Common Crowding Agents Have Only a Small Effect on Protein-Protein Interactions

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ABSTRACT Studies of protein-protein interactions, carried out in polymer solutions, are designed to mimic the crowded environment inside living cells. It was shown that crowding enhances oligomerization and polymerization of macromolecules. Conversely, we have shown that crowding has only a small effect on the rate of association of protein complexes. Here, we investigated the equilibrium effects of crowding on protein heterodimerization of TEM1-β-lactamase with β-lactamase inhibitor protein (BLIP) and barnase with barstar. We also contrasted these with the effect of crowding on the weak binding pair CyPet-YPet. We measured the association and dissociation rates as well as the affinities and thermodynamic parameters of these interactions in polyethylene glycol and dextran solutions. For TEM1-BLIP and for barnase-barstar, only a minor reduction in association rate constants compared to that expected based on solution viscosity was found. Dissociation rate constants showed similar levels of reduction. Overall, this resulted in a binding affinity that is quite similar to that in aqueous solutions. On the other hand, for the CyPet-YPet pair, aggregation, and not enhanced dimerization, was detected in polyethylene glycol solutions. The results suggest that typical crowding agents have only a small effect on specific protein-protein dimerization reactions. Although crowding in the cell results from proteins and other macromolecules, one may still speculate that binding in vivo is not very different from that measured in dilute solutions.

INTRODUCTION

Virtually all aspects of cellular and multicellular activities are dependent on macromolecular interactions. Their study in dilute homogeneous solutions containing purified components fails to address the crowded in vivo environment (1). Crowding has both thermodynamic and kinetic effects on the properties of macromolecules, a fact that has been known for at least five decades since the systematic studies conducted by Ogston (2) and by Laurent (3). It has become appreciable that crowding in living systems might have a dramatic effect on biochemical processes such as enzymatic activity (4–6) and protein stability (7,8). It also promotes precipitation and crystal growth (9). Crowded solutions made of macromolecules such as polyethylene glycol (PEG), Ficoll, dextran, or proteins (such as ovalbumin or hemoglobin) were used to simulate cellular crowding effects in the test tube. The advantage in working with those solutions is that, unlike the in vivo environments, they are easy to manipulate and are chemically and physically well defined.

Molecular crowding is more accurately termed the excluded-volume effect, because the mutual impenetrability of all solute molecules is its most basic physical characteristic. A theoretical model, in which colloids and polymers are mutually impenetrable, was first proposed by Asakura and Oosawa (10) and by Vrij (11). Nonadsorbing polymers added to a colloidal suspension induce an attractive interaction between colloidal particles called the depletion interaction. The depletion force can be described as the effective attraction between two spheres in a crowded solution induced by the inability of the crowder molecules to enter the volume between the spheres (i.e., depletion of crowder) when the sphere separation is smaller than the size of the crowder. Minton (12) used the scaled particle theory, first formulated by Lebowitz (13) and Gibbons (14), to account for the influence of crowding on the thermodynamic activities of proteins. Accordingly, crowding was shown to always enhance the association reaction equilibria. Association reactions are entropically preferred in crowded solutions due to the partial overlap of excluded volumes, which renders more space to the crowder (15). More recently, Chatterjee and Schweizer developed the Polymer Reference Interaction Site Model (16), which was used by Kulkarni et al. to predict the global variations of lysozyme and bovine serum albumin (BSA) second virial coefficients as a function of PEG mass and concentration (17). Additional approaches, making use of osmotic stress and water activity to rationalize the crowding effects, exist as well (18). Parsegian et al. showed that all of these approaches are equivalent to each other (19).

Most experiments report the enhancement of association reactions by crowding. These include:

- Self-oligomerization (for example, decamers of the bacterial cell division protein FtsZ in the presence of hemoglobin or BSA (20) and decamers of bovine pancreatic trypsin inhibitor in the presence of dextran (21)).
- Hetero-oligomerization (for example, the assembly of *Escherichia coli* ribosomal subunits in the presence of dextran, Ficoll, and PEG (22)).
- Polymerization (for example, of deoxyhemoglobin S (23), and actin (24) in the presence of dextran).

