A Pulsed-Field Gradient NMR Study of Bovine Pancreatic Trypsin Inhibitor Self-Association†

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ABSTRACT: Previous studies have produced conflicting interpretations regarding the aggregation state of BPTI in solution. Here, pulsed-field gradient NMR self-association measurements have been performed with BPTI under a variety of temperature, pH, salt, urea conditions, and protein concentrations. Relative to the standard proteins, lysozyme, ribonuclease, and ubiquitin, diffusion constants indicate that BPTI dimerizes at concentrations above about 3 mg/mL and below 280 K. At higher temperatures, a marked self-association is observed above 10 mg/mL. The apparent lack of significant effects from variations in pH and NaCl concentration suggests minimal contribution to the aggregation process from charge−charge interactions. In contrast, in nondenaturing concentrations of urea (2 M), BPTI behaves as a monomer, suggesting that hydrophobic and polar residues modulate BPTI association. The BPTI surface shows that while one side is highly charged, the opposite side, composed mostly of hydrophobic and some hydrophilic residues, is feasible as an interface for BPTI self-association.

Bovine pancreatic trypsin inhibitor (BPTI)[1] is a widely investigated protein of 58 residues with a well-established crystal structure. Its small size, great stability, and high solubility make it an appealing model system for numerous experimental and theoretical studies on folding and dynamics of globular proteins. Although it is important to interpretation of solution studies, BPTI self-aggregation behavior is not fully understood. An early ultracentrifugation study of Kraut et al. (1960) suggested the presence of dimeric BPTI, and since then conflicting reports concerning BPTI self-association have appeared in the literature. Preliminary calorimetric data suggested that the ratio of calorimetric to van't Hoff enthalpies is less than 1, possibly reflecting aggregation (Privalov, 1979). However, later studies on BPTI indicated that this ratio is approximately unity, indicating the absence of significant self-association (Moses & Hinz, 1983; Makhatadze et al., 1993). Other papers on the subject before 1989 are reviewed in Gallagher and Woodward (1989). Dynamic light scattering studies (Wills & Georgalis, 1981; Gallagher & Woodward, 1989) indicated that although BPTI may undergo a rapid association−dissociation in solution, it is essentially monomeric. The absence of a BPTI concentration dependence in hydrogen/deuteron exchange kinetics of surface NHs has been interpreted as indicating the absence of aggregation (Tüchsen & Woodward, 1985; Tüchsen, 1987). Based on molecular modeling studies, however, Zielenkiewicz et al. (1991) proposed that BPTI does possess a self-complementary surface that could be the site of intermolecular interactions. In part, these conflicting reports may be resolved by considering the fact that various solution conditions (e.g., salt, pH, temperature, BPTI concentration), which may affect BPTI self-association, were used in different studies. This is especially critical if BPTI forms weak aggregates as indicated by an apparent association constant of 460 M^{-1} (Kraut et al., 1960).

To reexamine the question of self-association, we measured the translational diffusion coefficient, *D*, for BPTI using pulsed-field gradient (PFG) NMR (Steiskal & Tanner, 1965; Gibbs & Johnson, 1991) under a variety of solution conditions. PFG NMR is a convenient method for characterizing self-association and complexation in protein systems (Altieri et al., 1995; Dingley et al., 1995; Lennon et al., 1994; Lin et al., 1995; Mayo et al., 1996). Values of *D* have been obtained as a function of temperature and concentration at different pH values, at low and high NaCl concentration, and in the presence of a nondenaturing concentration of urea. BPTI dimerization is indicated at temperatures below 280 K and at protein concentrations greater than about 3 mg/mL. At higher temperatures, self-association is observed at higher protein concentrations. Changing the ionization state of BPTI or the solution dielectric has little if any effect on dimerization. In 2 M urea solution, self-association is not observed at 10 mg/mL even at temperatures below 280 K. The results of this study define the solution conditions under which BPTI dimerization becomes a significant concern.

