance is essentially independent of hydration level while the mobile fraction of hydrogen atoms increases strongly at $h > 0.2$ [Fig. 4(a)]. The mobile fraction reflects the number of hydrogen atoms involved in the slow process on our experimental time scale ~20 ps–1 ns and essentially correlates with inte-

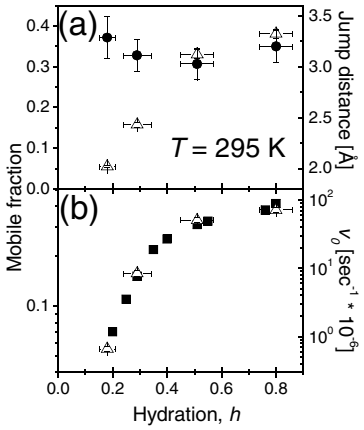

FIG. 4. (a) Mobile fraction (△) and jump distance (●) of the slow process as a function of hydration. (b) mobile fraction (△) of the slow process and initial rate of the lysozyme-catalyzed reaction, ν_0, (■) as a function of hydration [13].

grated QES intensity. It appears that the dependence of the mobile fraction on the level of hydration correlates with catalytic activity [Fig. 4(b)]. These results suggest that the additional relaxation process correlates with biological activity of the protein.

The microscopic mechanism of this slow process remains unclear. The width of the QES spectra does not show any significant Q dependence, suggesting that this is a local process with atomic displacements smaller than $R \sim 2\pi/Q_{\max} \sim 3.5$ Å. The latter is consistent with estimates of the jump distance from $EISF(Q)$ and with the fact that internal motions are expected to be limited as long as the protein stays folded. Recently, Tournier and Smith proposed [25] that motions of secondary structures might be the modes that are activated above the dynamical transition temperature. This is consistent with our results because most of the hydrogen atoms are involved in this motion and the scale of their displacements is consistent with the proposed picture. We want to emphasize that our results are inconsistent with a recent NMR study [26]. Our data for lysozyme, as well an earlier study of RNAse A [27], show no evidence for a trimodal distribution of methyl dynamics; moreover, while the low-T anharmonic dynamics is dominated by methyl protons they are not the sole contributor above T_D.

In conclusion, we report the existence of two onsets of anharmonicity in the dynamics of lysozyme. The first onset appears at $T \sim 100$ K in all samples regardless of hydration level and is consistent with methyl group rotation. Methyl groups appear to make a significant contribution to the quasielastic neutron scattering spectra of proteins at low hydration. The second onset is the well-known dynamical transition at $T_D \sim 200$–230 K that appears only in samples with $h > 0.2$ and is ascribed to activation of an additional slow process. Dependence of the intensity of the slow process on hydration correlates well with catalytic activity. This suggests that the relaxation process is directly related to the biochemical activity of the protein and that its activation is a prerequisite for protein function. The microscopic picture of the process remains unclear.

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