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Homodimerization (for example, apomyoglobin forms dimers in the presence of other proteins serving as crowding agents (25)).

In all of these cases, there is a qualitative, and sometimes semiquantitative, agreement with theoretical predictions (26). Interestingly, no experimental data was reported for the effect of macromolecular crowding on heterodimerization equilibrium.

Although the excluded volume and depletion force theories relate principally to the equilibrium state of reactions, they should be applicable to association rates as well, since the structure of the transition state complex preceding association is very similar to the structure of the complex (27). Thus, association is predicted to be enhanced in crowded solutions (5), as was reported for polymerization or filament growth (28). However, crowding also reduces the rate of diffusion of proteins, and hence it is expected to have a more complicated effect on the association rate (5). Indeed, protein-dimerization reactions do not show enhanced association rates. It was found that for barnase and its inhibitor barstar, the rate of association did not change in solutions of the polymer Povidone (29). Similarly, a lack of effect was reported for cytochrome *f*-plastocyanin interaction in Ficoll 70 solution (30). For the association of TEM1-β-lactamase and its inhibitor β-lactamase inhibitor protein (BLIP) in the presence of up to 35% of PEGs of different sizes, there was actually a decrease in association rate relative to buffer, albeit only by two- to fourfolds (31,32).

Here, we wish to broaden our view and obtain a detailed correlation of the effects of crowding on both kinetics and thermodynamics of protein-protein dimerization, using two high-affinity heterocomplexes (the enzyme TEM1 binding its inhibitor BLIP and the enzyme barnase binding its inhibitor barstar) and one low-affinity complex (the GFP variants CyPet and YPet). Electrostatic steering has only a marginal role in the association reaction of TEM1-BLIP, resulting in an association rate constant of $3 \times 10^5\ M^{-1}\ s^{-1}$, dissociation rate constant of $2 \times 10^{-4}\ s^{-1}$, and affinity in the nM range (33). Conversely, the barnase-barstar pair is one of the fastest-binding and highest-affinity protein complexes, with an association rate of $4 \times 10^8\ M^{-1}\ s^{-1}$, dissociation rate of $4 \times 10^{-6}\ s^{-1}$, and an affinity of 0.01 pM (34). This fast binding is due to strong electrostatic forces acting between barnase and barstar.

MATERIALS AND METHODS

Protein expression and purification

Expression and purification of TEM1, BLIP, barnase, barstar, CyPet and YPet were all previously described (33–36). Unless indicated otherwise, measurements were preformed in 10 mM HEPES, pH 7.3 at 25°C.

Viscogens

Ethylene glycol (EG), PEG600, PEG1000, PEG8000, and dextran6000 were purchased from Sigma Chemical (St. Louis, MO). Glucose was purchased from BDH Merck (Poole, Dorset, UK). All chemicals were used without further purification. See Supporting Material for details.

Viscosity measurements

Viscosity measurements were done using a Cannon-Fenske Routine Viscometer 150/I750 (Cannon, State College, PA) as previously described (31).

Association rate measurements

Association rates were measured using a stopped-flow fluorescence spectrometer (Applied PhotoPhysics, Leatherhead, UK) under second-order conditions as described earlier (31). See Supporting Material for details.

Binding measurements using surface plasmon resonance (SPR)

Binding constants were determined by surface plasmon resonance (SPR) spectroscopy, as applied in the ProteOn XPR36 Protein Interaction Array System (BioRad, Hercules, CA). See Supporting Material for details.

Enzyme inhibition assay

Binding constants were determined as previously described (33). See Supporting Material for details.