MATERIALS AND METHODS

BPTI Purification. BPTI purchased from Novo Corp. (Denmark) was further purified on a preparative reverse-phase HPLC C-18 column using an elution gradient of 0−60% acetonitrile with 0.1% trifluoroacetic acid in water. Eluted BPTI was dialyzed against water and lyophilized. Lysozyme, BSA, ribonuclease, and ubiquitin were used as obtained from Sigma Chemical Co. (St. Louis, MO). The average molecular weights of proteins were determined from

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[1] Abbreviations: NMR, nuclear magnetic resonance; PFG, pulsed-field gradient; rf, radio frequency; FID, free induction decay; BPTI, bovine pancreatic trypsin inhibitor.

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the amino acid sequence as 6500 (BPTI), 14 300 (lysozyme), 66 000 (BSA), 13 600 (ribonuclease), and 8600 (ubiquitin).

Pulsed-Field Gradient NMR Self-Diffusion Measurements. For NMR measurements, proteins were dissolved in 0.6 mL of unbuffered D_2O, and the pH was adjusted by adding microliter quantities of NaOD or DCl. Pulsed-field gradient (PFG) NMR self-diffusion measurements were made on a Bruker AMX-600 using a GRASP gradient unit. NMR spectra for measurement of diffusion coefficients, *D*, were acquired using a 5 mm triple-resonance probe equipped with an actively shielded z-gradient coil. The maximum magnitude of the gradient was calibrated using the manufacturer's standard procedure based on the frequency spread of the applied gradient and was found to be 100 G/cm. This was consistent with the value of 98 G/cm obtained from analysis of PFG data on water using its known diffusion constant (Mills, 1973). The linearity of the gradient was checked by performing diffusion measurements on water over different ranges of the gradient. The PFG longitudinal eddy−current delay pulse-sequence (Gibbs & Johnson, 1991) was used for all self-diffusion measurements which were performed in D_2O over the temperature range 271−310 K. The concentration of standard proteins is 10 mg/mL; BPTI concentration is varied as indicated in the figure legends.

For unrestricted diffusion of a molecule in an isotropic liquid, the PFG NMR signal amplitude, *A*, normalized to the signal obtained in the absence of gradient pulses, is related to *D* by

$$A = \exp[-\gamma^2 g^2 \delta^2 D(\Delta - \delta/3)] \quad (1)$$

where *g* is the gyromagnetic ratio of the observed nucleus; *g* and *d* are the magnitude and duration of the magnetic field-gradient pulses, respectively, and *D* is the time between the gradient pulses (Stejskal & Tanner, 1965). For these experiments, $d = 4$ ms, $g = 1-75$ G/cm, $D = 34.2$ ms, and the longitudinal eddy−current delay $T_e = 100$ ms. Each diffusion constant, *D*, was determined from a series of 15 one-dimensional PFG spectra acquired using different *g* values. Diffusion coefficient measurements were calibrated by performing the PFG NMR self-diffusion measurements on the well-studied proteins BSA, lysozyme, ribonuclease, and ubiquitin. Here, PFG measurements yield *D* values at 293 K of 10.1×10^{-7} cm^2/s for lysozyme (10 mg/mL), 10.2×10^{-7} cm^2/s for ribonuclease (10 mg/mL), and 14.3×10^{-7} cm^2/s for ubiquitin (10 mg/mL). These *D* values agree reasonably well with those obtained from light scattering by extrapolation to infinite dilution: 10.6×10^{-7} cm^2/s for lysozyme (Dubin et al., 1971) and 10.7×10^{-7} cm^2/s for ribonuclease (Squire & Himmel, 1979), and obtained by using similar PFG NMR measurements on ubiquitin, 14.9×10^{-7} cm^2/s (Altieri et al., 1994). The *D* values calculated from our measurements at 298 K (11.1×10^{-7} cm^2/s for lysozyme and 5.8×10^{-7} cm^2/s for bovine serum albumin) were also close to the literature data: 11.5×10^{-7} cm^2/s (Dubin et al., 1967) and 11.0×10^{-7} cm^2/s (Mikol et al., 1990) for lysozyme and 5.8×10^{-7} cm^2/s (Wattenbarger et al., 1992) for BSA. The relatively good agreement in diffusion coefficients indicates that the PFG longitudinal eddy−current delay pulse sequence allows derivation of accurate diffusion constant values.

