The Inverse Relationship between Protein Dynamics and Thermal Stability

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ABSTRACT Protein powders that are dehydrated or mixed with a glassy compound are known to have improved thermal stability. We present elastic and quasielastic neutron scattering measurements of the global dynamics of lysozyme and ribonuclease A powders. In the absence of solvation water, both protein powders exhibit largely harmonic motions on the timescale of the measurements. Upon partial hydration, quasielastic scattering indicative of relaxational processes appears at sufficiently high temperature. When the scattering spectrum are analyzed with the Kohlrausch-Williams-Watts formalism, the exponent β decreases with increasing temperature, suggesting that multiple relaxation modes are emerging. When lysozyme was mixed with glycerol, its β values were higher than the hydrated sample at comparable temperatures, reflecting the viscosity and stabilizing effects of glycerol.

INTRODUCTION

In the context of molecular dynamics, questions regarding the relationship among enzyme activity, solvent effects, protein stability, and flexibility often arise. For instance, it has been shown that thermophilic proteins tend to be less flexible than their mesophilic homologs, suggesting an inverse relationship between stability and flexibility (Wrba et al., 1990; Gershenson et al., 2000). However, a recent study found no correlation between enzyme activity and global dynamics (Daniel et al., 1999). To meaningfully address these issues, it is important to acknowledge the differences between global and local events that may govern various phenomena in a protein. For instance, because thermal stability is a description of how a protein unfolds globally in response to thermal energy, measuring the global rather than the local motions is more likely to explain how protein dynamics may determine thermal stability. In contrast, enzyme activity is a less global, if not entirely local, phenomenon, especially when induced fit is involved. Therefore, it is more appropriate to probe localized motions before attempting to describe how enzyme activity is determined by molecular flexibility.

We will examine the relationship between thermal stability and protein flexibility in two protein powders, and how flexibility may in fact determine the thermal stability of a molecule. Because it is well known that protein powders become more thermally stable at decreasing hydration level and in the presence of glass-forming compounds such as glycerol (Bell et al., 1995a,b), we set out to measure and compare the atomic motions of two proteins under various solvation conditions. Incoherent neutron scattering measures the self-correlation of single particle motion of individual atoms. Hydrogen has by far the largest scattering cross-section of any atoms commonly present in proteins. Therefore, incoherent neutron-scattering measurements on these systems are almost exclusively sensitive to motions of the protons, making neutron scattering very well suited for measuring the dynamics of proteins (Lovesey, 1984; Cusack et al., 1988; Smith, 1991). Incoherent neutron scattering can take place in the elastic, quasielastic, and inelastic regimes. For elastic scattering, there is no exchange of energy in the scattering process, and changes in the elastic intensity as a function of temperature indicate the emergence of thermally excited motions. In quasielastic scattering, there is a small change in energy because of relaxations of the proteins, and the nature of the relaxations are described by the lineshape of the scattering spectrum. Finally, inelastic scattering reflects the vibrational modes of the system. In this paper, we characterize the dynamics of lysozyme and ribonuclease (RNase) A powders as a function of temperature and different solvation conditions by measuring their incoherent quasielastic scattering, and we quantify the change in dynamics in terms of the integrated elastic peak intensity and quasielastic peak shape.

MATERIALS AND METHODS

Chicken egg white lysozyme and bovine pancreas RNase A type III-A were obtained from Sigma (St. Louis, MO); deuterium oxide and deuterated glycerol were obtained from Cambridge Isotope Laboratory (Cambridge, MA). Because we were interested in only the protein dynamics, it was important to minimize the scattering from the solvents in our measurements. This can be accomplished for a neutron-scattering measurement by using deuterated solvents because the scattering cross-section of deuterium is more than 10 times less than that of hydrogen. However, minimizing the H_2O scattering requires deuterating the exchangeable protons on the backbones and side chains. In practice, this was accomplished by dissolving 0.4 grams of a protein in 45 ml of D_2O and leaving the solution overnight at room temperature for the deuterium exchange to complete. The sample was then freeze-dried into powders. Prompt γ activation analysis (Paul, 1997; Paul and Lindstrom, 2000) of the deuterated material showed no appreciable amount of residual exchangeable hydrogen. To