Isothermal titration calorimetry (ITC)

Measurements were done using an iTC_{200} instrument (MicroCal, Northampton, MA). Approximately 260 μL of 9 μM barstar was used to fill the cell of the calorimeter and titrated with a total of ~40 μL barnase at 50 μM. See Supporting Material for details.

Fluorescence resonance energy transfer (FRET)

CyPet (FRET donor) and YPet (FRET acceptor) were used in equal concentrations of 1 μM. The solution was excited at 420 nm, and the emission spectrum (between 430 and 650 nm) was recorded. See Supporting Material for details.

Fluorescence correlation spectroscopy (FCS)

Translational diffusion time of 25 nM YPet with and without 500 nM CyPet was measured using a homebuilt fluorescence correlation spectrometer (FCS) as described in Sherman et al. (37) at a constant temperature of 21°C. See Supporting Material for details.

Dynamic light scattering (DLS)

DLS experiments were performed on a model No. 802 DLS instrument (Viscotek, Houston, TX) at an incident-light wavelength of 830 nm. See Supporting Material for details.

RESULTS

Throughout the experiments described in this article, PEG and dextran were used as high-molecular-weight crowding agents, whereas the monomeric compounds EG and glucose were used as the corresponding low molecular-weight controls. Results of affinity measurements are mostly reported as relative association constants, i.e., the ratio between K_A in buffer and K_A in a solution containing the viscogen. Values >1 indicate that the affinity in the viscogen solution is lower than in buffer.

TEM1-BLIP

Effect of crowding agents on the rate of association

The association rate constants of TEM1-BLIP interaction in various PEG and small viscogens solutions have been previously determined (1,31,32). Here we performed the same measurements using dextran and glucose solutions. Fig. 1 *A* shows the relative association rate constants (buffer/viscogenic solution) of wt TEM1 and BLIP +4 (D163K, N89K, and V165K) in solutions of viscogens of various size and concentration. For a diffusion-controlled reaction of two similar-sized particles, the rate of collision (k_{coll}) is given by the Smoluchowski relation

$$k_{coll} = 4\pi DR, \tag{1}$$

with R being the sum of the effective radii of the particles and D the sum of their translational diffusion coefficients (D_t) calculated according to the Stokes-Einstein (SE) relation,

$$D_t = \frac{k_B T}{6\pi\eta r}, \tag{2}$$

with η being the viscosity of the medium and r the hydrodynamic radius of the particle. As the rate of collision is linear with D_t, and D_t is inversely linear with viscosity (1), we may assume that k_{coll} is inversely proportional to viscosity. Therefore, by multiplying the measured values of k_a by η, we obtain a measure of the effects of viscogens on the rate of binding that is essentially independent of the rate of collision. We define the deviation from the SE behavior as

$$\alpha = \frac{k_{a(crowd)} \times \eta_{(crowd)}}{k_{a(buffer)} \times \eta_{(buffer)}} - 1, \tag{3}$$

where $k_{a(buffer)}$ and $k_{a(crowd)}$ refer to the rate of association in buffer and in solution of a viscogen agent, respectively, and $\eta_{(buffer)}$ and $\eta_{(crowd)}$ are the respective viscosities. The viscogen concentration dependence of α can be roughly attributed to events along the association pathway occurring between collision and complex formation. In Fig. 1 *C*, values of α are plotted as a function of the mass percent of the viscogen. In dextran the TEM1-BLIP association was faster than expected from SE ($\alpha > 0$), whereas in glucose the association was slower than expected from SE ($\alpha < 0$). These results are in line with those observed for PEG and EG (32).

Effect of crowding agents on the rate of dissociation

Dissociation rate constants of the TEM1-BLIP wt and mutant complexes were measured in buffer and in the presence of viscogens using SPR spectroscopy (Fig. 2 *A*). In polymer and glucose solutions, a slight decrease in dissociation rate was observed. Table 1 summarizes the relative dissociation rates in the different solutions. In addition to the mass-percent data of the different viscogens, we provide the c/c^* ratio of the different PEG solutions in Table 1. The value c^* is the dilute-to-semidilute crossover mass concentration calculated from $N^{-4/5}$, where N is the number of monomer units in the polymer chain (38). We have previously shown that these calculated c^* values were in good correlation with the different phases for association rates measured for TEM1 binding BLIP (32).

