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Translational and Rotational Diffusion of a Small Globular Protein under Crowded Conditions

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Abstract

Protein protein interaction is the fundamental step of biological signal transduction. Interacting proteins find each other by diffusion. To gain insight into diffusion under the crowded conditions found in cells, we used nuclear magnetic resonance spectroscopy (NMR) to measure the effects of solvent additives on the translational and rotational diffusion of the 7.4 kDa globular protein, chymotrypsin inhibitor 2. The additives were glycerol and the macromolecular crowding agent, polyvinylpyrrolidone (PVP). Both translational diffusion and rotational diffusion decrease with increasing solution viscosity. For glycerol, the decrease obeys the Stokes Einstein and Stokes Einstein Debye laws. Three types of deviation are observed for PVP: the decrease in diffusion with increased viscosity is less than predicted, this negative deviation is greater for rotational diffusion, and the negative deviation increases with increasing PVP molecular weight. We discuss our results in terms of other studies on the effects of macromolecules on globular protein diffusion.

Keywords

macromolecular crowding; nuclear magnetic resonance; viscosity

1. Introduction

Understanding the details of protein complex formation is important because protein assemblies transmit, integrate, and transduce biological signals. To form a complex, the partner proteins must not only encounter one another, but also find each other's binding site. These events involve translational and rotational diffusion, respectively.

Theory provides molecular level insight into complex formation in dilute solution,[1] but these conditions are far from those found in cells. Macromolecules can occupy up to 30% of a cell's volume and reach concentrations of 100 g/L to 400 g/L.[2] Such large volume occupancies will affect diffusion, but relatively little attention has been paid to the effects of crowding, despite the fact that crowding can increase protein stability,[3] accelerate folding,[4] and promote aggregation.[5]

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Supporting Information Available: Rotational and translational diffusion measurements in PVP. This material is available free of charge via the Internet at http://pubs.acs.org.

Diffusion is quantified as the translational (D_t) diffusion and rotational (D_r) diffusion coefficients. D_t and D_r are related to viscosity *via* the Stokes Einstein law, $D_t=\kappa T/6\pi\eta r$, and Stokes Einstein Debye law $D_r=\kappa T/8\pi\eta r^3$, where η is viscosity, κ is the Boltzmann constant and r is the protein's radius. [6-8] These relationships, however, apply only to spherical proteins in homogenous solution. Solution homogeneity means that the test protein is dramatically larger than the solute(s) controlling the viscosity. These criteria are not likely to apply to the cellular interior. Although it may sometimes be reasonable to treat a globular protein as a sphere, the cytosol is not homogeneous, and test proteins will be about the same size as the crowding molecules.

It seems likely that macromolecular crowding will cause deviation from these simple relationships. Deviation comes in two forms, negative and positive. Negative deviation means that increased viscosity decreases diffusion less than is predicted by the Stokes equations. Positive deviation is the opposite. Studies of globular protein diffusion in synthetic macromolecular crowding agents reveal negative deviation for both translational and rotational diffusion.[9-15] In studies of globular protein diffusion in solutions of globular proteins, negative deviation is observed for rotation,[16] but only slight negative deviation and positive deviation are observed for translation.[10,16,17] In summary, both globular proteins and synthetic polymers affect the rotational diffusion of globular proteins less than is expected, synthetic polymers affect transitional diffusion less than is expected, but globular proteins have either a small negative or a positive effect on translational diffusion.

Up to now, fluorescence has been the main tool for quantifying protein diffusion under crowded conditions. Here we report the first use of NMR with an ^{15}N enriched protein to study globular protein diffusion under crowded conditions. Specifically, we quantify the translational and rotational diffusion of chymotrypsin inhibitor 2, a 7.4 kDa globular protein, in solutions of a small molecule, glycerol, and the synthetic polymer poly(vinylpyrrolidone) (PVP). PVP is a random coil polymer[18] that interacts only weakly with chymotrypsin inhibitor 2.[3] We use PVP solutions in the dilute to semidilute range (i.e., up to concentrations where the PVP molecules overlap[19]).

