



Published in final edited form as:

J Am Chem Soc. 2010 July 14; 132(27): 9392–9397. doi:10.1021/ja102296k.

Effects of proteins on protein diffusion

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Abstract

Despite increased attention, little is known about how the crowded intracellular environment affects basic phenomena like protein diffusion. Here, we use NMR to quantify the rotational and translational diffusion of a 7.4-kDa test protein, chymotrypsin inhibitor 2 (CI2), in solutions of glycerol, synthetic polymers, proteins, and cell lysates. As expected, translational diffusion and rotational diffusion decrease with increasing viscosity. In glycerol, for example, the decrease follows the Stokes-Einstein and Stokes-Einstein-Debye laws. Synthetic polymers cause negative deviation from the Stokes Laws and affect translation more than rotation. Surprisingly, however, protein crowders have the opposite effect, causing positive deviation and reducing rotational diffusion more than translational diffusion. Indeed, bulk proteins severely attenuate the rotational diffusion of CI2 in crowded protein solutions. Similarly, CI2 diffusion in cell lysates is comparable to its diffusion in crowded protein solutions, supporting the biological relevance of the results. The rotational attenuation is independent of the size and total charge of the crowding protein, suggesting that the effect is general. The difference between the behavior of synthetic polymers and protein crowders suggests that synthetic polymers may not be suitable mimics of the intracellular environment. NMR relaxation data reveal that the source of the difference between synthetic polymers and proteins is the presence of weak interactions between the proteins and CI2. In summary, weak but non-specific, non-covalent chemical interactions between proteins appear to fundamentally impact protein diffusion in cells.

Keywords

In-cell NMR; Macromolecular crowding; Protein diffusion; Weak interactions

Protein diffusion affects many aspects of cell biology, from metabolism to signal transduction. The intracellular environment, however, is complex and difficult to study directly. Most work is performed in solutions where the total protein concentration is less than 10 g/L. These dilute solutions give optimal signals, but may lack biological relevance. Macromolecules occupy up to 30% of a cell's volume and reach concentrations of 100 to 400 g/L.¹ Such large volume occupancies affect protein stability,² folding,^{3,4} and aggregation,⁵ but only recently has attention been directed to the effects of macromolecular

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Supporting Information Available. R1R2, Rotational and translational diffusion measurements in synthetic polymers and proteins. This material is available free of charge via the Internet at <http://pubs.acs.org>.

crowding on protein diffusion.^{6,7} Furthermore, many studies of macromolecular crowding use synthetic polymers rather than natural proteins.

Diffusion is described by the Stokes-Einstein Law, $D_t = \kappa T / 6\pi\eta r$, and the Stokes-Einstein-Debye law, $D_r = \kappa T / 8\pi\eta r^3$, where D_t is the translational diffusion coefficient, D_r is the rotational diffusion coefficient, η is the solution viscosity, κ is the Boltzmann constant, and r is the radius of protein being studied. These relationships are based on the assumption that the protein is much larger than the molecule used to increase the viscosity.⁸⁻¹⁰ High concentrations of macromolecules are expected to cause deviations from the Stokes Laws as the macromolecules approach the size of the test protein. Deviations come in two forms. Negative deviation means that increased viscosity decreases diffusion less than predicted, and positive deviation means that increased viscosity decreases diffusion more than predicted. Studies of protein diffusion with synthetic polymers as crowding agents show negative deviation for both translational and rotational diffusion.¹¹⁻¹⁶ For protein diffusion in protein solutions, most efforts have focused on translation, where both positive deviation^{17,18} and negative deviation¹¹ have been observed.

The ability to detect a protein by using NMR spectroscopy depends on its rotational dynamics, which are reflected in the protein's rotational correlation time (τ_c). Increasing the viscosity or the protein size increases the τ_c , resulting in a longer longitudinal relaxation time, T_1 , and a shorter transverse relaxation time, T_2 . Long T_1 values decrease the sensitivity of experiments and short T_2 values broaden the resonances.¹⁹⁻²¹ Since D_r is proportional to $1/\tau_c$, rotational motion is reflected in the width of its resonances.