Dissociation rates in PEG1000 solutions were measured for 20 different mutant pairs (Fig. 2 *B*). In the 10 and 30%

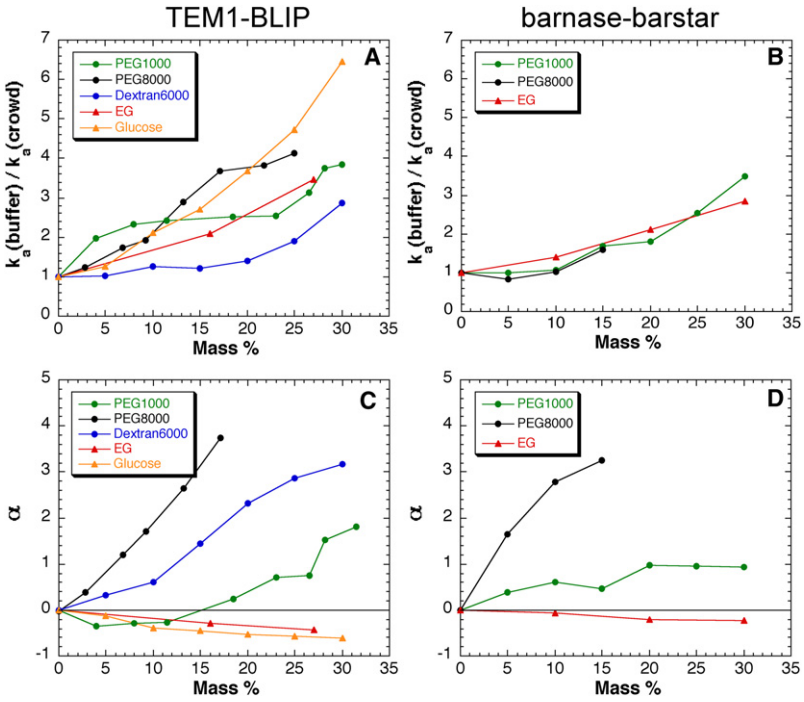

FIGURE 1 Relative association rate constants in viscogenic solutions. (*A*) Relative rates (buffer/crowded solution) of TEM1 wt with BLIP +4. Data for PEG and EG solutions are from Kozer et al. (32). (*B*) Relative rates of wt barnase with wt barstar. Association rates were measured in the presence of 50 mM NaCl. (*C* and *D*) Deviations from SE behavior calculated according to Eq. 3 for TEM-BLIP and for barnase-barstar. Viscosity values of PEG and EG solutions were taken from Kozer et al. (32). The horizontal line at $y = 0$ represents the SE limit, in which association rates are dictated by viscosity alone.