2. Experimental Section

^{15}N enriched chymotrypsin inhibitor 2 was expressed and purified as described.[3,20] Viscosities were measured with a Viscolite 700 viscometer (Hydramotion Ltd., England). Experiments were performed on a 600 MHz Varian Inova spectrometer equipped with a standard triple resonance HCN probe with three axis gradients. Translational diffusion was measured by using a heteronuclear stimulated echo sequence.[21] Gradient strengths ranged from 1.2 G/cm to 58.0 G/cm.[22] Rotational diffusion was assessed from the ^{15}N T_1/T_2 ratio.[23] The pulse sequences used were from the Biopack software supplied with the instrument. The ^1H dimension was acquired with a sweep width of 12000 Hz and consisted of 1024 complex points. The ^{15}N dimension was acquired with a sweep width of 2500 Hz and consisted of 64 and 128 complex increments for chymotrypsin inhibitor 2 in PVP and in glycerol, respectively. For T_1 measurements in PVP, relaxation delays were set to 0.01, 0.5, 0.9, 1.1, 1.3 and 1.5 s. Delays of 0.01, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0 s were chosen for the glycerol experiments. For T_2 measurements in PVP, the delays were 0.01, 0.03, 0.05, 0.07, 0.09, 0.11 s. Delays of 0.01, 0.07, 0.11, 0.15, 0.17, 0.21, 0.25 s were used for the glycerol experiments. Eight transients were acquired per spectrum. The data were processed with NMRPipe [24]and NMRView.[25] T_1 and T_2 values were averaged.

3. Results and Discussion

^1H based pulsed field gradient methods are widely used to measure the D_t of biological macromolecules in dilute solution. Making these measurements in viscous, crowded solutions,

however, is difficult for two reasons. First, the ^{1}H longitudinal magnetization decays too quickly to quantify test protein diffusion. Second, background signals from the crowding agent dominate the spectra. Heteronuclear stimulated echoes overcome these problems by using a longer lived heteronucleus,[21] in this instance, ^{15}N, which is absent from the crowding molecule. Figure 1 shows the amide ^{1}H signals from ^{15}N enriched chymotrypsin inhibitor 2 in PVP solution. Their intensity decreases with increasing gradient strength. Figure 2 shows plots of the logarithm of the relative intensity *versus* gradient strength. The slopes are the translational diffusion coefficients.

NMR can also provide information about rotational dynamics. These dynamics affect the longitudinal relaxation time T_1 and the transverse relaxation time T_2.[26] Both relaxation times are captured in the rotational correlation time, τ_m, which is proportional to $1/D_r$. We obtained τ_m from the value of T_1/T_2.[23,27]

The results for glycerol, 10 kDa PVP, 40 kDa PVP, and a mixture of the two are shown in Figure 3, where the ratio of the diffusion coefficients in buffer to those in the solvent additive is plotted against the macroscopic viscosity relative to that of water.

In glycerol, both rotational and translational diffusion follow the Stokes laws. This result is expected because the experimental system is in accord with the assumption used to define the laws. Specifically, glycerol (93 Da) and water (18 Da) are much smaller than chymotrypsin inhibitor 2. Additionally, the close agreement with theory suggests that chymotrypsin inhibitor 2 can be treated as a sphere.

Macromolecular crowding causes dramatic negative deviations from the Stokes Laws (Figure 3), and PVP reduces translational diffusion more than rotational diffusion. The size of the crowding molecule is also important. The same weight concentration of 40 kDa PVP slows both types of diffusion more than 10 kDa PVP. The data for the 1:1 mixture of 10 kDa and 40 kDa PVP show that the larger polymer dominates the negative deviation. These observations agree with results from studies using synthetic polymers with fluorescence detection,[9,11] showing that NMR is a useful tool. Most importantly, our results support the idea that the nature of the solvent additive is important for understanding the diffusional effects.

In terms of protein complex formation, our data and those of others,[11,14] show that macromolecular crowding has a small effect on the ability of one protein to find the other's binding site because crowding has a small effect on rotational diffusion. This observation makes intuitive sense because connecting monomers into a polymeric crowding agent should have a small effect of the rotational freedom of the test protein, provided that chemical interactions between the crowder and the test protein are weak.