Here, we use NMR spectroscopy to quantify both the rotational and translational diffusion of a 7.4 kDa ¹⁵N-enriched globular protein, chymotrypsin inhibitor 2 (CI2), as a function of crowder concentration. These crowders include the glycerol, synthetic polymers, globular proteins, and *Escherichia coli* cell lysates. We find that proteins and synthetic polymers have dramatically different effects on CI2 diffusion. The difference is caused by weak interactions between the proteins that dramatically decrease the rotational motion of CI2. The results not only provide new information about protein diffusion under physiologically relevant conditions, but also explain the difficulty in obtaining in-cell NMR spectra of globular proteins^{20,21} and suggest that synthetic polymers are not suitable systems for assessing the biological effects of crowding.

Materials and Methods

¹⁵N-enriched CI2 was expressed and purified as described.^{2,19} Chicken lysozyme, chicken ovalbumin, bovine serum albumin (BSA), Ficoll 70 (Ficoll) and polyvinylpyrrolidone 40 (PVP) were purchased from Sigma-Aldrich and used without further purification. Viscosities were measured with a Viscolite 700 viscometer (Hydramotion Ltd., England). Glycerol, PVP and Ficoll were dissolved in 50 mM sodium acetate (pH 5.4). A more concentrated buffer was required for proteins crowders. Lysozyme, ovalbumin and BSA were dissolved in 200 mM sodium acetate (pH 5.4).

E. coli Lysates

Cultures of strain BL21 (DE3) Gold (Stratagene) containing an empty pET28a plasmid (Novagen) were grown at 37 °C with shaking in a New Brunswick Scientific I26 incubator at 250 rpm in twelve 250 mL Erlenmeyer flasks, each containing 100 mL of Luria-Bertani (LB) media (10 g Bacto-Tryptone, 5 g Bacto-yeast extract and 10 g NaCl in 1 L of H₂O) and 50 µg/mL kanamycin. Each overnight culture was diluted into 1 L of LB media containing 50 µg/mL kanamycin. After 12 h at 37 °C with shaking at 250 rpm, the cultures were harvested by centrifugation at 4,000g (Sorvall RC-3B, H6000A) for 30 min at 4 °C.

The pellets were stored at -20°C overnight. Pellets were resuspended in 10 mL of distilled and deionized water. The suspensions were sonicated (Fisher Scientific, Sonic Dismembrator Model 500) on ice for 10 min with a duty cycle of 2 s on, 2 s off. The lysate was collected after centrifugation at 14,000g (Sorvall RC-5B, SS-34) for 30 min and lyophilized (Labconco, 7740020). The protein concentration in the re-dissolved lysates (pH 7.4) was determined with a modified Lowry assay (Thermo Scientific).

Relaxation and Diffusion

The experiments were performed on a 600 MHz Varian Inova spectrometer equipped with a standard triple resonance HCN probe with three axis gradients at 25°C . The relaxation and diffusion experiments were performed as described.^{22,23} Briefly, translational diffusion was measured by using a heteronuclear stimulated echo sequence.²⁴ Gradient strengths ranged from 1.2 G/cm to 58.0 G/cm. Rotational diffusion was assessed from the ^{15}N T_1/T_2 ratio acquired with pulse sequences from the Biopack software supplied with the instrument.²⁵ The ^1H dimension was acquired with a sweep width of 12000 Hz and comprised 1024 complex points. The ^{15}N dimension was acquired with a sweep width of 2500 Hz and comprised 64 complex increments. For T_1 measurements in solutions of 50 and 100 g/L crowders, the relaxation delays were 0.01, 0.4, 0.6, 0.7, 0.9, and 1.2 s. Delays of 0.01, 0.3, 0.4, 0.6, 0.9, 1.2, and 1.5 s were chosen for 200 g/L, and delays of 0.01, 0.4, 0.6, 0.9, 1.3, and 1.8 s were used for 300 g/L. For T_2 measurements in solution of 50 and 100 g/L, the delays were 0.01, 0.03, 0.07, 0.09, 0.15, and 0.21 s. Delays of 0.01, 0.03, 0.07, 0.09, 0.11, and 0.19 s were used for the 200 g/L. Delays of 0.01, 0.04, 0.05, 0.07, 0.09, and 0.11 s were used for 300 g/L. Eight transients were acquired per spectrum. The data were processed with NMRPipe²⁶ and NMRView.²⁷

Results

Crowders

The properties of CI2 and the crowders are given in Table 1. The synthetic polymers comprise PVP and Ficoll. PVP is a random coil polymer.²⁸ Its backbone structure is shown in Figure 1. Ficoll, a crosslinked and branched derivative of sucrose, is more globular.²⁹ The proteins include BSA, ovalbumin, and lysozyme.