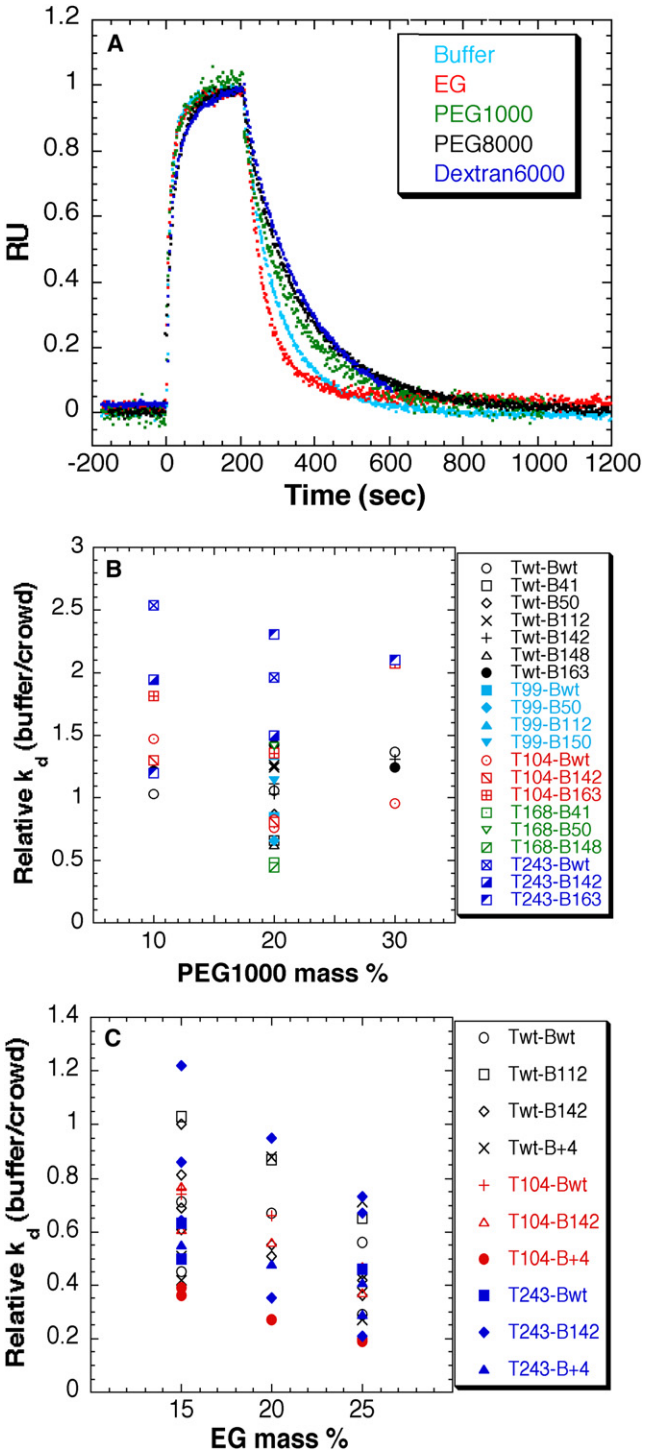

FIGURE 2 Dissociation rate of TEM1-BLIP. (*A*) SPR binding curves for TEM1 wt-BLIP F142A in buffer, 15% EG, 10% PEG1000, 15% PEG8000, and 10% dextran6000. (*B* and *C*) Relative dissociation rates (rate in buffer/rate in viscogen) of different TEM1-BLIP (T-B) mutants. All mutations introduced (except D163K) are to Ala, with the respective residue number written. The label "+4" is the fast-associating BLIP mutant of N89K, D163K, and V165K.

TABLE 1 Relative rates and association constants for TEM1 binding BLIP in crowded solutions

Solution	Mass concentration*	c/c^*†	Relative k_d‡	Relative k_a§	Relative K_A¶	Relative K_A‖
PEG600	30	2.4				2.9**
	45	3.6				1.4**
PEG1000	10	1.2	1.5	2.4	1.6	1.9††
	20	2.4	1.1	2.5	2.3	1.3††
	30	3.6	1.5	3.7	2.5	1.3**
PEG8000	8	5.1	1.2	1.6	1.3	0.9††
	15	9.6	1.6	1.7	1.1	0.6**
	15	9.6				1.0††
	20	12.8				1.0**
Dextran6000	10		1.9	1.3	0.7	
	15		2.3	1.2	0.5	
	20		2.7	1.4	0.5	
EG	15		0.7	2.0	2.9	1.8††
	20		0.6	2.6	4.3	4.9††
	25		0.4	3.2	8.0	7.7††
	40					6.3**
	50					23.9**
Glucose	10		1.6	2.1	1.3	
	15		2.1	2.7	1.3	
	20		2.3	3.7	1.6	
	25		2.4	4.7	2.0	
	30		2.7	6.5	2.4	

Values of standard errors for the different measurements are given in the Supporting Material.