The Stokes−Einstein equation $D = k_B T/6\pi\eta R$ was used to relate *D* to the macromolecular radius, *R*, which in turn was considered to be proportional to a power, *a*, of the apparent molecular weight, M_{app}. For solid spheres, *R* is proportional to $M_{app}^{1/3}$, while for random coils *R* is proportional to $M_{app}^{1/2}$ (Cantor & Schimmel, 1980). Typically $a = {}^1/_3$ is used for compact globular proteins. In general, when comparing diffusion coefficients for two proteins, 1 and 2, under the same conditions:

$$(D_1/D_2)^a = M_{2\ app}/M_{1\ app} \quad (2)$$

Therefore, the diffusion coefficient for a monomeric protein of known molecular weight can be used as a standard to evaluate M_{app} for another protein. Use of the Stokes−Einstein relationship has been derived specifically for a hard sphere. The actual molecular shape and the shape of the molecular aggregate are expected to affect the diffusion coefficient. The maximum change, for example, in D_{dimer} due to dimer geometry compared to *D* of a spherical molecule of equal volume would be about 5% (Teller et al., 1979). M_{app} for BPTI was calculated by using *D* values for lysozyme, ribonuclease, and ubiquitin as standard monomers of known molecular weight.

RESULTS

An exemplary stack plot of pulsed-field gradient (PFG) NMR spectra for BPTI is shown in Figure 1. Arrows indicate the three resonances used to calculate values of *D*. Typical PFG decay curves for BPTI and lysozyme are shown in Figure 2. Data are presented as resonance intensities normalized to those at $g = 1$ G/cm. Fifteen data points, each with a different PFG strength (only five such points are shown in Figure 1), were routinely acquired for each set of solution conditions. Curves were approximated as single exponentials. The slope of each linear least-squares fit line is related to the self-diffusion coefficient by eq 1. For each reported value of *D*, the decay of each of the three resonances indicated in Figure 1 was measured, and the three resulting *D* values were averaged with a standard deviation of about 3%. For experiments done at lower protein concentrations, this deviation remained essentially unchanged since additional transients were averaged to maintain the same NMR spectral signal to noise ratio.

Since diffusion data acquired at one temperature and at one concentration allow for a limited interpretation, the temperature dependence of the diffusion coefficients for the protein standards and BPTI was investigated. In the absence of aggregation at low protein concentration, a plot of log *D* versus $1/T$ is expected to follow the Arhhenius relation showing a linear slope reflecting the activation energy for the self-diffusion of water, i.e., 4.8 kcal/mol (Mills, 1973). Figure 3 shows that the inverse temperature dependence of *D* (plotted in a semilogarithmic scale) for the standard proteins lysozyme, ribonuclease, and ubiquitin, is linear above 280 K with a slope corresponding to the activation energy of water self-diffusion. The error in determining individual *D* values is within the confines of each symbol in the figure. The apparent molecular weights calculated for standard proteins from their *D* values are in good agreement with their actual molecular weights, indicating that all are monomers at 10 mg/mL and at temperatures above 280 K. Interestingly, at temperatures below 280 K, decreasing *D* values for ribonuclease and ubiquitin deviate