Spectra

^{15}N - ^1H heteronuclear single quantum correlation (HSQC) spectra of CI2 were acquired in aqueous solutions containing 350 g/L glycerol and 300 g/L synthetic polymers, proteins, and in rehydrated *E. coli* lysate. Different crowders have different effects on the spectra. A typical high quality spectrum²² was obtained in glycerol (Figure 1A). High quality spectra were also observed in 300 g/L solutions of the synthetic polymers PVP and Ficoll (Figure 1B and C). The effect of protein crowders of increasing size (Table 1) is shown in Figure 1, panels D–F. Low quality spectra were obtained in 300 g/L BSA, and only side chain resonances from mobile asparagines and glutamines were observed in lysozyme, ovalbumin and cell lysate (Figure 1G).

Diffusion Data

The pulsed field gradient experiment used to quantify D_1 ³⁰ makes no assumption about CI2 size. The method to assess rotational diffusion [*i.e.*, T_1/T_2]²⁵ relies on the assumptions that CI2 is rigid and can be treated as a sphere. The first assumption is known to be valid.³¹ Inspection of the structure shows that CI2 has the shape of a typical globular protein,³² and, as discussed below, NMR data indicate it can be treated as a sphere.

Figure 2 shows the ratios of the diffusion coefficient in buffer (D_b) to that under crowded conditions (D_c) as a function of the relative viscosity for various crowders. In these plots, large y-values reflect a large impediment to diffusion. As expected, translational diffusion and rotational diffusion of CI2 decrease with increasing viscosity. The behavior in terms of the Stokes Laws, however, depends on the crowder. As observed previously,²³ both rotational and translational diffusion follow the Stokes Laws in glycerol (Figure 2A). Dividing the Stokes-Einstein-Debye equation by the Stokes Einstein equation yields $D_r/D = 3/4 r^{-2}$, where r is the apparent CI2 radius. Consistent with the Stokes Laws, the radius from the glycerol data, 1.7 nm, is independent of glycerol concentration and compares favorably with the 1.4 nm estimated from the molecular weight and partial specific volume of CI2. This similarity provides confidence that CI2 can be treated as a sphere. Next, we examine the effects of macromolecular crowders where diffusion can deviate from the Stokes Laws.

The synthetic polymers generate negative deviation for both translational and rotational diffusion (Figure 2B and C). That is, diffusion is affected less than predicted by the Stokes Laws. Furthermore, the polymers impede CI2's translational motion more than its rotational motion. Proteins have the opposite effect (Figure 2D, E and F). They cause positive deviation for rotational diffusion and either positive or no deviation for translation. Also in opposition to observations on synthetic polymers, rotational diffusion is impeded more than translational diffusion. Consistent with our conclusion that protein crowders severely impede rotation, we are unable to acquire rotational diffusion data in 300 g/L solutions of lysozyme, ovalbumin and lysates because the resonances broaden beyond detection.

To our knowledge, there is only one report on the rotational diffusion of a protein in solutions crowded with proteins.¹⁸ In that report, the test protein apomyoglobin shows negative deviation, which is opposite to what we observe. If negative deviation were general, we would expect to observe high quality HSQC spectra in solutions crowded with globular proteins and in cells. This expectation, however, is not fulfilled; solutions crowded by globular proteins yield poor quality or no spectra (Figure 1), and none of the five globular proteins we have studied by in-cell NMR yield useful spectra.²⁰ Others report findings similar to ours.^{33,34} Perhaps apomyoglobin is not a good model protein because it is not completely globular.³⁵

Figure 2G shows that diffusion in cell lysates is similar to diffusion in solutions crowded by proteins. This similarity suggests that concentrated proteins solutions are physiologically relevant models.

Relaxation Data

The average ^{15}N line width [$1/(\pi T_2)$] of backbone CI2 resonances in different crowders was assessed from relaxation data (Figure 3). The average width increases with glycerol concentration. The resonances broaden in PVP and Ficoll. The widths are larger in solutions crowded by proteins, and similar to the widths obtained in cell lysates. Linewidth, however, is affected by both viscosity and binding. The product of longitudinal relaxation rate R_1 ($1/T_1$) and transverse relaxation rate R_2 ($1/T_2$) can be made independent of viscosity (see Discussion) and is hence a good method for assessing weak binding.²² A histogram of the average $R_1 R_2$ values for various crowders is shown in Figure 4. Smaller average values are observed for glycerol and synthetic polymers than for protein crowders and the cell lysates.