*Mass concentration is expressed as weight-percent of the viscogenic agent from the total weight of the solution.

†Note that c is the mass concentration in w/w, and c^* is the dilute-semidilute crossover mass concentration (32).

‡Relative k_d of buffer/viscogen. Mean values of wt TEM1 and BLIP F142A, except for PEG1000 and EG, in which the values are mean of different mutant pairs as indicated in Fig. 2, *B* and *C*, respectively. Measurements were done in the ProteOn XPR36.

§Relative k_a of buffer/viscogen. Values for PEG and EG are taken from the literature (31,32) and are for wt-TEM1 binding BLIP+4. Measurements were done in a stopped-flow.

¶Calculated as the ratio between relative k_a and relative k_d.

‖Relative association constants (in buffer/viscogen) for wt-TEM1 BLIP-F142A as determined by equilibrium methods.

**Values determined by inhibition assay.

††Values determined by SPR steady-state measurements.

solutions there was a slight slowdown of ~50% in the dissociation rate constant compared to buffer. However, this change was within the standard deviation of these data. In the 20% solution there was no change in dissociation rate compared to buffer (p-value > 0.1). The measurements in 20% solution involved a larger number of samples. The mean value represents measurements made on different days (i.e., using different ProteOn XPR36 chips).

Dissociation rate constants in EG were measured for 10 different mutant pairs, in solutions of concentrations up to 25% (Fig. 2 *C*). In 25% solutions a significant (p-value < 0.05) twofold increase in k_d compared with buffer was measured. The relative dissociation rate constants of eight

different wt and mutant complexes in 25% EG were compared to evaluate whether the effect of EG is a general characteristic of the proteins, or relates to specific residues on their binding surfaces (Table S1 in Supporting Material). The mutant complexes have k_d values between 7.8×10^{-5} s^{-1} and 1.6×10^{-2} s^{-1}. The influence of EG on the different mutant complexes was found to be similar using an ANOVA test (data not shown). This suggests a nonspecific effect of EG on dissociation, which does not depend on the detailed chemistry of the interface.

Effect of crowding agents on affinity

The effects of crowded solutions on k_a and k_d values of TEM1-BLIP interaction suggest that crowding by polymers (at least up to a concentration of 30%) has only a small effect on the affinity of complex formation. Table 1 shows the expected relative K_A values as calculated from k_a/k_d. In dextran the affinity increased up to twofold, whereas in PEG8000 the affinity was not affected. In PEG1000, a smaller polymer, the affinity decreased by a factor of 2.5. In EG solutions the affinity decrease was quite prominent, reaching approximately eightfold in the 25% solution. As these results contradict many previous findings (20–25), we felt it necessary to confirm those using complementary methods. In Table 1, rightmost column, we show the results of affinity determination using equilibrium methods, including SPR spectroscopy and an enzyme inhibition assay (see also Fig. S1 and Fig. S2). The experiments were done using the pair wt-TEM and BLIP-F142A. The ~10-fold weaker affinity of this pair compared to wild-type provides a larger dynamic range for measurements of increased and decreased affinities. The binding affinity in buffer was 47 ± 7 nM according to the enzyme inhibition assay, and 160 ± 17 nM according to the SPR data. A threefold difference in binding constants between different methods is common (33), particularly as the enzyme inhibition assay involves also the contribution of the substrate as competitor to the inhibitor. Therefore, only relative results (i.e., change in values measured in viscogen solutions versus those measured in buffer for each method separately) are provided.