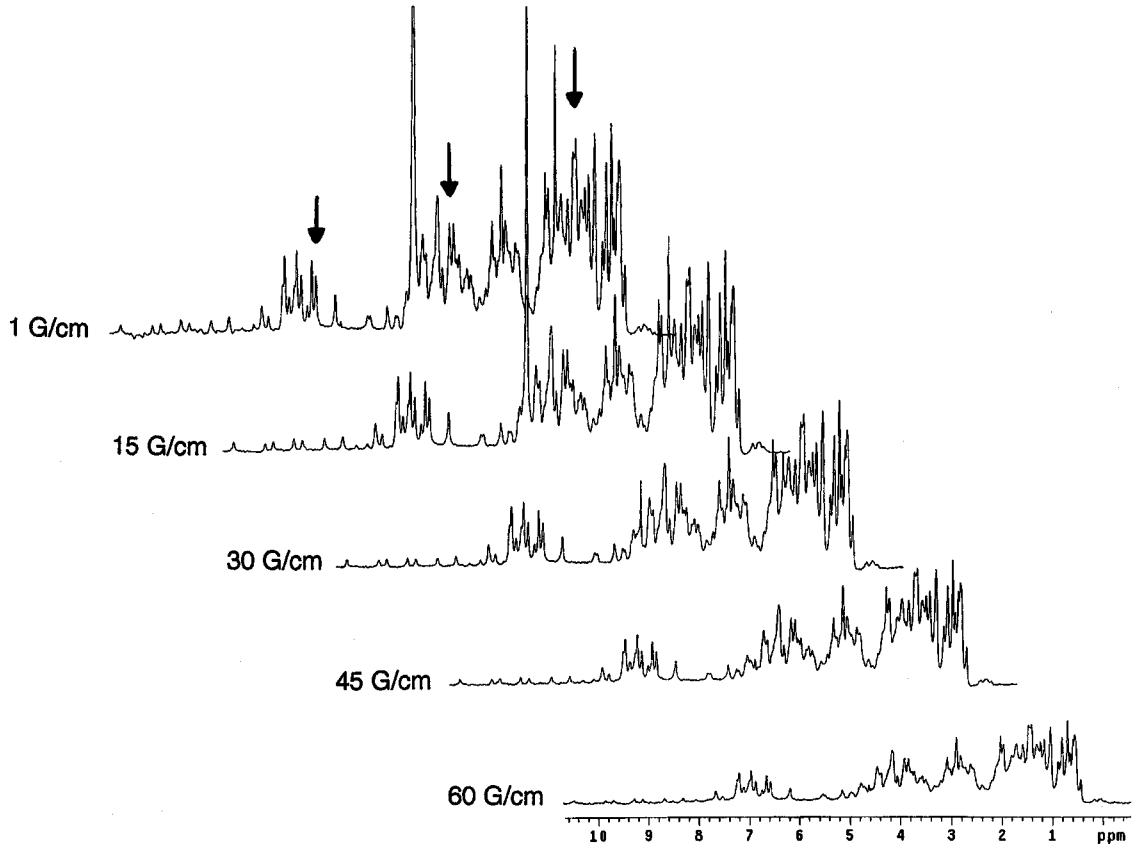

FIGURE 1: Stack plots of pulsed-field gradient NMR spectra for BPTI are shown for five values of gradient strength: 1 G/cm, 15 G/cm, 30 G/cm, 45 G/cm, and 60 G/cm. These data have been acquired at pH 5.5, 293 K, and 10 mg/mL BPTI. Arrows indicate which three resonances have been used to derived diffusion coefficient data.

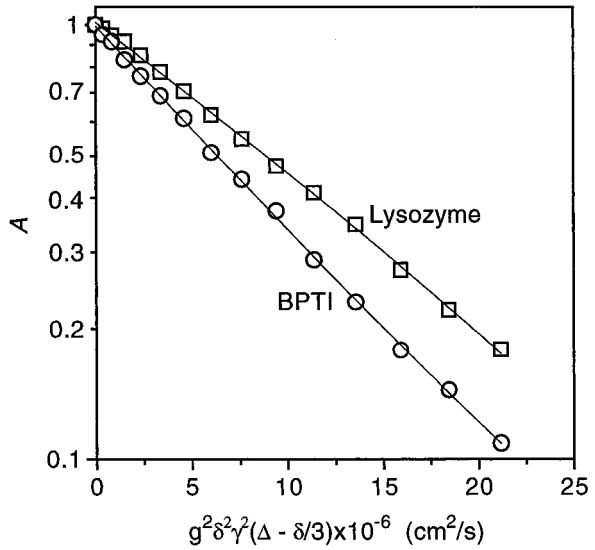

FIGURE 2: Representative pulsed-field gradient NMR measurements. The normalized resonance amplitude, *A*, of BPTI and lysozyme is shown as a function of $g^2\gamma^2\delta^2(\Delta - \delta/3) \times 10^{-6}$ s/cm^2. Data shown were acquired at pH 5.5 and 293 K on sample concentrations of 10 mg/mL.

