

Separating the Contribution of Translational and Rotational Diffusion to Protein Association

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Abstract: The association of two proteins is preceded by a mutual diffusional search in solution. The role of translational and rotational diffusion in this process has been studied theoretically for many years. However, systematic experimental verification of theoretical results is still lacking. We report here measurements of association rates of the proteins β -lactamase (TEM) and β -lactamase inhibitor protein (BLIP) in solutions of glycerol and poly(ethylene glycol) of increasing viscosity. We also measured translational and rotational diffusion in the same solutions, using fluorescence correlation spectroscopy and fluorescence anisotropy, respectively. It is found that in glycerol both translational and rotational diffusion rates are inversely dependent on viscosity, as predicted by the classical Stokes–Einstein relations, while the association rate depends nonlinearly on viscosity. In contrast, the association rate depends only weakly on the viscosity of the polymer solutions, which results in a similar weak dependence of k_{on} on viscosity. The data are modeled using the theory of diffusion-limited association. Deviations from the theory are explained by a short-range solute-induced repulsion between the proteins in glycerol solution and an attractive depletion interaction generated by the polymers. These results open the way to the creation of a unified framework for all nonspecific effects involved in the protein association process, as well as to better theoretical understanding of these effects. Further, they reflect on the complex factors controlling protein association within the crowded environment of cells and suggest that a high concentration of macromolecules does not significantly impede protein association.

Introduction

Many aspects of cellular and multicellular activities are dependent on macromolecular interactions, which are a result of diffusion and specific recognition. The biochemical understanding of such processes has been achieved by extrapolating the results obtained from studies of dilute homogeneous solutions containing purified components. It has been suggested that the association of a pair of proteins can be described as a three-step reaction, which starts with the collision of the two proteins through diffusion, and is then followed by the formation of an encounter complex; structural rearrangement and desolvation promote the creation of the final associated complex.¹ The mutual search of two proteins for each other can be guided by specific long-range interactions between the active sites, such as electrostatic forces. Not less important for this search are force-free translational and rotational diffusion, as well as nonspecific solution interactions.

The role of rotational diffusion in association has been appreciated theoretically for many years.² Since the active sites

typically form small patches on the associating proteins, most encounters between the two proteins will be unproductive, unless they can quickly find the correct mutual orientation. Indeed, considering the time and length scales for translational and rotational Brownian motion of a typical protein, it is reasonable to postulate that a pair of proteins which have just encountered each other will have ample time to reorient and associate before they separate again. Theories of diffusion-limited association (DLA)^{3–5} attempted to provide a sound physical description for this process. Computer simulations, typically using Brownian dynamics methodology, also showed how rotational motion can enhance the association rate.^{6,7} Any attempt to gauge the success of theories and simulations in describing the crucial physical aspects of the problem should rely, in principle, on our ability to carry out experiments where the various variables can be separated. To the best of our knowledge, such an attempt has not been carried out so far in a systematic fashion.

The dependence of the translational and rotational diffusion coefficients on solution viscosity is traditionally described by the so-called Stokes–Einstein (SE) relation, $D_t = k_B T / 6\pi\eta R$,

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and Stokes–Einstein–Debye relation, $D_r = k_B T / 8\pi\eta R^3$, respectively. In these relations, η is the solution viscosity, T its temperature, k_B is the Boltzmann constant, and R is the hydrodynamic radius of the diffusing species. The limits of these relations (both of which will be referred to below as the SE relations) have been tested many times. In particular, it is well-known that in polymer solutions translational and rotational diffusion might have a different functional dependence on bulk viscosity. Here we demonstrate how deviations from SE behavior provide an opportunity to test the relation between translation, rotation, and the association rate of the protein TEM1- β -lactamase (TEM) and its protein inhibitor, β -lactamase indicator protein (BLIP). Kozer and Schreiber recently showed that in solutions of several high molecular weight additives (selected to mimic the effect of crowding on association⁸) the association reaction between TEM and BLIP is very weakly dependent on macroscopic viscosity.⁹ A similar observation was reported by Ladurner and Fersht for the interaction of barnase with barstar.¹⁰ We now focus on solutions of poly(ethylene glycol) of a molecular weight of 8000 (PEG 8000) and explain this observation by the weak dependence of rotational diffusion on viscosity, in conjunction with the depletion interaction operating in these solutions. This behavior is found to be very different than that in solutions of the small viscogen glycerol, where a super-linear dependence of the association rate on viscosity is observed, and is attributed to short-range repulsive forces operating between the proteins close to contact. Our observations allow us to critically test the predictions of current theories and provide a benchmark for further refinement of the physical understanding of protein association.

Materials and Methods

Chemicals. Glycerol, ultrapure grade, was purchased from ICN. Poly(ethylene glycol) 8000 (MW 8000) was purchased from Sigma. They were used without further purification.