No significant change in binding affinity was detected in PEG solutions of up to 30% mass compared to the affinity in buffer (Table 1). This observation is in agreement with the effect of PEG on binding rates; since both association and dissociation rates are slowed down to a similar extent, no overall change in the binding affinity should be seen. Conversely, a large decrease in binding affinity was measured for high-mass-percent EG solutions (Table 1). At 50% EG, the affinity was ~24-fold weaker than in buffer. This observation is in agreement with our results for the effect of EG solutions on binding rates; since association rates are getting slower and dissociation rates are getting faster, an overall decrease in the binding affinity should be observed.

Fig. 3 *A* shows a comparison between the relative affinities as determined from binding rate constants (Table 1) to the relative affinities as determined by equilibrium measurements (Table 1, *rightmost column*). The data obtained by the different methods are in good agreement. This is despite the substantial differences between the methods used; SPR spectroscopy probes the thickness of the binding layer on the surface while the enzyme inhibition

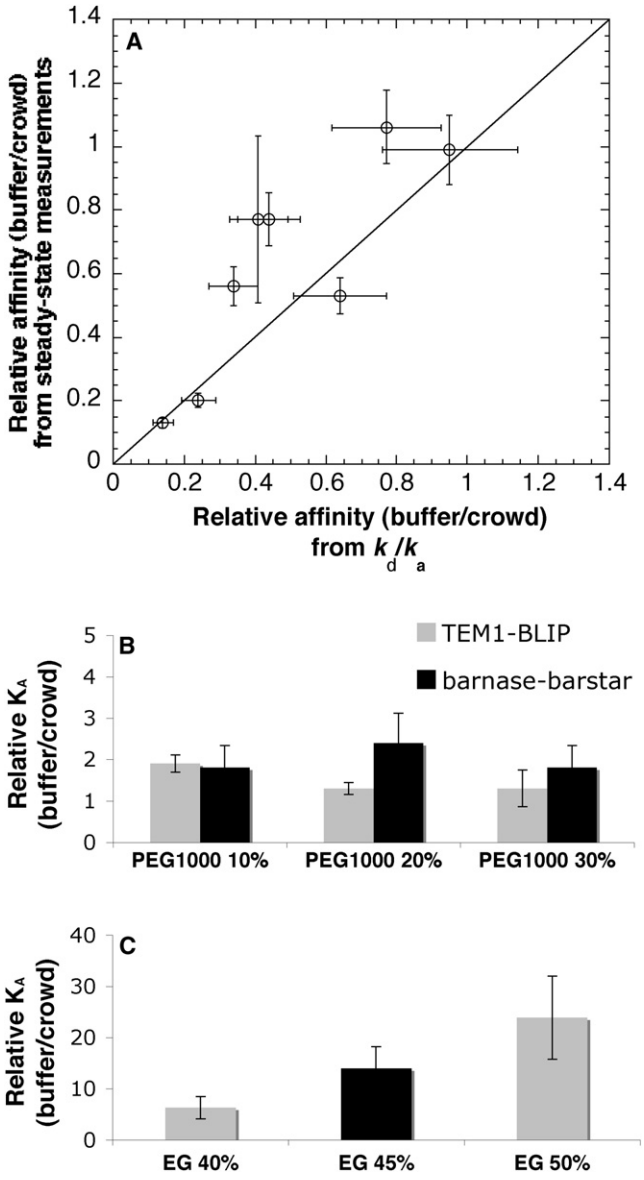

FIGURE 3 Comparison between experimental methods and protein pairs. (*A*) Relative binding constants of TEM1-BLIP as determined from the kinetic measurements or by steady-state measurements. The abscissa is the relative affinity that was calculated according to the relative rate constants (k_d/k_a). Values are from Table 1. The ordinate is the relative affinity in the same solution that was determined by steady-state measurements. Values are from Table 1, rightmost column. The line of $Y=X$ represents the expected position of the data points assuming a 1:1 relation between the values. (*B* and *C*) Comparison between relative association constants (K_A) of TEM1-BLIP and barnase-barstar in crowded PEG solutions (*B*) and in EG solutions (*C*). The TEM1-BLIP data is from the SPR steady-state experiments and from the inhibition assay (Table 1, *rightmost column*). The barnase-barstar data is from the ITC experiments (Table 2).

method is in solution and probes enzymatic activity. This emphasizes the reliability of these methods in determining the relative affinity of protein complexes, whereas absolute values differ.

