Molecular Dynamics of Solid-State Lysozyme as Affected by Glycerol and Water: A Neutron Scattering Study

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ABSTRACT Glycerol has been shown to lower the heat denaturation temperature (T_m) of dehydrated lysozyme while elevating the T_m of hydrated lysozyme (Bell, Hageman, and Muraoka, 1995. J. Pharm. Sci. 84:707–712). Here, we report an in situ elastic neutron scattering study of the effect of glycerol and hydration on the internal dynamics of lysozyme powder. Anharmonic motions associated with structural relaxation processes were not detected for dehydrated lysozyme in the temperature range of 40 to 450K. Dehydrated lysozyme was found to have the highest T_m by Bell et al. (1995b). Upon the addition of glycerol or water, anharmonicity was recovered above a dynamic transition temperature (T_d), which may contribute to the reduction of T_m values for dehydrated lysozyme in the presence of glycerol. The greatest degree of anharmonicity, as well as the lowest T_d, was observed for lysozyme solvated with water. Hydrated lysozyme was also found to have the lowest T_m by Bell et al. (1995b). In the regime above T_d, larger amounts of glycerol lead to a higher rate of change in anharmonic motions as a function of temperature, rendering the material more heat labile. Below T_d, where harmonic motions dominate, the addition of glycerol resulted in a lower amplitude of motions, correlating with a stabilizing effect of glycerol on the protein.

INTRODUCTION

The ability to decipher the relationship between protein structure, function, and stability continues to be an important research area in the biological sciences. It is widely recognized that proteins have many conformations, and conformational dynamics are crucial to protein functionality (Yon et al., 1998). It has been suggested that protein dynamics and reactivity can be controlled by embedding a protein within a glassy solid (Franks et al., 1991; Levine and Slade, 1992; Hageman, 1992; Hagen et al., 1995; Gottfried et al. 1996; Kleinert et al., 1998; Cordone et al., 1999; Lichtenegger et al., 1999). For example, Gottfried et al. (1996) showed that hemoglobin dynamics were restricted in dehydrated trehalose glasses. The entrapment of a particular protein conformation in glasses is linked to the high internal viscosity of the system (Ansari et al., 1992; Hagen et al., 1995). Thus, high viscosity results in the slowing of protein relaxation near and below the glass transition temperature of the solvent.

In aqueous solutions, Timasheff (1992) has reported that the addition of cosolvents or osmolytes results in preferentially hydrated protein molecules due to steric exclusion of the osmolyte and changes in surface tension around the protein. This results in a reduction in the protein's ability to undergo conformational changes as compared to that in water alone, thereby stabilizing the protein structure in solution. Many polyols promote the preferential hydration of proteins, including sucrose (Lee and Timasheff, 1981), glycerol (Gekko and Timasheff, 1981), and sorbitol (Xie and Timasheff, 1997) among other mixed solvents (Inoue and Timasheff, 1968; Timasheff and Inoue, 1968). Protein unfolding in the presence of these added osmolytes is thermodynamically unfavorable due to the higher chemical potential of the denatured molecule (Wang and Bolen, 1997; Qu et al., 1998). Therefore, under thermodynamic equilibrium in such solvents, the native protein structure is favored.

The accumulation of evidence for the stabilizing effects of polyols (e.g., sugars) has led to their routine usage in formulating biopharmaceuticals in order to improve their long-term storage and delivery (Prestrelski et al., 1993; Remmele et al., 1997; Shamblin et al., 1999; Hancock and Zografi, 1997; Franks et al., 1991; Bell et al., 1995b). Proteins are frequently produced via lyophilization, and polyols are often utilized as cryoprotectants. Factors that affect the physical and chemical stability of lyophilized peptides and proteins include temperature, pH, residual moisture content, and the presence of excipients (Bell, 1997). Differential scanning calorimetry (DSC) has shown that the thermal stability of dehydrated lysozyme and somatotropin decreases with increasing moisture, irrespective of the excipients (Bell et al., 1995b). The degree of change in thermal stability elicited by moisture was, however, dependent on the type of excipient and the moisture content. Polyols (e.g., glycerol, sucrose) lowered the denaturation temperature (T_m) of dry lysozyme while increasing the T_m of hydrated lysozyme (Bell et al., 1995b). Thus, although polyols may function as cryoprotectants, they paradoxically have both destabilizing and stabilizing effects with respect to protein thermal stability. Definitive explanations for these discrepancies are lacking.