Proteins. A detailed description of the proteins TEM and BLIP, as well their production protocol, can be found in ref 11. TEM has a molecular weight of 29 kDa and a hydrodynamic radius of 19.7 Å, and BLIP has a molecular weight of 17.5 kDa and a hydrodynamic radius of 16.4 Å (hydrodynamic radii given here are the radii of spheres of equal volumes to those of the proteins, as calculated from their crystal structure). In this study, we used wt-TEM and the following BLIP analogues: wt, +4 (D163K, N89K, V165K). Proteins were kept in 10 mM Hepes buffer adjusted to pH 7.2, and all measurements were conducted in the same buffer and pH. For diffusion experiments, we used the BLIP A1C mutant specifically labeled with the maleimide derivative of the dye Alexa 488 (Molecular Probes) and shown to be active after the labeling procedure.

A pBAD/HisA (invitrogen) plasmid containing pEGFP (Clontech) was a kind gift of Johan Hofkens (Leuven). The expression of the protein was induced for 12 h in *Escherichia coli* Top10 cells grown to an optical density of 0.5 at 600 nm using 0.2% arabinose. The His-tagged protein was subsequently isolated and purified under native conditions by Ni-chelation chromatography (Pharmacia). Purity of the samples was confirmed by polyacrylamide gel electrophoresis. The molecular weight of the protein is 27.7 kDa, and its hydrodynamic radius is 18.8 Å.

Fluorescence Anisotropy Measurements. The rotational dynamics of proteins in various viscous solutions were obtained from steady-

state fluorescence anisotropy measurements, carried out on a commercial spectrofluorimeter (ISS PC1) equipped with rotating calcite polarizers, and a thermostated bath, which allowed maintaining the sample temperature at $25(\pm 0.2)$ °C. Each measurement was repeated at least three times and averaged. The rotational correlation time, θ , was calculated from the fluorescence anisotropy using Perrin's equation:

$$\frac{r_0}{r} = 1 + \frac{\tau}{\theta} \quad (1)$$

where τ is the fluorescence lifetime, r is the measured anisotropy, and r_0 is the limiting anisotropy in a solution of infinite viscosity. This description of rotational dynamics in terms of a single depolarization process is valid for a molecule that can be approximated as a spherical object, in which case the rotational correlation time is related to the molecular rotational diffusion coefficient by $\theta = 1/6D_r$. To obtain the rotational correlation times of proteins, such as TEM and BLIP, one needs to either rely on intrinsic fluorescence (typically of tryptophan residues) or use extrinsic labeling. Both options present difficulties since, in most cases, the rotational motion of intrinsic or extrinsic fluorophores is dominated by local motions rather than by the overall motion of the protein. Thus, even in time-resolved fluorescence anisotropy studies, the component due to overall protein rotation can be small, limiting the accuracy of the measurement. We therefore chose to measure the fluorescence anisotropy of eGFP molecules, in which the chromophore is held rigidly within the protein scaffold, so that the only rotational process is the overall rotation of the protein. Indeed, the rotational decay of eGFP molecules was shown to be monoexponential.¹² Since the volume of eGFP is not very different than that of TEM and BLIP, the rotational dynamics of these proteins are expected to be similar. The fluorescence lifetime of a molecule also influences the measured anisotropy, and we were therefore careful to separately determine the relative quantum efficiency of eGFP in each solution. We found that both glycerol and PEG have a negligible effect on the quantum efficiency of eGFP. A similar result was reported by Phillips and co-workers.¹² Great care was taken to accurately determine r_0 of eGFP (0.393 ± 0.002), which was measured in a $\sim 100\%$ glycerol solution at 5 °C. A limited set of rotational anisotropy measurements with Alexa-labeled BLIP showed a similar trend to the results reported below with eGFP.

Fluorescence Correlation Spectroscopy. Translation diffusion was measured using a home-built fluorescence correlation spectrometer (see Supporting Information). The fluorescence correlation function was fitted to the following equation, valid for a single diffusing species moving through a Gaussian-shaped sampling volume:¹³

$$G(t) = \frac{1}{N} \left(1 + \frac{t}{\tau_D} \right)^{-1} \left(1 + \frac{t}{\omega^2 \tau_D} \right)^{-1/2} \quad (2)$$

In this equation, τ_D is the translational correlation time given by $\tau_D = \omega_{xy}^2 / 4D_r$, where ω_{xy} is the lateral dimension of the observation volume. ω is the ratio between the axial and lateral dimensions of the observation volume, and N is the average number of molecules in this volume, which is of the order 1 when the molecular concentration is kept at the nanomolar range. The correlation function computed from experimental measurements was fitted to eq 2 for times $> 10 \mu\text{s}$, to avoid complications due to triplet-state dynamics, which we carefully assessed to be negligible at these times. Performance of the instrument was checked by measuring the diffusion of rhodamine 6G molecules. Using the known diffusion coefficient of this dye, $(2.9 \pm 0.7) \times 10^{-6} \text{ cm}^2/\text{s}$,¹⁴ we were able to routinely show that the observation volume in our experiment, $\pi^{3/2} \omega_{xy}^2 \omega_z$, is $1.2 \pm 0.2 \text{ fL}$.