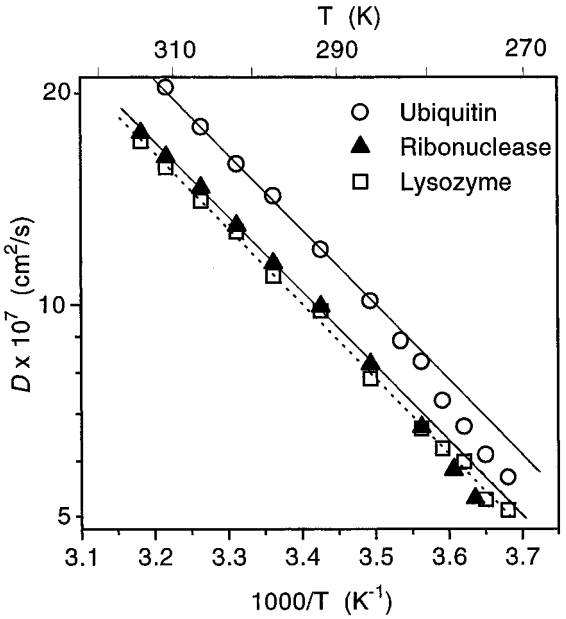

FIGURE 3: Temperature dependence of the diffusion coefficients of standard proteins, lysozyme, ribonuclease, and ubiquitin. The slope of the lysozyme Arrhenius curve, shown as a dotted line, is the same as for the self-diffusion of water (see text). Solid parallel lines are drawn through data for ribonuclease and ubiquitin to show low-temperature deviations from linearity as discussed in the text.

from the expected slope (solid lines), whereas those for lysozyme (dashed line) are linear over this entire range of temperatures. Apparently ribonuclease and ubiquitin have a tendency to associate at lower temperatures, while lysozyme does not. The increase in apparent molecular weight for ribonuclease and ubiquitin accounts for no more than 20% from their respective monomeric values. From these data, we conclude that lysozyme can be used as a standard protein for molecular weight calibration over the temperature range from 271 K to 310 K, while ribonuclease and ubiquitin are also appropriate standards at temperatures above 280 K.

For BPTI at 1, 10, and 30 mg/mL semilogarithmic plots of *D* versus 1/*T* are presented in Figure 4. For reference, the lower solid line shows the behavior of lysozyme. At a BPTI concentration of 1 mg/mL (triangles in Figure 4), the