Discussion

CI2 is Invisible in HSQC Spectra in Cells, 20 at High Protein Concentrations, and in Cell Lysates

Even in a 350 g/L glycerol (93 Da) solution, which has a relative macroscopic viscosity of 2.9, the CI2 spectrum looks like it does in dilute solution (Figure 1A). The viscosities of synthetic polymer solutions are 10-times larger than those of glycerol at similar g/L-concentrations (Table S1), yet we still obtain typical CI2 spectra (Figure 1B, and C).

Using proteins as crowding agents leads to dramatically different results. The spectral quality is extremely low in concentrated protein solutions (Figure 1D–F), despite the fact that these solutions have viscosities similar to those of the glycerol samples, and 10-fold lower than those of the synthetic polymers. The spectra are so severely degraded in BSA that only CI2 glutamine and asparagine side chain resonances and a few backbone resonances are detected. Backbone resonances are completely absent in spectra acquired with lysozyme and ovalbumin. The side chain resonances are observed because they have internal motion that is independent of overall rotational motion.¹⁹ Importantly, we observe the same effect with cell lysates (Figure 1G), suggesting that our results are biologically relevant. Our results are also consistent with those from in-cell NMR experiments where resonances become too broad to give useful HSQC spectra.^{21–23,33}

We cannot blame bulk viscosity for the poor quality of the spectra in protein solutions because the viscosities are far lower than those of the synthetic polymers. We also can rule out inhomogeneity as a factor because the solutions are homogenous. To understand the difference between the effects of synthetic polymers and proteins we used NMR to quantify CI2 diffusion.

Synthetic Polymers and Proteins Have Opposite Effects

The synthetic polymers PVP and Ficoll are much larger than CI2 (Table 1). At the concentrations used here (≥ 100 g/L), molecules of these polymers overlap to form a mesh.³⁶ If the chemical interactions between the polymers and CI2 are extremely weak, we expect CI2 to experience less than the macroscopic viscosity. This expectation is borne out (Figure 2B and C). We also note that PVP and Ficoll slow CI2's rotational diffusion less than its translational diffusion. This result is expected because rotation in the mesh should be easier than translation through the mesh. It is interesting to compare the PVP results to the Ficoll results. The stronger deviation observed in Ficoll is expected because its molecule weight is larger (Table 1). It is also of interest to estimate the apparent size of CI2 from D_r/D_t as described above for glycerol solution. In 200 g/L solution of synthetic polymers, the apparent radius is 1.1 nm in PVP and 1.0 nm in Ficoll, which, assuming a partial specific volume of 0.73 mL/g, corresponds to apparent molecular weights of 4.7 and 3.6 kDa, respectively. Thus CI2 acts like a smaller protein in solutions of synthetic polymers.

Assuming that non-specific, non-covalent chemical interactions between the proteins and CI2 are extremely weak, the concentrated solutions of globular proteins should act like a collection of spheres. Negative deviation is also expected for these systems as long as the protein remains mobile. Inert spheres should remain mobile up to near the close-packing limit, which for practical purposes occurs at a volume occupancy of $\sim 64\%$.³⁷ The volume occupancy here is only $\sim 21\%$ at the highest concentrations (300 g/L). Nevertheless, we observe not the expected negative deviation, but positive deviation for rotational diffusion and positive or negligible deviation for translational diffusion for proteins solutions (Figure 2D–F) and in cell lysates (Figure 2G). This strong attenuation of rotational diffusion does not depend on the size or charge of the protein (Table 1), suggesting the generality of our results. We suggest that the dramatically different effects of synthetic polymers and proteins

arise because of non-specific, non-covalent chemical interactions between the proteins and CI2. We also estimated the effective size of CI2 under these conditions from D_r/D_t . In 200 g/L protein solutions, the apparent radius of CI2 is 2.5 nm in BSA and 2.4 nm in lysozyme, corresponding to apparent molecular weights of 56.7 kDa and 45.4 kDa, respectively. These apparent molecular weights are more than seven times those calculated from CI2's amino acid sequence. The increase in size suggests that CI2 interacts with other proteins in solutions. Put another way, even weak favorable interactions between CI2 and the protein crowders should lead to the observed larger effects on rotation compared to translation because rotational diffusion depends on volume, r^3 , while translational diffusion depends only on size, r .