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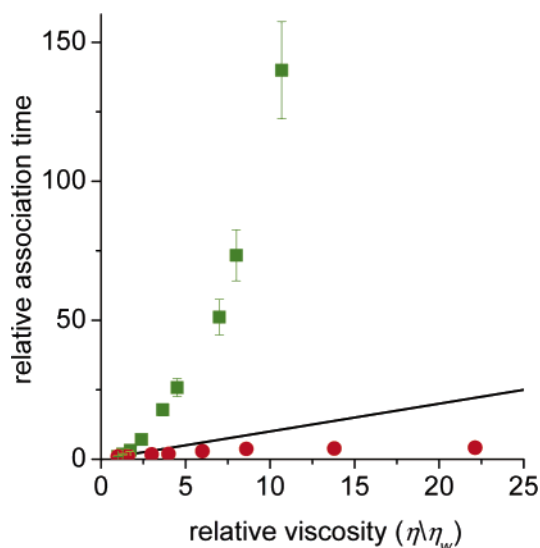


Figure 1. Dependence of the relative association time ($k_{a,w}/k_a$) of the TEM–BLIP pair on relative viscosity in glycerol solution (green squares) and PEG 8000 solutions (red circles). Association times were measured using a stopped-flow apparatus. The line is the SE prediction for the relative association time (see discussion following eq 3). The error bars for the PEG 8000 measurements are smaller than the symbol size.

Association Rate Measurements. The association reaction of TEM and BLIP in various solutions was measured following the methods of Kozer and Schreiber,⁹ under second-order kinetic conditions, with equal concentrations ($0.5 \mu\text{M}$) of both proteins. A thermostated bath allowed maintaining the sample temperature at $25(\pm 0.2) \text{ }^\circ\text{C}$. The data were fitted to a standard equation describing association under the condition of equal reactant concentrations (see Supporting Information for more details).

Results

Methodology. To obtain a full picture of the nonspecific dynamic processes affecting the association reaction, we studied the association reaction of TEM and BLIP in water solutions of the small viscogen glycerol, as well as in water solutions of the polymer PEG 8000. The concentration of the additives was systematically varied, and the macroscopic viscosity at each concentration was assessed. We then determined in each solution the translational and rotational correlation times, as well as the association times (inverse rates). We found it convenient to relate measured times to the corresponding values in water. Thus, all values given below are relative and unitless.

Association Rates. The association rates of TEM and BLIP were extracted from fits to stopped-flow measurements in glycerol and PEG 8000 solutions of increasing viscosity, as described in the Materials and Methods section. We define a *relative association time* $\bar{\tau}_a = k_{a,w}/k_a$ (where $k_{a,w}$ is the association rate constant in water), and in Figure 1, we show the dependence of $\bar{\tau}_a$ on relative viscosity, $\bar{\eta} = \eta/\eta_w$ (η_w is the viscosity of water), for the two types of solutions (glycerol, squares, and PEG 8000, circles); $\bar{\tau}_a$ shows a strong, nonlinear dependence on viscosity in glycerol. Surprisingly, it is found to depend only weakly on viscosity in PEG 8000 solutions.

Translational Diffusion. Correlation functions calculated from FCS data taken in glycerol and PEG 8000 solutions were fitted to eq 2. Figure 2 shows the relative translational correlation time, $\bar{\tau}_D = \tau_D/\tau_{D,w}$ (where $\tau_{D,w}$ is the correlation time in water),

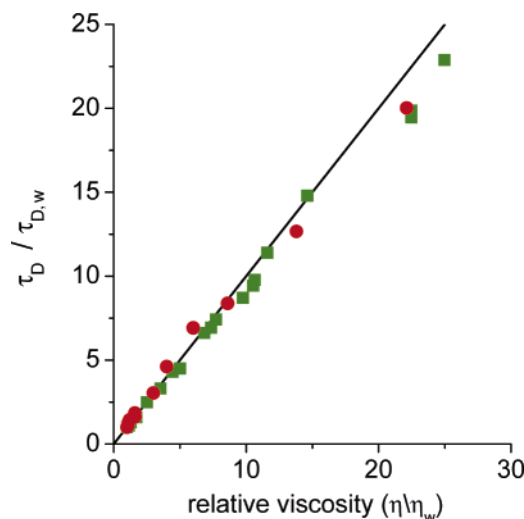


Figure 2. Dependence of the relative translational correlation time of Alexa488–BLIP molecules on relative viscosity in glycerol solution (green squares) and PEG 8000 solutions (red circles). The line is the SE prediction. Translational correlation times were obtained by fitting FCS correlation functions.

obtained from the fits as a function of relative viscosity, both in glycerol solutions (squares) and in PEG 8000 solutions (circles).