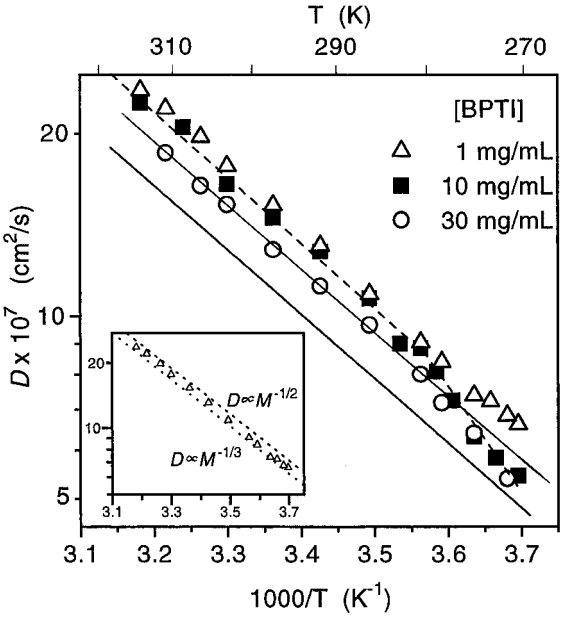

FIGURE 4: Temperature dependence of PFG NMR-derived diffusion coefficients is shown for BPTI concentrations of 1, 10, and 30 mg/mL at pH 5.5, in 150 mM sodium chloride and with no additional salt. For reference, the standard curve for lysozyme (solid line) also has been redrawn. Solid and dashed lines have been drawn through data for 30 and 10 mg/mL, respectively, to show low-temperature deviations from linearity as discussed in the text. The insert shows the data at 1 mg/mL where the dotted lines represent hypothetical *D* values for monomeric BPTI calculated from the *D* values of standard proteins by using eq 2 with $a = {}^1/_2$ (upper line) and ${}^1/_3$ (lower line).

curve is linear throughout the entire temperature range, indicating that the aggregation state does not change with temperature. The insert at the lower left corner replots these 1 mg/mL data with the dotted lines representing the trend in *D* values for BPTI calculated relative to those for lysozyme by using eq 2 with $a = {}^1/_2$ (upper dotted line) and ${}^1/_3$ (lower dotted line). The experimental values for BPTI at 1 mg/mL lie between both dotted lines, indicating that, in reality, the value of *a* is neither ${}^1/_2$ nor ${}^1/_3$ but somewhere in between and supports the conclusion that BPTI at this concentration can be considered monomeric. At temperatures above 280 K, increasing the BPTI concentration to 10 mg/mL (Figure 4) only slightly decreases *D* values, implying at most weak self-association.

Below 283 K, however, for BPTI at 10 and 30 mg/mL the slope increases significantly, and *D* values reflect an increasing apparent molecular weight. At 271 K, *D* is not much larger than that for lysozyme and is consistent with an apparent molecular weight of mostly dimeric BPTI. The temperature dependence of *D* at the highest concentration, 30 mg/mL, is also shown in Figure 4. Lower *D* values indicate that at temperatures above 280 K, BPTI is more aggregated at 30 mg/mL than at 10 mg/mL. A line has been drawn through the 30 mg/mL data points to show that there is significant deviation from linearity at lower temperatures. As mentioned above, the error in determining individual *D* values lies within the confines of the symbol. It is noteworthy that the concentration dependence of *D* values for lysozyme is very weak for concentrations not exceeding 30 mg/mL (Mikol et al., 1990). *D* values for lysozyme at 5 mg/mL, 10 mg/mL, 20 mg/mL, and 30 mg/mL were also checked here using PFG NMR diffusion measurements and found to be essentially identical. This implies that the

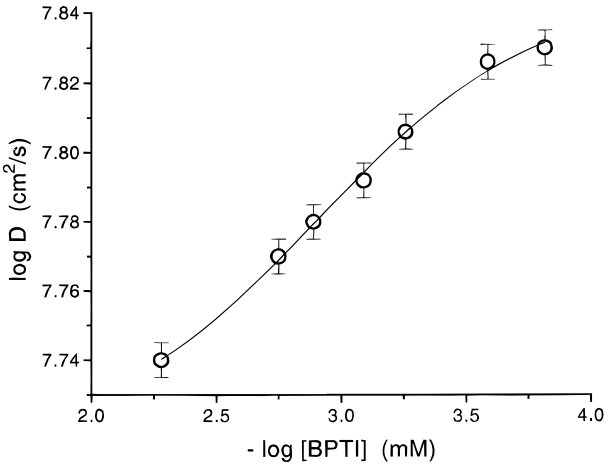

FIGURE 5: Concentration dependence of the diffusion coefficients of BPTI at 271 K, pH 5.5, and 150 mM sodium chloride. The circles with the error bars represent theexperimental values with the corresponding standard errors. The solid line is the theoretical fitting from the monomer−dimer model with $k = 0.7 \times 10^{-3}$ M, $D_{monomer} = 7.1 \times 10^{-7}$ cm^2/s, $D_{dimer} = 5.0 \times 10^{-7}$ cm^2/s.

difference in BPTI *D* values at 10 and 30 mg/mL does not arise from a viscosity effect.