Relaxation Data Indicate Non-specific, Non-covalent Chemical Interactions Involving Proteins

NMR is useful for investigating weak protein interactions in dilute solution³⁸ and under crowded conditions.²² The simplest quantitative experiment is to examine the average resonance widths under different conditions. We used T_2 data to assess line widths [$1/(\pi T_2)$]. Favorable interactions between CI2 and the crowders will broaden resonances by impeding rotation. The data in Figure 3 show not only that widths increase with crowder concentration, but also that protein crowders have the most dramatic effect. Lysozyme, ovalbumin, and lysates have such a strong effect that we can only estimate the widths at the highest concentration. The data are consistent with the presence of favorable CI2-crowder interactions, especially between the protein crowders and CI2. Unfortunately, width also increases with viscosity, so this method alone cannot provide definitive information on CI2-crowder interactions.

T_1 and T_2 are affected by viscosity, global correlation time, and temperature, but Kneller et al.³⁹ showed that the product of $1/T_1$ and $1/T_2$ (R_1R_2) is constant when the product of the Larmor frequency and the global correlation time is much greater than unity at a given temperature and magnetic field. In addition, the protein must lack extensive ms internal motion, which is known to be true for CI2.³¹ This viscosity independence makes R_1R_2 a useful tool for assessing intermolecular interactions.²²

The R_1R_2 data are shown in Figure 4. Provided CI2 has a rotational correlation time >7 ns (assured by the viscosity of all our samples), R_1R_2 should equal 19.6 s^{-2} at 600 MHz for unbound CI2.²² As we have shown, R_1R_2 values from 19.6 s^{-2} to 24.0 s^{-2} are consistent with CI2 dimerization.²² Larger values indicate involvement in larger assemblies, most likely with the crowding molecules.²²

The average value of R_1R_2 data for glycerol and the synthetic polymers (Figure 4) are consistent with extremely weak interactions with CI2. Nevertheless this sensitive method indicates that interactions in PVP are stronger than interactions in Ficoll. We cannot state with certainty that these are exclusively CI2-PVP interactions, but NMR pulsed-field gradient experiments indicate that CI2 can be no more than a dimer in solutions containing 300 g/L of 40 kDa PVP.²

Protein crowders give different results and show that they interact more strongly with CI2. The R_1R_2 values in concentrated protein solutions and in lysates exceed those for monomeric or dimeric CI2 and depend strongly on crowder protein concentration. In summary, the data point to a non-specific affinity of proteins for one another as the source of the difference between the diffusion of CI2 in solutions crowded with synthetic polymers and proteins. The chemical origin of these non-covalent interactions may reside in the local distribution of complementary CI2-protein charges and in the repeating nature of polypeptide amide nitrogen H-bond donors and carbonyl oxygen acceptors.

Although proteins interact more strongly with CI2 than do synthetic polymers, results of previous work show that the dissociation constant for CI2-protein complexes is large, 10 mM or greater.²² Another indication that these are weak interactions is that the value of R_1R_2 does not depend in a predictable way on the charge of the crowding protein (Table 1). Most importantly, our data show that even weak protein-protein interactions severely impede rotation.

Conclusions

The intracellular environment is crowded and inhomogeneous, and weak interactions are a special and critical feature of living cells.⁴⁰ For instance, weak interactions are thought to organize metabolic paths and protein-protein interaction networks.⁴¹⁻⁴² The importance of weak protein-protein interactions under crowded conditions has also been highlighted in a recent computational study and a recent review of the crowding literature.⁴³⁻⁴⁴ Our study provides quantitative data supporting these hypotheses and methods for assessing weak but physiologically important interactions.

From a practical point of view, the results explain why ^{15}N - ^1H HSQC spectra of globular proteins are difficult to detect in cells.²¹⁻⁴⁵ Although we focused on a single protein, our difficulty in observing in-cell HSQC spectra of six globular proteins suggests that weak interactions are universal.²⁰ Augustus *et al.* also suggest that weak interactions between proteins and DNA result in disappearance of the MetJ spectrum in ^{15}N -HSQC experiments.³³ The fact that synthetic polymer crowders and globular proteins have such different effects on diffusion suggests that synthetic polymers may not be the best choice for modeling the effects of the intracellular environment on protein diffusion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Gregory B. Young for spectrometer maintenance, Michael Rubinstein, Andrew Miklos and Matthew Redinbo for useful discussions, and Elizabeth Pielak and the reviewers for helping to improve the manuscript. Supported by National Institutes of Health grant 5DP1OD783 and National Science Foundation grant MCB-051647.