The line in Figure 2 is the linear prediction of the SE relation (i.e., $\bar{\tau}_D = \bar{\eta}$). The translational correlation times follow this relation in both types of solutions up to $\bar{\eta} \sim 20$. The close adherence to the linear relation in PEG 8000 solutions is rather unexpected, considering that at polymer concentrations greater than 2.5% (w/v), equivalent to $\bar{\eta} > 1.5$, the solution is in the semidilute regime, with significant overlap between adjacent polymer molecules. However, we remember that PEG 8000 molecules, with a radius of gyration $R_g = 4.2 \text{ nm}$, are not much larger than the protein molecules studied here. It is therefore likely that their translational dynamics are still the dominant factor in determining the motion of a tracer protein on the time scale probed in FCS, from $\sim 10 \mu\text{s}$ to $\sim 1 \text{ ms}$. This behavior contrasts with what is usually found for translational diffusion in solutions of (typically larger) polymers.^{15–17} In a recent example, Koenderink et al. used dynamic light scattering to measure translational diffusion in solutions of xanthane, a polymer of a molecular weight of $4 \times 10^6 \text{ g/mol}$,¹⁸ and found a significant deviation from SE behavior. In this case, only local motions of the polymer network were important on the time scale probed by dynamic light scattering, and overall translation of the giant polymer molecules was too slow to affect the result.

It is important to note here that, while some recent studies suggest attractive interactions between PEG and protein molecules,^{19,20} our translational diffusion results overrule such interactions in the present case. Any association of PEG and protein molecules would lead to species with higher hydrodynamic radii and longer diffusion times than predicted by the

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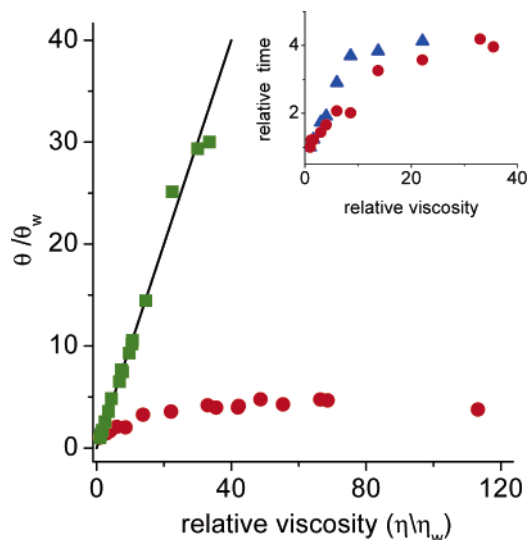


Figure 3. Dependence of relative rotational correlation time of eGFP molecules on relative viscosity in glycerol solution (green squares) and PEG 8000 solutions (red circles). The line is the SE prediction. Rotational correlation times were calculated from fluorescence anisotropy measurements. Inset: comparison of the experimental relative rotational correlation times in PEG 8000 solutions (red circles) with the relative association times (blue triangles).

SE relation. Our result agrees with the work of Bhat and Timasheff,²¹ who showed that PEG induces preferential hydration of proteins.

Rotational Diffusion. Rotational correlation times were calculated from fluorescence anisotropy measurements of eGFP (the rationale behind using eGFP to measure rotation was discussed in Materials and Methods and was checked with a limited set of measurements on Alexa-labeled BLIP). Figure 3 shows the relative rotational correlation time, $\bar{\theta} = \theta/\theta_w$ (θ_w is the correlation time in water) in glycerol solutions (squares) and in PEG 8000 solutions (circles), as a function of relative viscosity, $\bar{\eta}$. As above, the line is the linear prediction of the SE relation (i.e., $\bar{\theta} = \bar{\eta}$). The rotational correlation times in glycerol solutions remain close to the SE prediction at least up to a relative viscosity of 30. However, the rotational correlation times in PEG 8000 strongly deviate from this relation, being much faster than predicted by viscosity. In fact, their dependence on viscosity is very similar to the dependence of the relative association times on viscosity (inset to Figure 3).

The success of the SE relation to capture the viscosity dependence of molecular rotational correlation times is well-known. This success can be attributed to the fact that the interaction of solvent molecules with the rotating molecule is well-described by the stick boundary condition used to derive the relation. It is likely that rotation in polymer solutions is qualitatively different. It occurs in a “solvent cage” made essentially of water molecules, into which the bulky polymer molecules cannot penetrate. This is why the macroscopic viscosity is not a good determinant of the rotational diffusion in these solutions, as already shown previously.²²