The objective of this project was to use neutron scattering techniques to examine the relationship between the physical

Received for publication 13 April 2000 and in final form 28 July 2000.

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0006-3495/00/11/2728/05 $2.00

stability (i.e., T_m) of protein powders and their internal molecular dynamics. In particular, the effects of moisture, glycerol, and temperature on the dynamics of lysozyme powder were examined.

MATERIALS AND METHODS

Sample preparation

Lysozyme from chicken egg white (Sigma, St. Louis, MO) was used without further purification. The scattering cross-section of hydrogen is 10 times greater than that of deuterium. Thus to maximize the scattering from the protein relative to the other components in our samples, we endeavored to minimize extraneous sources of hydrogen, including the exchangeable protons on the protein itself. Lysozyme was D-exchanged by dissolving 1 g lysozyme in 10 g D_2O. After allowing hydrogen exchange to occur overnight at room temperature, the sample was freeze-dried into a powder. A neutron prompt gamma-ray trace analysis of the D exchanged lysozyme indicated the amount of residual exchangeable hydrogens was negligible (Paul, 1997; Paul and Lindstrom, 2000). Some samples were prepared using deuterated glycerol (Cambridge Isotope Laboratories, Andover, MA) and D_2O to minimize the scattering from these compounds.

Four samples were prepared: dry D-exchanged lysozyme, lysozyme powder hydrated with 30% D_2O, and dehydrated lysozyme-glycerol samples prepared with 20% and 50% deuterated glycerol. The dry sample was used after the initial deuterium-hydrogen exchange step described previously. For preparation of the other samples, 800 mg of dry D-exchanged lysozyme were used. The hydrated sample was prepared by equilibrating dry lysozyme against saturated K_2SO_4 (prepared in D_2O) for 7 days in a vacuum desiccator. The lysozyme-glycerol mixtures were prepared in the proper proportions (by weight) in 10 ml D_2O. The mixture was quenched in liquid nitrogen and freeze-dried for 36 h. Residual water content in these two samples was not detectable by prompt gamma-ray measurements.

Neutron scattering experiments

Neutron scattering probes molecular dynamics directly. The scattering of neutrons by hydrogen atoms is very intense compared to that of most other elements. In addition, the available range of observable time scales makes this technique selectively sensitive to the change in internal motions of hydrogenated materials, such as proteins. The backscattering technique has been shown to be capable of detecting the change in the motions of the hydrogen atoms in myoglobin powder as a function of temperature (Doster et al., 1989). In this study, the same technique was applied to study the effect of glycerol on the dynamics of lysozyme.

The current study used the neutron backscattering instrument located at the National Institute of Standards and Technology (NIST) Center for Neutron Research (Gehring and Neumann, 1998). The incident wavelength is at 6.271 Å with an energy resolution of 1 μeV. The accessible range of momentum transfer, Q, on this spectrometer is 0.25 to 1.75 $Å^{-1}$. For this study, the instrument was used in the elastic scattering mode. The measured elastic intensity, I_{el}, is then proportional to the value of the scattering function S(Q, E) at E = 0. For incoherent scatters such as hydrogen, I_{el} can be used to estimate the root mean squared (rms) amplitude of motions $\langle \Delta u^2 \rangle$ according to:

$$I_{el} \propto \exp[-Q^2 \langle \Delta u^2 \rangle] \quad (1)$$

For each sample, elastic scattering was measured starting at 40K in 2K increments to a temperature above the sample's endothermic denaturation temperature, T_m, which was previously determined by DSC (Bell et al., 1995). The highest temperature measured for hydrated lysozyme was lower due to moisture loss during the experiment. All measurements were performed inside a closed cycle helium refrigerator, which was modified for high temperature operation. At each temperature, scattering data were collected for 3 min. All the data were normalized to the average of the 40K and 50K data, meaning that the values of $\langle \Delta u^2 \rangle$ presented are offset by the values at ~45K.