The effects of macromolecular crowding on collisional frequency are harder to rationalize because synthetic polymers and globular proteins have opposite effects on translational diffusion.[9-17] Whereas synthetic polymers slow diffusion less than is expected by the Stokes Einstein law, globular proteins can have a larger than expected effect. Strong crowder test protein interactions, which are expected in cells, will only exasperate this trend.

4. Conclusions

In summary, we report the first application of heteronuclear NMR to quantify the translational and rotational diffusion of a globular protein in crowded solutions. Consistent with the results using other techniques, our data show that both types of diffusion are less affected than predicted by the Stokes laws, with rotational diffusion being more negatively affected than translational diffusion. The size of the crowding molecule compared to the test protein also influences diffusion, with the larger crowding molecules playing a dominate role.

Our work also highlights a difference between synthetic polymers and globular proteins as crowding agents; synthetic polymer crowding agents cause negative deviation for the translation of globular proteins, whereas globular protein crowding agents cause nearly negligible or even positive deviation. Additional studies using independent techniques are needed to address this difference, but understanding its molecular origin is key to understanding protein movement in cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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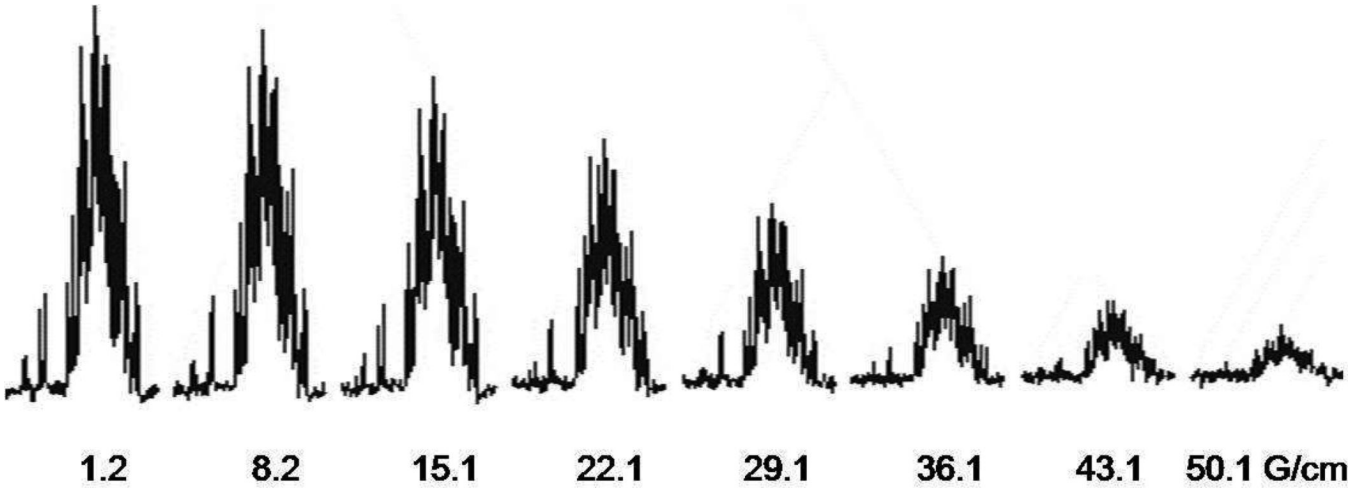

Figure 1.

^{15}N filtered ^{1}H spectra of 1mM ^{15}N enriched chymotrypsin inhibitor 2 in 100 g/L 10 kDa PVP (50 mM acetate buffer; pH 5.4; 25°C; 0.3 s diffusion time; 0.02 ms gradient duration) with increasing gradient strength (G/cm).