Discussion

Diffusion in Polymer Solutions. The effect of polymer solutions on translation and rotation, in general, and in the

context of proteins, in particular, has been studied by many authors (see, e.g., ref 23 for a compilation of many experimental results on protein diffusion) and is not the subject of this work. Rather, our main interest in the diffusion results is in using them to understand association kinetics. We will therefore not attempt a comparison of our results to the extensive literature on protein diffusion in polymers. One point is nevertheless worthy of discussion here. The concurrent measurement of *both translational and rotational diffusion* within the same matrix can, in principle, pave the way to better understanding of the microscopic structure of a polymer network as it influences various motions. While several theories of translational diffusion in polymer matrices exist (see ref 17 for a recent review), it is difficult to find a consistent first-principles theoretical treatment of both rotational and translational motion of a small probe in semidilute polymer solutions. One such approach is provided by the theory of diffusion in a Brinkman fluid,^{24,25} which was used successfully to model measurements of translational and rotational diffusion of a probe particle in xanthane solutions, already mentioned above.¹⁸ The important parameter in the theory is the hydrodynamic screening length of the medium, commonly equated to the correlation length of the polymer solution. The Brinkman fluid theory does predict a much weaker effect on the rotational motion of a probe in PEG 8000 solutions than on its translational motion, although a quantitative agreement with the experiment is not achieved (see Supporting Information). We will leave this important problem to a future study and focus on the main issue of this work, association kinetics.

Understanding Association Kinetics. To interpret the association rate results, we turn to the theory of diffusion-limited association (DLA). The theory starts from the combined rotation–translation diffusion equation for a pair of particles (spheres) moving in a liquid. To account for nonuniform reactivity of the spheres, the diffusion equation is augmented by boundary conditions that guarantee reaction at the proper mutual orientation.³ Szabo and co-workers showed how this formalism can be used to obtain an analytic expression for the association reaction rate.⁴ Using the Szabo approach, together with a simple approximation due to Berg,²⁶ Zhou⁵ wrote down an explicit formula for the fully diffusion-controlled rate (which is the rate in the case that each encounter between the reactive patches leads to association) of two spheres with spherically symmetric reactive regions of sizes δ_1 and δ_2 :

$$k_a = 4\pi(D_{t,1} + D_{t,2})a[F_1\xi_2 \tan(\delta_2/2) + F_2\xi_1 \tan(\delta_1/2)] \quad (3)$$

where $F_i = \sin^2(\delta_i/2)$, $\xi_i = \sqrt{(1 + D_{r,i}a^2/(D_{t,1} + D_{t,2}))/2}$, $D_{t,i}$ and $D_{r,i}$ are the translational and rotational diffusion coefficients of protein i ($i = 1, 2$), respectively, and a is the sum of the hydrodynamic radii of the two proteins. If we assume that the two proteins have equal-sized reactive patches, we can use eq 3 to calculate the relative association time. It is easy to see that in the SE case, when both translational and rotational diffusion coefficients are inversely proportional to the viscosity, the relative association time is equal to the relative viscosity, $\bar{\tau}_a = \bar{\eta}$. As shown in Figure 1, this simple behavior is obtained neither in solutions of the small viscosogen glycerol, where the relative

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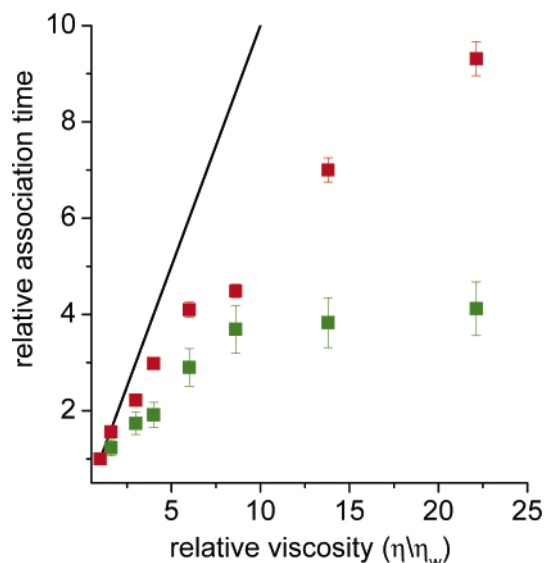


Figure 4. Comparison of the experimental relative association times in PEG 8000 solutions (green squares) to values calculated from translational and rotational diffusion times using diffusion-limited association (DLA) theory (eq 3, red squares). The line is the SE prediction.

association times are much larger than the relative viscosities, nor in polymer solutions, where they are much smaller. Can we understand the sources of deviation from the SE behavior?