RESULTS AND DISCUSSION

Fig. 1 shows the natural log of elastic peak intensity, I_{el}, plotted as a function of Q^2 for the 30% hydrated sample at several temperatures. According to Eq. 1, the slope yields the rms displacement, $\langle \Delta u^2 \rangle$. As expected, the slope increases with increasing temperature, reflecting increased thermal motion. As will be shown, the change in $\langle \Delta u^2 \rangle$ as a function of temperature may be used to predict the thermal stability of the protein. Fig. 2 shows the temperature dependence of $\langle \Delta u^2 \rangle$ for all four samples. With the exception of the data for the dry lysozyme, each curve displays a change in slope at a temperature termed the dynamic transition temperature (T_d; Doster et al., 1989). The value of T_d depends on the nature and amount of solvent present, shifting from 210K for hydrated lysozyme to 330K when the solvent was 20% deuterated glycerol (Table 1). More interestingly, T_d was completely suppressed when the protein powder was dry. Previous neutron scattering results (Fitter, 1999) demonstrated that the observed motions of dry α-amylase remained predominately vibrational up to 300K, and slower relaxational motions did not occur to an appreciable extent at the picosecond time scale. By analogy with these results, the dynamics of dehydrated lysozyme observed must also be predominantly vibrational on the nanosecond time scale up to its T_m (i.e., 429K). The change in slope observed at T_d for the other samples is interpreted as the onset of anharmonic motions resulting from structural relaxations within the solvated lysozyme. According to our results and those of Reat et al. (1998), the amplitude of these

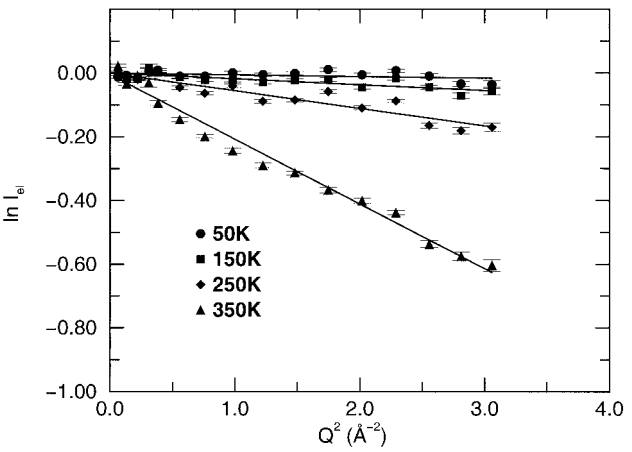

FIGURE 1 Natural log of elastic scattering intensity as a function of Q^2 for the 50:50 mixture of lysozyme and deuterated glycerol at four different temperatures. The slope of each line is taken as a measure of $\langle \Delta u^2 \rangle$ at the corresponding temperature.

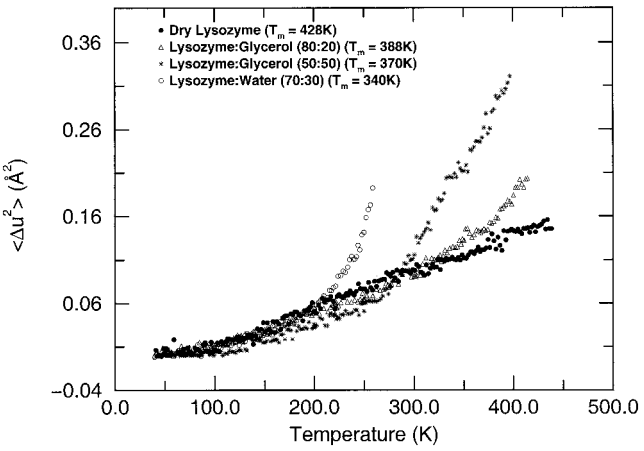

FIGURE 2 $\langle \Delta u^2 \rangle$ as a function of temperature for lysozyme samples. T_d was determined by visual inspection to be the temperature at which the slope of these curves changes.

anharmonic motions is reduced as the amount of solvation in the system is decreased, and the protein displays characteristics close to a harmonic solid when it is completely dehydrated. This result contradicts molecular dynamics simulations, which suggested dynamic transitions for water-soluble proteins are possible even without water (Steinbach et al., 1991; Smith, 1991).