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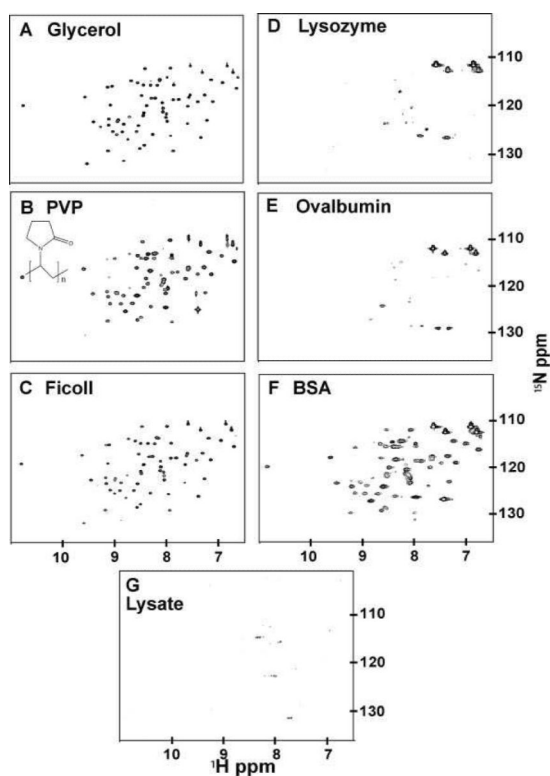


Figure 1. ^{15}N - ^1H HSQC- spectra of Cl_2 solutions (1 mM, 25 °C, pH 5.4) containing 350 g/L glycerol (A) and 300 g/L PVP (B), Ficoll (C), lysozyme (D), ovalbumin (E), BSA (F), and *E. coli* lysate (G). The backbone structure of PVP is shown in panel B.

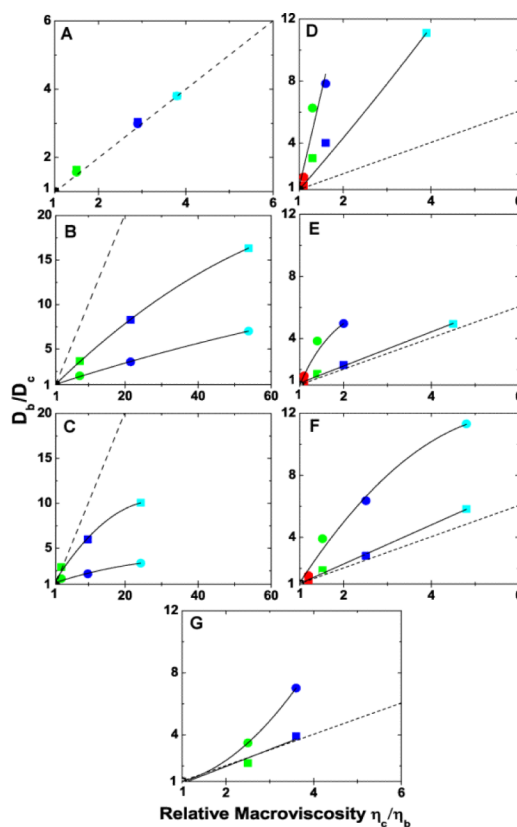


Figure 2. Ratio of translational (squares) and rotational (circles) diffusion coefficients of CI2 in dilute buffer (D_b) to its diffusion coefficients in crowded solutions (D_c) (25 °C, pH 5.4) containing glycerol (A), PVP (B), Ficoll (C), lysozyme (D), ovalbumin (E), BSA (F), and *E. coli* lysate (G) as a function of relative viscosity (Glycerol: green, 200 g/L; blue, 350 g/L, cyan, 420 g/L. Other crowdors: red, 50 g/L; green, 100 g/L; blue, 200 g/L; cyan, 300 g/L.). The smooth curves are polynomial fits of no theoretical significance. The dashed lines illustrate the unitary slope and origin-intercept expected for Stokes Laws. Points below and above dashed line indicate negative deviations and positive deviations, respectively. The uncertainties are smaller than the symbols. The PVP data have been published.²¹