Association in PEG Solutions and the Depletion Interaction. In PEG 8000 solutions, translational diffusion obeys the SE relation, but rotational motion strongly deviates from this relation and depends only weakly on viscosity. A deviation from the prediction $\bar{\tau}_a = \bar{\eta}$ is expected in this case. Figure 4 compares the values calculated from eq 3, using the measured translational and rotational diffusion coefficients (red symbols), with the experimentally measured relative association times (green symbols). Since only the translational diffusion coefficients of BLIP and the rotational diffusion coefficients of eGFP were measured, the other required values were obtained by scaling the measured values with the ratios of volumes or radii obtained from the crystal structures of the proteins. The DLA values reasonably match the measured values at low viscosities, but clearly deviate from them at higher viscosities, where the experimental relative association time plateaus, similar to the behavior of the rotational correlation time with viscosity (see inset to Figure 3), while the calculated time continues to grow. It is obvious that some of the mismatch between the calculation and the measurement is due to the rather naïve assumptions made in deriving and using eq 3, mainly that the reactive patches are symmetric and of equal size. Observation of the crystal structure of the TEM–BLIP complex shows, though, that these assumptions are not grossly incorrect, and we therefore believe that they explain only a small fraction of the deviation observed.

One important factor which is absent from the above description and might significantly affect the association reaction in polymer solutions is the depletion interaction, or excluded volume effect. This interaction can be described as the effective attraction between two spheres in a polymer solution induced by the inability of the polymer molecules to enter the volume between the spheres, when their separation is smaller than the size of a polymer molecule. The strength of the depletion interaction should therefore depend on the size of the spheres and the polymer, as well as on the polymer concentration. The

effect is well-appreciated in polymer physics²⁷ and was measured both indirectly,²⁸ using scattering techniques, and directly, by probing the relative fluctuations of a pair of colloids held in optical tweezers with a polymer solution.²⁹ This effect has also been extensively discussed by Minton and co-workers in relation to the crowding phenomenon in biological environments,^{30,31} and also using a slightly different terminology by Bhat and Timasheff²¹ and Parsegian and co-workers.³²

We can use the experimental data together with the DLA theory calculation to obtain the energy involved in the depletion interaction. For that purpose, we model the effect of depletion on the association rate in the following manner:

$$\bar{\tau}_{a,\text{measured}} = \bar{\tau}_{a,\text{DLA}} e^{U/k_B T} \quad (4)$$

In this relation, we ignore the spatial dependence of the depletion energy, U . It was shown that when the reactive regions on the two proteins are small and the interaction potential is centrosymmetric its effect on the association rate can be calculated using its value at contact only.³³ This approximation was used successfully to predict the effect of an electrostatic potential on association rates.³⁴ Equation 4, which is written in the same spirit, can therefore be used to *directly calculate the depletion energy U from the experimental results*. The depletion energy is found to depend linearly on polymer concentration, as shown in Figure 5A (see Supporting Information for the parameters of the linear fit). At the highest concentration of PEG 8000 (~25 gr%), the depletion energy is $-0.8 k_B T$.

The analytical treatment of the depletion potential goes back to Asakura and Oosawa,³⁵ who modeled the macromolecular solution as a solution of hard spheres. The Asakura–Oosawa theory is valid only in the limit of small polymer concentrations, below the semidilute regime, as well as when $R \gg R_g$ (the so-called “colloid limit”). Interestingly, this theory, in which the depletion potential depends on the “ideal gas” osmotic pressure of the solution and a geometric factor, predicts a linear dependence on polymer concentration, similar to our experimental result. However, we found that the geometric coefficient in this theory is much too large to properly fit the experimental results, which is not surprising considering that most PEG 8000 association measurements are within the semidilute regime, and that the protein and polymer molecules are of similar size. Schweizer and co-workers^{36,37} have developed in recent years an integral equation theory, which takes into account the polymeric nature of high concentration solutions and is also valid when $R \leq R_g$. The theory was applied to measurements of the second virial coefficients of PEG solutions of proteins and was shown to semiquantitatively reproduce the experimental results.^{19,38} The

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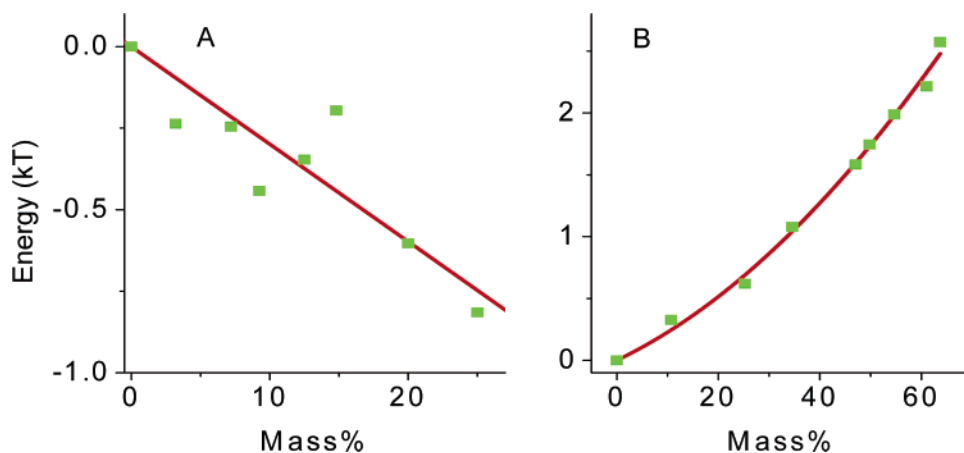


Figure 5. (A) The depletion energy in PEG solutions calculated from the experimental data and eq 4, and shown as a function of polymer concentration. The red line is a linear fit. (B) The repulsion energy in glycerol solutions calculated from the experimental data and eq 4, and shown as a function of polymer concentration. The red line is a fit to a second-order polynomial.