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achieve the desired levels of hydration, the deuterium-exchanged, lyophilized powders were equilibrated against a saturated K_2SO_4 solution prepared in D_2O in a vacuum desiccator for up to 3 days. The moisture content of the samples was measured both before and after each scattering experiment by the Karl Fisher method or simple weighing and was determined to be 30 and 24 wt % for the hydrated lysozyme and RNase A, respectively. The glycerol-lysozyme mixture was prepared by freeze-drying a solution of equal amount of deuterium-exchanged lysozyme and deuterated glycerol dissolved in D_2O into a homogenous paste. Afterward, each sample was wrapped in aluminum foil formed into an annular shape and inserted into an aluminum cylindrical (2 cm × 5 cm, i.d. × length) can for scattering measurements from 40 to 300 K in 10-K increments.

The Fermi Chopper spectrometer at the NIST Center for Neutron Research (Gaithersburg, MD) was used for this study. Measurements were carried out at 4.8 Å incident wavelength. This provides an energy resolution of ~140 μeV, allowing us to observe picosecond dynamics. At each temperature, scattering data were collected for between 1.5 and 2.5 h, corresponding to counting statistics around the elastic and quasielastic peaks of better than 90 to 99%. RNase A measurements were collected for each sample between 40 K and the highest temperature at 10-K increments. For the lysozyme studies, data were first collected at 40 K for the purpose of data normalization, then the temperature was switched immediately to their individual so-called dynamic transition temperature T_d determined in an earlier paper (Tsai et al., 2000) and increased at 10-K increments.

The raw data were converted into the usual scattering function $S(Q, \omega)$ where Q and ω are the momentum and energy transferred in the scattering event. Note that for an incoherent scatterer, $S(Q,\omega)$ is the time and space Fourier transform of the van Hove self-correlation function (Squire, 1978). The mean-square displacement, $\langle u^2 \rangle$, averaged over all hydrogen atoms was calculated from $S(Q,\omega)$ according to:

$$I_{el} \approx \int_{-\delta\omega}^{\delta\omega} S(Q,\omega)\, d\omega\delta\omega \approx \exp(-\langle u^2 \rangle Q^2) \qquad (1)$$

where I_{el} is the integrated elastic intensity and $2\delta\omega$ is the spectrometer's energy resolution (140 μeV). The data were normalized to the lowest temperature measured (40 K). Then the slope of ln (I_{el}/I_{el}^{40k}) versus Q^2 yields $\Delta\langle u^2 \rangle$ which is the change in $\langle u^2 \rangle$ between the temperature of interest and 40 K. Although the thermal dependence of $\langle u^2 \rangle$ for lysozyme under different solvation conditions has already been determined in an earlier paper (Tsai et al., 2000), similar data are also obtained in this study for RNase A according to Eq. 1.

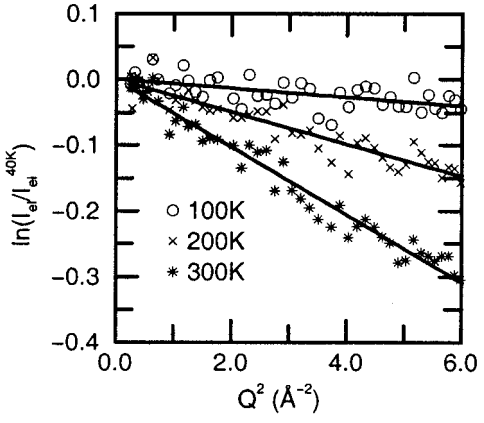

FIGURE 1 ln (I_{el}/I_{el}^{40k}) versus Q^2 for dry RNase A at different temperatures and their linear fits.