The shift in T_d for lysozyme with respect to glycerol content is similar to the effect of water on protein dynamics. Therefore, even though glycerol is highly viscous, it is capable of facilitating protein motions at temperatures above T_d. As suggested by Fitter (1999), the existence of a dynamic transition is not a property unique to proteins. Pure glycerol also displays such a transition around 210K (Wuttke et al., 1995). Thus, our data indicate the importance of noncovalent interactions between the protein and additive (i.e., water or glycerol) in determining the value of T_d. Because protein structures are the result of a delicate balance between a population of noncovalent forces, interactions with a polyhydroxy compound, such as glycerol, may allow conformational averaging that is not possible in dehydrated lysozyme alone. The lowering of T_d with increasing glycerol content provides evidence for such a possibility, since anharmonic motions are crucial for conformational switching.

TABLE 1 Dynamic transition temperatures (T_d) and thermal denaturation temperatures (T_m) for lysozyme

Treatment	T_d (K)	T_m (K)
30% D_2O	210	343
50% glycerol	270	370
20% glycerol	330	388
dehydrated	n.d.	429

Data from Bell et al. (1995b).
n.d., not detected.

Though glycerol appears to facilitate anharmonic motions above T_d, it limits the amplitude of harmonic motions below T_d. From 40 to 200K, the values of $\langle \Delta u^2 \rangle$ were somewhat lower in the presence of glycerol than those observed in pure lysozyme (Fig. 2). More importantly, $\langle \Delta u^2 \rangle$ decreased with increasing glycerol concentration. Since the data in Fig. 2 are normalized to those at 45K, the values of $\langle \Delta u^2 \rangle$ plotted here are reduced by the magnitude of $\langle \Delta u^2 \rangle$ at 45K. Because the values of $\langle \Delta u^2 \rangle$ are approaching their zero-point limit at temperature as low as 45K, the values of $\langle \Delta u^2 \rangle$ are likely to be similar for the different samples. Thus, the reduction of $\langle \Delta u^2 \rangle$ observed for the glycerol-lysozyme mixtures are almost certainly real. In addition, the damping of $\langle \Delta u^2 \rangle$ has also been observed for two other proteins in a trehalose glass (Gottfried et al., 1996; Cordone et al., 1999). At first glance, one may be tempted to explain these results in terms of the viscosity of the mixtures. Below T_d, however, the pure lysozyme samples display similar values of $\langle \Delta u^2 \rangle$ whether dry or hydrated, arguing against a viscosity effect. Instead, Gottfried et al. (1996) suggested that in the dehydrated glassy solid, trehalose and glycerol may substitute for water in the protein's hydration shell. The polyols interact more strongly with the exposed moieties in the solid than does water. These enhanced interactions with polyols could stabilize the surface moieties to a greater degree than water, thereby reducing the protein vibrational dynamics. Thus, the lower harmonic amplitude seen in the current study could be due to tighter packing of the protein structure.

One of the objectives of this study was to explore the relationship between protein dynamics and protein stability. According to Tang and Dill (1998), an inverse correlation exists between protein flexibility and protein stability. They further pointed out that protein flexibility can be characterized statically or dynamically. In the static description, flexibility is reflected by the number of structural conformations in the equilibrium ensemble. In the dynamic description, flexibility is described by how quickly a protein can carry out its conformational switching, and the rate constant at which conformational switching takes place is a measure of the energy barriers between native and nonnative conformations. Based on these concepts, we believe that by measuring the $\langle \Delta u^2 \rangle$ of lysozyme powders, we arrive at an accurate description of how their thermal stability is affected by the presence of different solvents at different temperatures.