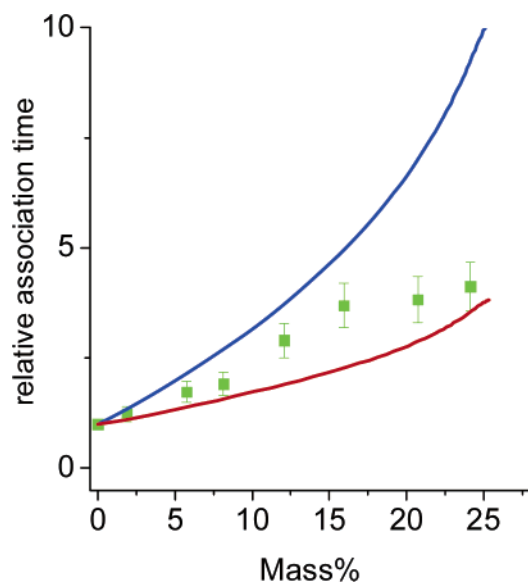


Figure 6. Comparison of the experimental relative association times, plotted as a function of the polymer concentration, to the times calculated from DLA theory (blue line) and further corrected by Schweizer's theory of the depletion interaction (red line).

depletion potential *at contact* is given in Schweizer's theory by:

$$U = -k_B T \ln(1 + \pi n_p R_g^2 a/2) \quad (5)$$

where n_p is the number density of the polymer, and R_g is the dilute solution value of the radius of gyration adjusted according to the polymer concentration.³⁹ We corrected the DLA relative association times using eq 5 together with eq 4. The result of this calculation is shown in Figure 6, in which the experimental results, plotted as a function of the polymer concentration in mass % (w/v), are compared to the DLA theory relative association times, shown here as a continuous curve (obtained from Figure 4 using a polynomial fit, blue line), and to Schweizer's theory-corrected relative times (red line). The numerical values are rather close to the measured ones, but the functional form is clearly not quite correct—it continues growing instead of leveling off. This is because of the logarithmic

dependence of U on concentration in Schweizer's theory, as opposed to the linear dependence found from the experimental values. Nevertheless, the combination of the DLA theory with Schweizer's theory for the depletion interaction accounts for the experimental results at least semiquantitatively. We hope our work will encourage further theoretical efforts that will make the agreement more quantitative.

Association in Glycerol Solutions. A nonlinear dependence of the relative association time on viscosity was found in glycerol solutions (Figure 1). Willson and co-workers⁴⁰ measured the association rates of lysozyme with monoclonal antibodies in glycerol solutions, and their results also deviated from the SE prediction, although the small range of viscosities they tested precludes an accurate determination of the observed functional dependence. Schreiber observed a nonlinear dependence of association rates of the proteins barnase and barstar in both glycerol and sucrose solutions (unpublished data). The deviation from the SE result found here is therefore not specific to the protein pair studied in the current work. This deviation may be linked to a short-range repulsive force between the proteins, induced by glycerol.

The occurrence of a glycerol-induced repulsive force between proteins is well-documented. Farnum and Zukoski⁴¹ and Blanch and co-workers⁴² measured the second virial coefficient in water/glycerol solutions of proteins and showed that it increases with the concentration of glycerol, indicative of increasing repulsion between protein molecules. The effect was attributed to the preferential hydration of proteins in glycerol solutions, which is due to the exclusion of glycerol from the hydration layer.⁴³ We suggest that the increased repulsion can also be understood in reference to lubrication theory,⁴⁴ which calculates a repulsive force between two approaching surfaces in a liquid due to the removal of the solvent from the gap between the surfaces.

We used eq 4 together with the experimental data to extract the repulsive energy as a function of glycerol concentration, shown in Figure 5B. In this case, the dependence on concentration deviates somewhat from linearity (see Supporting Informa-

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tion). At a glycerol concentration of 35 mass %, the repulsive energy is $\sim 1 k_B T$, but at 70 mass %, it is already larger than $2.5 k_B T$. From the second virial coefficient data of Liu et al.,⁴² it is possible to deduce a similar value for the repulsive energy in glycerol.

Conclusions

We presented in this work a combined measurement of protein association rate and protein translational and rotational diffusion in two different types of solutions. In solutions of the small viscogen glycerol, both translation and rotation follow the predicted SE behavior at least up to a relative viscosity of 20. The association time grows nonlinearly with viscosity. This was attributed to the effect of glycerol on the specific stage of the association reaction, inducing short-range repulsion due to preferential hydration of the interface and perhaps also due to a lubrication force.