To better characterize the self-association of BPTI at low temperature, log *D* vs log of the BPTI concentration is plotted in Figure 5. These diffusion data were accumulated at 271 K where differences in *D* values for BPTI (Figure 4) appear to be greatest. For a monomer−dimer equilibrium, the fraction of monomer BPTI, α, can be evaluated by using a simple relationship between the experimental value of *D* and the weighted sum of diffusion coefficients for monomer, D_1, and dimer, D_2, molecules:

$$D = \alpha D_1 + (1 - \alpha) D_2 \qquad (3)$$

Experimental diffusion coefficients at 1 mg/mL BPTI were used for D_1. To obtain D_2 , two approaches were used: (1) take $0.72 \times D_1$ which is theoretically predicted for the ratio $D_{dimer}/D_{monomer}$ for a two-sphere dimer (Wills & Georgalis, 1981); or (2) average D_2 values calculated from eq 2 with an apparent molecular weight for BPTI of 13 000 for both $a = {}^1/_2$ and ${}^1/_3$. The solid line represents the best fit of these data to a monomer−dimer equilibrium with a dissociation constant of 0.7×10^{-3} M using the second approach. The first approach also gave a good fit to the data. This confirms the conclusion initially based on molecular weight comparisons to standard proteins that BPTI does form dimers under these conditions.

For insight into the forces modulating BPTI self-association, the influence of pH, salt, and urea on the diffusion constant was investigated. The only distinguishable difference in *D* values at pH 1.5, 5.5, and 7.5 is a small decrease in apparent molecular weight at low temperature and pH 1.5 or 7.5 when compared to pH 5.5. This observation suggests a small contribution to dimerization from electrostatic forces. Addition of up to 200 mM NaCl, however, produces no measurable differences in *D*. In contrast, addition of a nondenaturing concentration of urea (2 M) effectively dissociates BPTI at all temperatures. Figure 6 shows diffusion constant data for BPTI in 2 M urea. The curve is linear through the entire temperature range. Moreover, relative to the lysozyme curve under the same urea conditions (Figure 6), BPTI remains monomeric even at temperatures

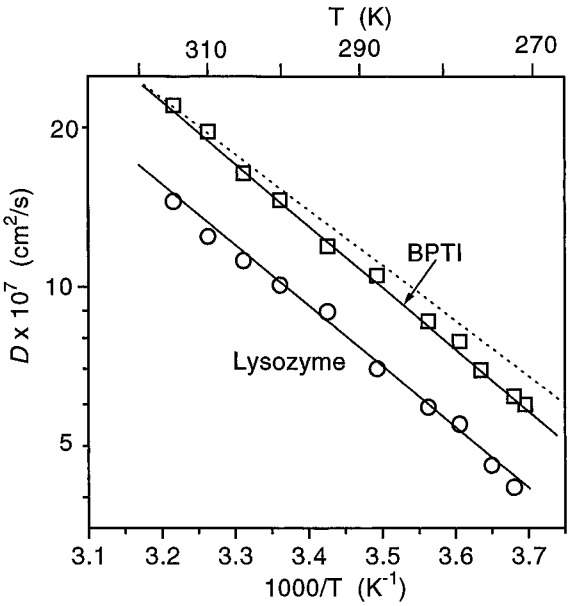

FIGURE 6: Temperature dependence of diffusion coefficients in 2 M urea for BPTI (10 mg/mL) and lysozyme, pH 5.5, in 150 mM sodium chloride. The dotted line shows the temperature dependence of the diffusion coefficient of lysozyme in the absence of urea.

below 280 K. The slope is somewhat steeper than that in water alone (dotted line Figure 6), reflecting differences in activation energies of different solutions.