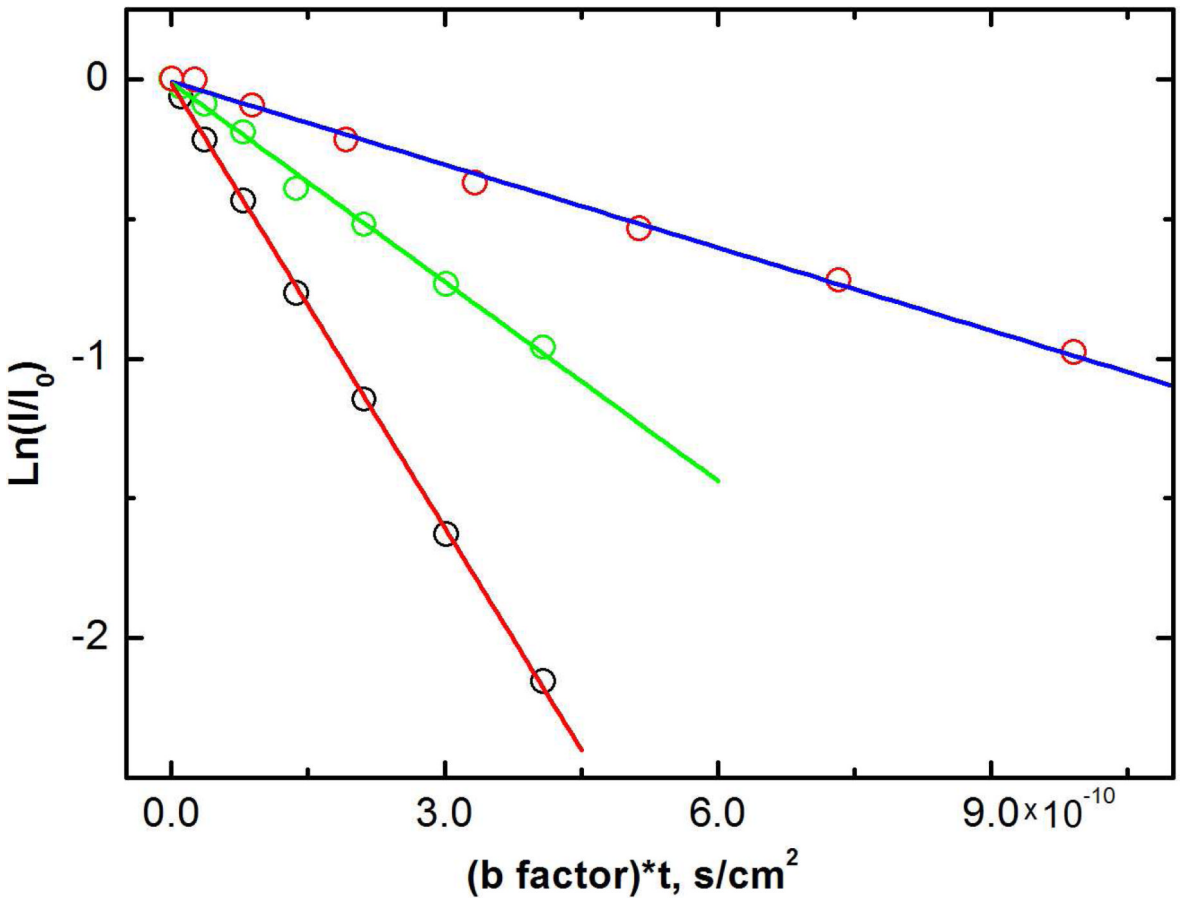

Figure 2.
Translational diffusion results for chymotrypsin inhibitor 2 in 1:1 (g/g) mixture of 40 kDa :10 kDa PVP [Red, 100 g/L; green, 200 g/L; blue, 300 g/L, $b = (\gamma G\delta)^2\Delta$]. The conditions are defined in the caption to Figure 1.