In PEG 8000 solutions, there is a dramatic difference between the dependence of translational and rotational diffusion on the bulk viscosity. While the former still presents the SE behavior, the latter depends only weakly on viscosity, a phenomenon which is akin to the rotational dynamics of colloidal particles in colloid solutions. We found that this difference in behavior has a profound effect on the rate of protein association, which also shows very weak dependence on macroviscosity. However, association times calculated from the DLA theory, taking into account the measured values of the diffusion coefficients, systematically deviate from the measured association times. These deviations are attributed to the depletion interaction, which induces effective attraction between protein molecules in polymer solutions.

Both association—stalling repulsion in glycerol solutions and association—enhancing attraction in PEG 8000 solutions can be seen as manifestations of a similar phenomenon, namely, preferential hydration.⁴³ We were able to extract the energies involved in the two processes from the experimental data.⁴⁵ The results of this paper therefore offer a unified picture for all nonspecific effects involved in the protein association process. In principle, they should allow the calculation of association rates of other proteins in various solutions, with only the rate in water solution measured, by using a combination of DLA theory and the preferential hydration energies.

In addition to the fundamental importance of our results as a means to validate theoretical concepts, they also shed new light on the possible influence of the high concentration of macromolecules within the so-called *crowded* environment of living systems³⁰ and on the interactions between them. Indeed, a major criticism of *in vitro* studies of protein association is that, *in vivo* the conditions are very different from the ones in the test tube; 20–30% of the cell mass and volume is occupied by macromolecules, such as membranes, proteins, ribosomes, and RNA.^{8,46,47} Our experiments show that two types of effects are important in determining the protein association rates in

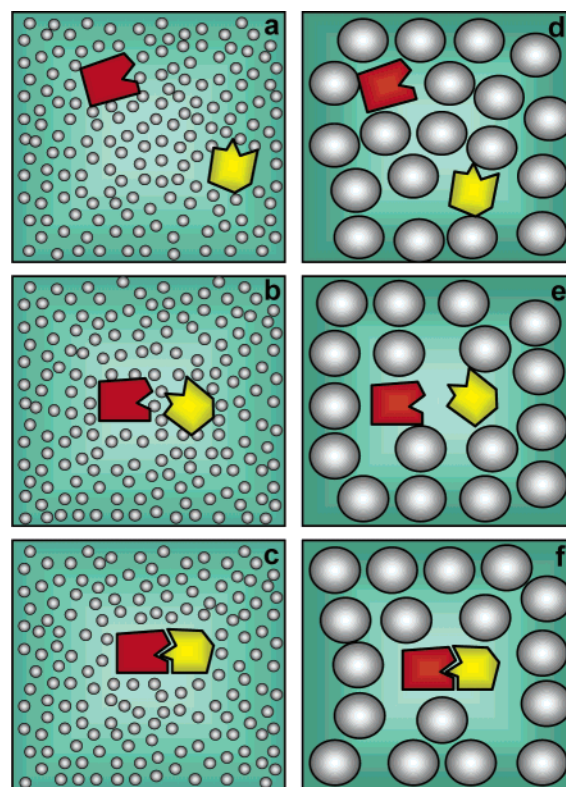


Figure 7. Scheme of the association reaction in glycerol (a–c) and PEG (d–f) solutions. In both solutions, association must involve both translational and rotational diffusion, which bring the two proteins into the correct mutual orientation. In glycerol solutions, the proteins have to overcome short-range repulsive forces (b). In PEG solutions, the proteins, once close together, are “trapped” in a cage of polymer molecules (e) which, in addition to allowing them almost free rotation, induces a short-range attractive force between them through the depletion interaction.

concentrated macromolecular solutions resembling the crowded cytoplasm of cells (Figure 7). The first and foremost is the dynamic effect, due to relative changes in translational and rotational diffusion of macromolecules. The second is the effective interaction between the two proteins induced by the solution at short distance. This interaction could be attractive, as in PEG solutions, or repulsive, as in glycerol solutions. Particularly interesting is the finding that the bulk viscosity of a macromolecular solution is not a good predictor of the association rate. The important implication of this finding is that, contrary to naïve thought, the crowded cellular environment does not significantly hinder protein association.

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Supporting Information Available: Additional experimental details, further analysis of translational and rotational diffusion in PEG solutions, and further details on energies of attraction and repulsion. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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(45) Some preliminary data with low molecular weight PEG solutions suggest that at high monomer concentrations of the additive a repulsive interaction similar to that of glycerol is regained. Thus it is possible that the depletion energies we report are somewhat underestimated due to some repulsion even at the lower monomer concentrations achieved with PEG 8000. This point awaits further study.

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