DISCUSSION

The results reported here demonstrate that at concentrations above 0.3−0.4 mM (2−3 mg/mL) and at temperatures below 280 K, BPTI undergoes significant self- association. Based on PFG NMR-derived translational diffusion constants which have been calibrated against those of standard lysozyme, the highest apparent molecular weight achieved for BPTI under any conditions is below 13 000. Moreover, the concentration dependence of diffusion constants measured at 271 K can be reasonably approximated using a simple monomer−dimer equilibrium with a dissociation constant of 0.7×10^{-3} M. This value is in good agreement with that reported earlier by Kraut et al. (1960) and Anderer and Hoernle (1965), i.e., 2.2×10^{-3} M at 298 K. Even though some small fraction of large aggregates cannot be excluded, we conclude that BPTI dimers form the dominant higher aggregation state.

At temperatures above 280 K, BPTI self-association is less pronounced, but detectable at high protein concentrations. At 10 mg/mL, a small increase in the apparent molecular weight, indicated by decreased values of *D*, is observed (Figure 4). At 30 mg/mL, a considerably larger fraction of BPTI molecules self-associate. This accounts for the further decrease in diffusion constants, corresponding to an average molecular weight of 10 200. For a monomer−dimer equilibrium, this effectively translates into a distribution of approximately 65% dimer and 35% monomer BPTI at 30 mg/mL.

The condition dependence of BPTI self-association observed in these studies helps explain the apparent discrepancies in the literature. For example, concordant with the PFG results, BPTI self-association is not indicated by differential scanning calorimetry experiments performed at temperatures above 280 K and protein concentrations in the range of 1−2 mg/mL (Hinz & Moses, 1983; Makhatadze et al., 1993). BPTI dimerization was also not apparent in studies of hydrogen isotope exchange rates of surface amide protons at pH <4.5 and 298 K over the range 1.3−26 mg/mL (Tüchsen & Woodward, 1985; Tüchsen, 1987), even though under these conditions at the higher BPTI concentrations significant dimerization may have been present.

Dynamic light scattering studies on BPTI were done at 293 K as a function of pH, ionic strength, and concentration. These results indicated that BPTI at infinite dilution behaves as a hydrated monomer having a degree of hydration less than a monolayer of water (Gallagher & Woodward, 1989), consistent with the results of this study. The BPTI concentration-dependent (5−70 mg/mL) light scattering was suggested to arise from weak attractive forces which affect the apparent diffusion coefficient by altering the radial distribution function in a way that leads to greater hydrodynamic interactions. At temperatures greater than 280 K, the PFG results show a minimal concentration dependence between 1 and 10 mg/mL, but at 30 mg/mL a significant decrease in the diffusion constant is observed (Figure 4). In contrast, the scattering-derived diffusion constant under these conditions (293 K, pH 5.5) shows a linear dependence on protein concentration from 5 to 70 mg/mL [see Figure 5 in Gallagher and Woodward (1989)]. Although the actual reason for this discrepancy is unknown, two possibilities may be offerred: (1) light scattering is least sensitive to size changes in globular proteins with molecular weights below about 10 000−15 000, and (2) buffer conditions are different since in the scattering study 20 mM succinate and 0.3 M KCl were used.

The associative behavior observed by PFG NMR is not dependent on salt concentration or pH, but is absent when the solution is 2 M in urea. This level of urea is well below denaturation concentrations for BPTI. In terms of the forces which contribute to dimer formation, therefore, these results strongly suggest that the BPTI dimerization process is not modulated primarily by electrostatic effects and likely involves mostly hydrophobic interactions. The linearity of the entire *D* versus temperature curve for BPTI in 2 M urea, having the same slope as the curve for lysozyme (Figure 5) which does not aggregate under these conditions, implies that 2 M urea preferentially disrupts BPTI intermolecular surface interactions rather than intramolecular contacts. Furthermore, the observations that dimers are the predominant low-temperature aggregation state for BPTI and that low concentrations of urea eliminate self-association support the dimer model of Zielenkiewcz et al. (1991). There it was proposed that a self-complementary, highly hydrophobic surface of BPTI constitutes the monomer−monomer interface.

In conclusion, these PFG NMR studies outline the condition dependence of BPTI self-association. Given the fact that BPTI dimerizes at concentrations used in some physical studies, caution must be exercised in interpreting those experimental data.

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