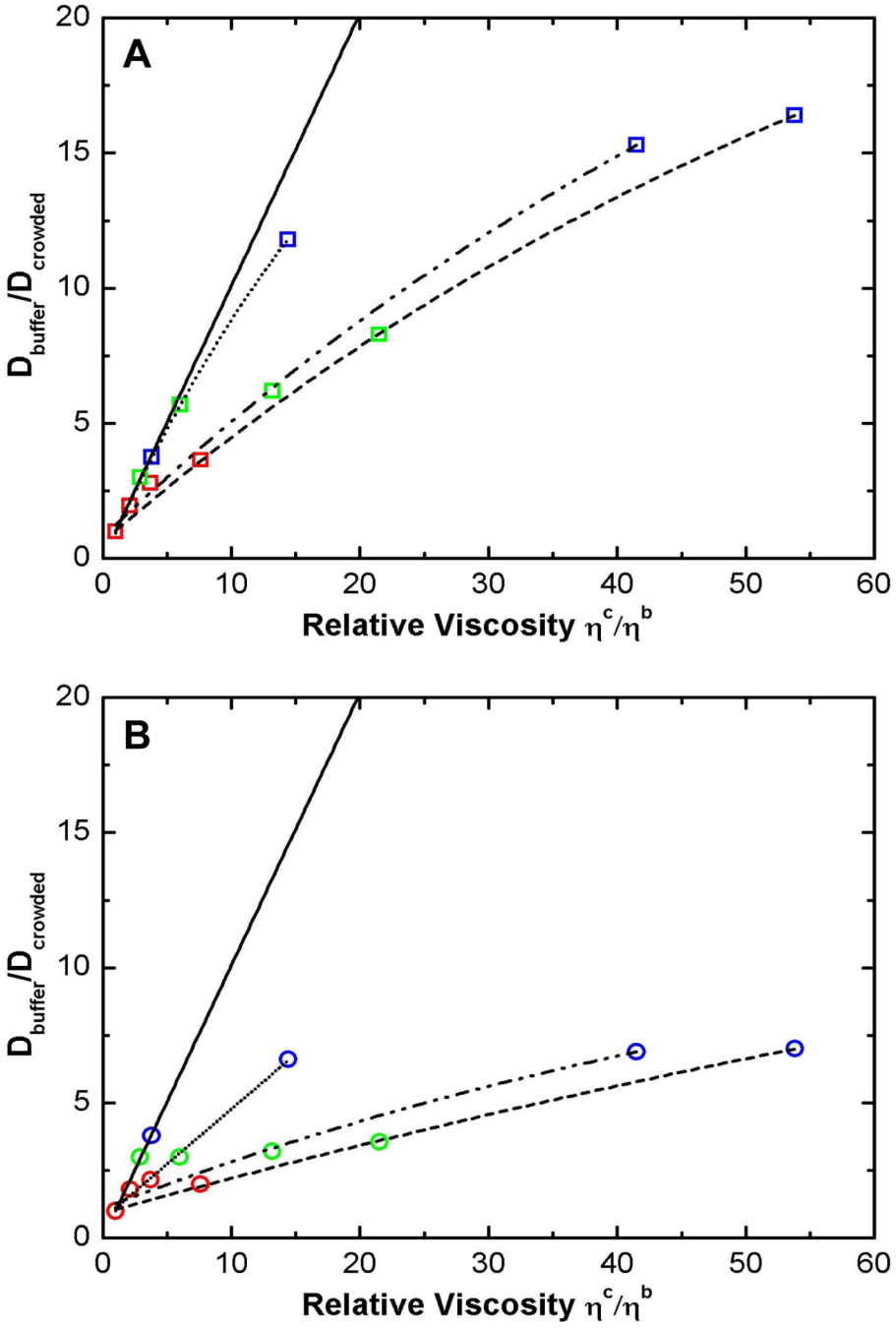

Figure 3.

Translational (A) and rotational (B) diffusion of chymotrypsin inhibitor 2 in a small molecule, glycerol (—) and family of differently sized polymers [10 kDa PVP (••), 40 kDa PVP (– –), 1:1 (g/g) mixture of 40 kDa :10 kDa PVP (– ••)]. The conditions are defined in the caption to Figure 1. The colors are defined in the caption to Figure 2. The curves for PVP are of no theoretical significance. For glycerol, the line extends beyond the points to illustrate the Stokes Einstein and Stokes Einstein Debye relationships. The data for 40 kDa PVP have been published.[28]