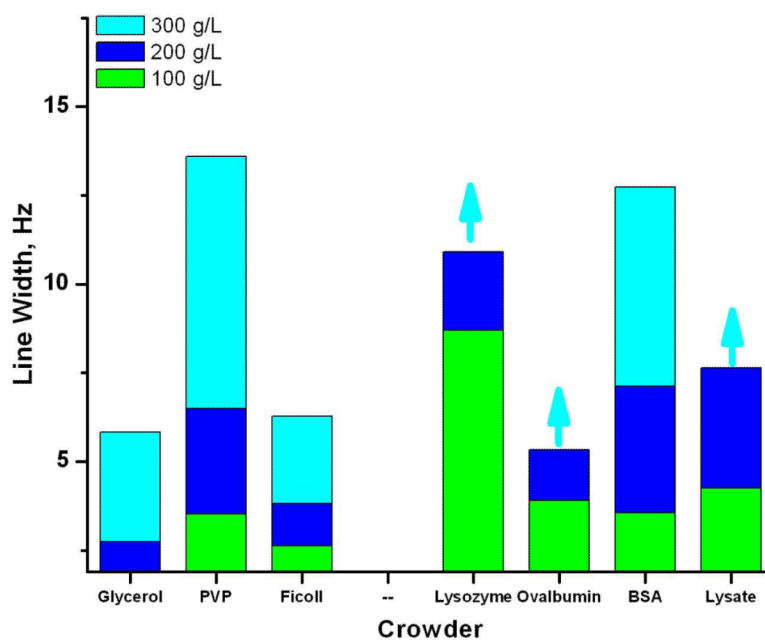


Figure 3. Average widths of CI2 backbone amide ^{15}N resonances (25 °C, pH 5.4) derived from T_2 measurements [line width = $1/(\pi T_2)$]. The starting point of Y-axis represents the average line width in dilute solution. The arrows indicate that the widths in 300 g/L are too broad to observe. The colors are defined in the caption to Figure 2.

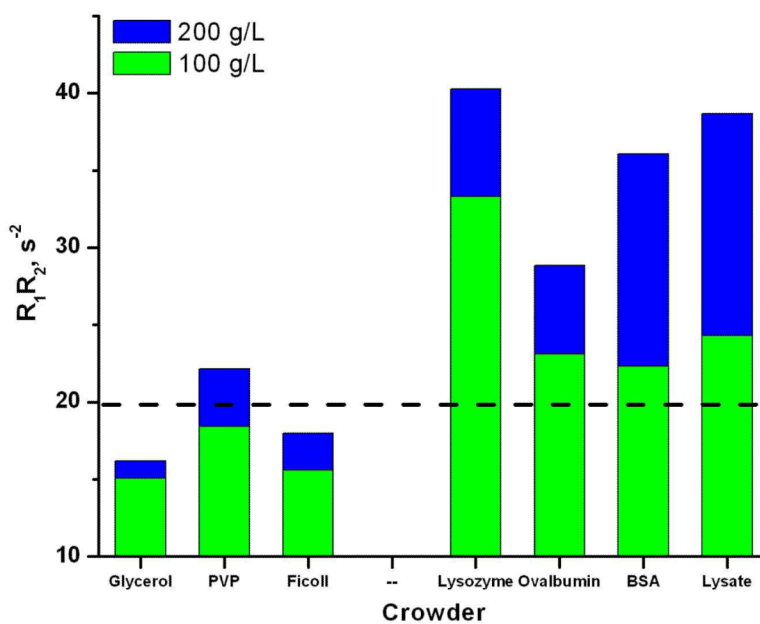


Figure 4. Histograms of average R_1R_2 values for CI2 in solutions of glycerol, synthetic polymers, globular proteins, and *E. coli* lysates (25 °C, pH 5.4). The dashed line is the theoretical maximum value for monomeric CI2 in the absence of conformational exchange. The colors are defined in the caption to Figure 2.

Table 1

Properties of CI2 and crowders

Molecule	Molecular Weight, kDa	pI	Charge at pH 5.4
CI2	7	6.5	Cation
Glycerol	0.09	NA*	Neutral
PVP	40	NA	Neutral
Ficoll	70	NA	Neutral
Lysozyme	15	11.0	Cation
Ovalbumin	45	4.6	Anion
BSA	66	4.7	Anion

* NA: Not Applicable