RESULTS AND DISCUSSION

Mean-square displacement of RNase A powders

Fig. 1 contains plots of ln (I_{el}/I_{el}^{40k}) versus Q^2 data for the dehydrated RNase A at different temperatures, and $\Delta\langle u^2 \rangle$ is plotted as a function of temperature for both the dehydrated and partially hydrated RNase A in Fig. 2. For the dehydrated sample, $\Delta\langle u^2 \rangle$ increases with T with constant slope between 60 and 300 K, indicating that harmonic motions that scale with k_BT account for most of the motions that take place within the sample in the absence of solvation. A similar trend has been seen for dehydrated lysozyme up to its thermal melting temperature of 450 K (Tsai et al., 2000). However, when a small amount of water was used to hydrate the RNase A sample, there is a change in slope near 250 K. This temperature has been termed the dynamic transition temperature T_d (Doster et al., 1989), the glass transition temperature T_g (Frauenfelder et al., 1991), or the conformation-motion temperature T_{cm} (Tang and Dill, 1998). The emergence of such a transition is often attributed to the onset of anharmonic vibrational motions and/or pure relaxational motions. This temperature has been reported to be ~200 K by many groups for "fully" hydrated powder (>30 wt %) and protein crystals (Doster et al., 1989; Andreani et al., 1995; Fitter et al., 1997; Fitter, 1999; Tilton et al., 1992; Zaccai, 2000; Tsai et al., 2000). The hydration level for RNase A is only 24 wt % in this case, therefore, our findings suggest that more thermal energy is required to facilitate anharmonic motions when the amount of solvation is less than what is considered the full hydration limit. Similarly, when water was replaced by different levels of glycerol as the solvent, T_d for lysozyme was reported to be at 320 K for a 80:20 wt % mixture of lysozyme:glycerol, versus 260 K for a 50:50 mixture, an upshift of 60 K when less glycerol was mixed with lysozyme (Tsai et al., 2000).

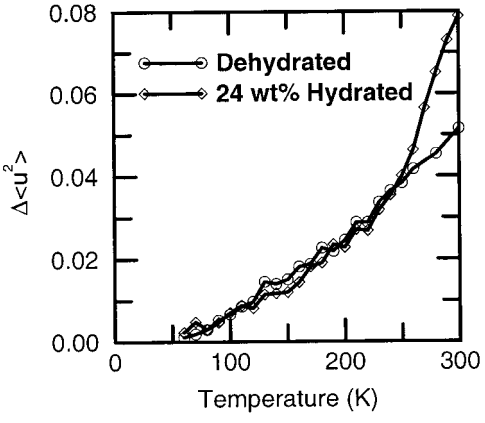

FIGURE 2 $\Delta\langle u^2 \rangle$ versus temperature for both the dry and partially hydrated RNase A powder.

Conformational sampling, flexibility, and stability

In their lattice model simulation with realistic protein sequence, Tang and Dill (1998) proposed that $\Delta\langle u^2\rangle$ for a protein rises above baseline at T_d because of the emergence of conformational fluctuations. They further proposed that such transition may be related to the so-called "entropy crisis" (Frauenfelder et al., 1991) in which the accessible conformations increase rapidly at temperatures above T_d. Such a view on the change in protein dynamics at T_d has been formulated around the idea of a protein being glassy. Below the glass transition temperature T_g, the heat capacity of a glassy compound experiences a second order transition. The compound's relaxations become nonexponential, and the relaxation rates become non-Arrhenius with respect to temperature (Green et al., 1994). Because a neutron-scattering measurement detects the T_d of a protein, we can characterize the change in the number of accessible conformations in terms of the number of relaxational processes taking place in a sample. In our simplistic representation, the more relaxational processes are detected, the more conformations are assumed to have become accessible.

In a quasielastic scattering experiment, information on the overall relaxation of the sample is contained in the intermediate scattering function $I(Q, t)$ which is related to $S(Q, \omega)$ through the Fourier transform:

$$S(Q, \omega) = \int_{-\infty}^{\infty} I(Q, t)\exp\{i\omega t\}dt \qquad (2)$$

If the sample exhibits only a single relaxation, $I(Q, t)$ can be modeled by a simple exponential with a single rate constant. However, if multiple relaxations emerge because of thermal excitation or solvation effects, $I(Q, t)$ becomes

$$I(Q, t) = \sum_{i=1}^{n}\exp\left[\frac{-t}{\tau_i}\right] \qquad (3)$$

where n is the total number of independent relaxational processes and the values of τ_i are the individual relaxation constants. According to Wong and Angell (1976), Eq. 3 can be approximated by the Kohlrausch-Williams-Watts (KWW) function (Williams and Watts, 1970):