The value of T_d and the change in $\langle \Delta u^2 \rangle$ as a function of temperature may be used to characterize the heat lability of a protein sample. As shown in Table 1, higher T_d tends to result in higher T_m. A higher T_d suggests the protein is less susceptible to thermally induced unfolding. Furthermore, in the region beyond T_d, $\langle \Delta u^2 \rangle$ versus temperature had the steepest slope for the D_2O hydrated lysozyme and shallowest for its dry counterpart (Fig. 2). Between these two extremes, as more glycerol was mixed with lysozyme, the slope became increasingly steep. The sample with the small-

est response to temperature change may be considered to be the most dynamically stable, which is reminiscent of the strong/fragile classification of glass-forming liquids (Angell et al., 1994; Angell, 1995). Fragile materials characterized by a high density of configurational states are more susceptible to thermally induced structural changes during heating than are strong materials, which have a low density of configurational states (Branca et al., 1999). Applying this concept to protein systems, dehydrated lysozyme would be described as strong, showing minimal changes in molecular dynamics during heating. Fragility increases with the addition of glycerol, which is consistent with our earlier notion that conformational averaging was facilitated by glycerol and that the amount of glycerol determined the extent of possible conformational averaging. Glycerol facilitates protein motions, as seen in our experiments, which make the protein more fragile and less stable above T_d.

In pure lysozyme, the molecular dynamics did not show an observable change at the denaturation temperature, T_m, as indicated by the constant slope in Fig. 2. Thermal denaturation of dehydrated globular proteins has been shown to be largely attributable to covalent modification of the protein structure, whereas for hydrated proteins, denaturation can be attributed to molecular unfolding (Bell et al., 1995a). Our current results suggest that the detectable motions for both native and covalently modified proteins in dehydrated solids are similar and lack anharmonicity. Such was not the case for phosphoglycerate kinase in solutions, where quasielastic neutron scattering indicated dynamic changes between the native and denatured states (Receveur et al., 1997). These contradictory observations of the dynamics of proteins in solutions as compared to that in solids confirm that protein stability for solids cannot be extrapolated from solution data (Bell, 1997).

In light of these arguments, data from this study also provide insight into glycerol's paradoxical effect on the thermal stability of lysozyme. As mentioned in the Introduction, glycerol lowers the T_m of dry lysozyme but raises T_m for hydrated lysozyme (Bell et al., 1995b). From Fig. 2, it becomes apparent that in the anharmonic regime (i.e., from T_d to T_m), the allowable amplitudes of vibrational motions are defined by the fully hydrated and dry lysozyme, with the dry lysozyme exhibiting the least amount of thermal motion. The addition of glycerol facilitates motions not possible in the dry protein at temperatures above T_d, making it less dynamically stable and more heat labile. However, the addition of moisture to the lysozyme-glycerol mixture cannot facilitate more motion than that maximally possible in hydrated lysozyme alone. As a consequence, a hydrated lysozyme-glycerol mixture would appear to be more dynamically stable and less heat labile when compared to an equally hydrated pure lysozyme sample. Although preferential hydration has been used to explain the stabilizing effect of polyols on proteins in solutions (Timasheff, 1992), a similar stabilizing phenomenon may also occur in hydrated lysozyme-glycerol solids.

CONCLUSIONS

This study suggests that values of T_m measured by DSC for lysozyme solids (Bell et al., 1995b) appear to be related to molecular dynamics as determined by neutron scattering. Glycerol affects the molecular dynamics of lysozyme differently depending upon the temperature relative to T_d. Below T_d, glycerol suppresses vibrational motions, whereas above T_d, it facilitates anharmonic motions. In addition, the response of lysozyme to heating differs depending upon the amount of glycerol. Above T_d, glycerol increased the heat lability of lysozyme, suggesting that glycerol may have an effect similar to water in these samples. In a mixed system of protein and cosolvent, the global dynamics of a protein is not a slave of the solvent, nor does it exhibit inherent features regardless of the solvent environment. Taken together, the results from this study have illuminated the complex interdependence between the motions of the protein, water of hydration, and cosolvent.

We thank Dr. Christopher Soles for his critical insight into our data interpretation, Dr. Robert Dimeo for his assistance in the back scattering spectrometer measurements, Dr. Rick Paul for the prompt gamma-ray measurement for residual water, and Dr. Jack Rush for careful reading of the manuscript.

All mentions of brand names in this paper are for the purpose of clarity and do not constitute endorsements from NIST.

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