$$I(Q, t) = \sum_{i=1}^{n}\exp\left[\frac{-t}{\tau_i}\right] \approx \exp\left[\frac{-t}{\tau}\right]^{\beta} \qquad (4)$$

with β reflecting the magnitude of n in Eq. 3. For $n = 1$, $\beta = 1$ and one has a simple relaxational process characterized by a simple exponential decay. As n increases, the relaxational processes become more diverse and β tends to decrease from 1. β can be determined by fitting the quasielastic scattering spectrum $S(Q, \omega)$ with the KWW function according to Eqs. 2 and 4. To maximize the signal to noise ratios in our data, $S(Q, \omega)$ were summed over a range of Q values between 0.35 and 2.4 Å^{-1} centered at 1.36 Å^{-1} at each temperature before any fittings were performed. A sample of the goodness of fit is shown in Fig. 3.

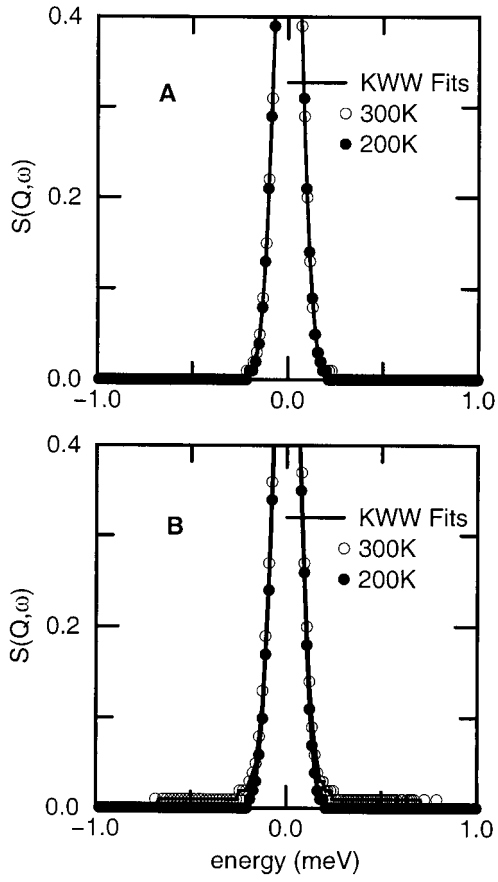

FIGURE 3 $S(Q,\omega)$ averaged over all values of Q for both the dry (A) and partially hydrated (B) RNase A powder at 200 and 300 K and corresponding KWW fits. The average Q value is 1.36 Å^{-1}. Existence of diffusive processes are suggested by the quasielastic scattering detected in $S(Q,\omega)$ at 300 K.

In Fig. 4, we show the values of β as a function of temperature for the 30 wt % hydrated lysozyme, the 50:50 wt % glycerol:lysozyme, and the 24 wt % hydrated RNase A samples. For both dehydrated lysozyme and RNase A, the scattering was resolution-limited and, thus, $\beta = 1$ at all temperatures. This is consistent with the notion that both proteins are trapped deep within a harmonic well between 50 and 300 K within the picosecond timescale, and it can only exhibit vibrational modes that result in the linear increase in $\Delta\langle u^2\rangle$ with temperature. For the 24 wt % hydrated RNase A, β begins to deviate from 1 at 240 K, close to the temperature at which T_d was detected in Fig. 2, and continues to drop with increasing temperature with corresponding increase in $\Delta\langle u^2\rangle$. Changes in $\Delta\langle u^2\rangle$ that begin at T_d result most likely from the emergence of relaxations facilitated by partial hydration. As more thermal energy becomes available, the protein begins to sample more con-

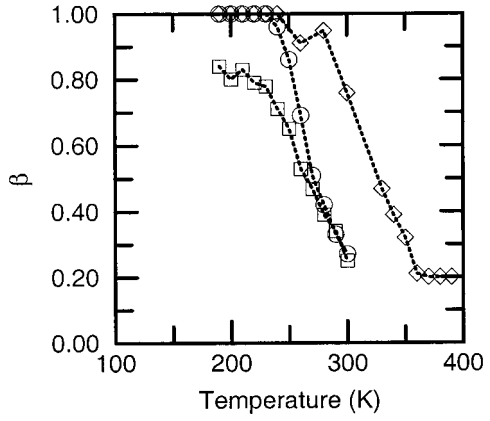

FIGURE 4 The values of β from the KWW fits for the 24wt % hydrated RNase A powder (○), the 30 wt % hydrated lysozyme powder (□), and a 50/50 wt % mixture of lysozyme and glycerol (◇). β is 1 for the dehydrated RNase A powder at all temperatures tested.

formational substates through additional relaxational processes, resulting in simultaneous decrease in β and increase in the overall mean-square displacement $\Delta\langle u^2\rangle$.

Because β for the dehydrated RNase A remains at 1 at all temperatures, water content is concluded to affect how a protein samples different conformations as temperature rises. To further illustrate this point, quasielastic measurements were carried out for the 30 wt % hydrated lysozyme near its T_d of ~200 K. As seen in Fig. 4, between 200 and 260 K, the 30 wt % hydrated lysozyme has β values lower than those associated with the 24 wt % hydrated RNase A. The extra water content enables lysozyme to exhibit more relaxational processes and, presumably, sample more conformational substates. Interestingly, such differences disappear at higher temperatures when the β values begin to converge, suggesting that at sufficiently high temperatures, although the solvent effects are still present, the water molecules become more mobile and probably somewhat decoupled from the proteins, and this allows the proteins to sample the conformational landscape more independently from the solvent.

We have provided evidence to illustrate the inverse relationship between protein flexibility and stability based on the β values seen so far and the knowledge that dehydated proteins tend to have higher thermal melting temperatures. To prove this point further, we present quasielastic data for lysozyme mixed with equal weight of the stabilizing agent glycerol. This mixture is more thermally stable and has a T_m of 370 K versus 340 K for the 30 wt % hydrated lysozyme (Bell et al., 1995b). The β values for the glycerol-treated lysozyme near and above its T_d of 260 K (Tsai et al., 2000) are also presented in Fig. 4. At each temperature, they are considerably higher than the β values seen in either of the hydrated proteins. By placing lysozyme in a viscous solvent such as glycerol, protein relaxations seem to become restricted and the conformational substates less accessible, resulting in lower $\Delta\langle u^2\rangle$ and higher β at all temperatures. However, the slope of β versus T for both lysozyme samples in Fig. 4 are very similar, suggesting that glycerol's viscous effect can be overcome by thermal energy, and that the conformational landscape of a protein may be independent of the solvent environment. Interestingly, β for the glycerol lysozyme mixture begins to level off at the sample's melting temperature of 370 K, indicating that upon denaturation, the conformational landscape remains more or less constant even at ever increasing temperatures. Based on these observations, we conclude that our data are in agreement with the findings of Tang and Dill (1998) which predicted that as the number of accessible conformation substates increases, the flexibility or dynamics of a molecule also increases, and that leads to a general decrease in protein stability.

CONCLUSION

An inverse relationship between atomic level dynamics and the thermal stability of a protein powder has been suggested from quasielastic neutron-scattering experiments with lysozyme and RNase A powders in this paper. The quasielastic scattering of each sample has been characterized using the KWW function where the exponent β inversely relates to the diversity of relaxations exhibited by a protein. For each of the dehydrated powder, their β values = 1 at all temperatures tested. This implies that the proteins are trapped in a deep harmonic well that prevents the protein from any significant level of conformational sampling, and the overall mean-square displacement simply scales with k_BT as expected for a harmonic solid. When the protein powders are solvated, we have shown that quasielastic scattering emerges near their dynamic transition temperatures observed by elastic scattering. This suggests that solvents facilitate conformational sampling when sufficient thermal energy is available. The decrease in β detected with increasing temperature in this study is considered to reflect the emergence of increasing conformational substates. We found that when a protein is mixed with a viscous solvent, the number of accessible conformations is greatly reduced, and these changes are readily reflected in the values of β in the KWW model. However, such viscous effect can be overcome by increased thermal energy, and in the case of lysozyme, the accessible conformational substates seem to be determined by its unique structure independently of its solvent environment.

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