Barnase-barstar

To establish that our results are not restricted to the protein pair TEM1-BLIP, we investigated a second heterodimerization model system, barnase binding to barstar.

Effect of crowding agents on the rate of association

Fig. 1 *B* shows the relative association rate constants of wt barnase and barstar in PEG and EG solutions. At 30% viscogen the decrease in rate was up to threefold. A representation of the data that also takes the viscosity and diffusion rates into account is presented in Fig. 1 *D*, where values of α are plotted as a function of the mass percent of the viscogen. The association behavior of barnase-barstar in different solutions was very similar to that measured for TEM1 and BLIP, with faster association than expected from SE ($\alpha > 0$) in PEG solutions, and slower association than expected from SE ($\alpha < 0$) in EG solutions.

Effect of crowding agents on affinity

Given these results, we speculated that, similarly to the case of TEM1-BLIP, the affinity of the complex would not be affected by crowding to a significant extent. To determine the affinity of barnase and barstar in viscogenic solutions, both an enzyme inhibition assay (Fig. S1) and ITC (Fig. S3) were used. Measurements were done on barnase K27A and barstar Y29A, since the affinity of the wild-type complex is too high to be directly measured by these methods. The affinity of the complex in buffer, as determined by the enzyme inhibition assay and by ITC, was 80 ± 14 nM, and 11.6 ± 1.7 nM, respectively. Table 2 shows that the affinities in PEG1000 solutions of various concentrations did not change significantly compared to buffer. Conversely, a large decrease in binding affinity was measured for high-mass-percent EG solutions.

TABLE 2 Relative association constants in EG and PEG1000 solutions of the barnase K27A barstar Y29A pair

Solution	Mass concentration (w/w)	Relative K_A $\frac{K_{A(buffer)}}{K_{A(crowd)}}$
PEG1000	10	1.8*
	20	2.4*
	25	1.7†
	30	1.8*
EG	45	14*

Values of SE for the different measurements are given in the Supporting Material.

*Values determined by ITC measurements. For EG, measurements were done with 50 mM NaCl to avoid nonspecific aggregation.

†Value determined by inhibition assay.

CyPet-YPet

The proteins CyPet and YPet, which are GFP variants that serve as a FRET pair (39), present weak binding affinity (~100 μM (39,40)). Their binding was measured using FRET, FCS, and DLS.

Change in FRET efficiency in different viscogen solutions was used to probe the change in the equilibrium distance distribution between the donor (CyPet) and the acceptor (YPet) molecules. To confirm that changes in FRET were related to the distances between the proteins, and not to change in their fluorescence properties, emission spectra of each protein were recorded in viscogen solutions and compared to that in buffer. No change in emission spectra was detected (see Fig. S4 *A* and Fig. S4 *B*). As only folded proteins emit light at these wavelengths, the results also indicate that the proteins retain their native structure and function even in high-mass-percent solutions, and that their relative quantum yields do not change.

The FRET efficiency of a 1 μM solution of CyPet and YPet was calculated from fluorescence spectra recorded using different buffer solutions, with different ionic strength and with various concentrations of PEG or EG. Fig. S4 *C* shows a typical spectrum of CyPet and YPet in buffer and in the presence of 40% PEG1000. Fig. 4 *A* shows the change in FRET in 10 mM HEPES buffer with increasing concentrations of viscogens. In PEG solutions, no change in FRET efficiency compared to buffer was detected up to 20% mass. In 25–30% there was a clear rise in FRET, which reached a maximum at ~40% mass. In EG solutions, no change in FRET efficiency compared to buffer was detected up to 60% mass. When repeating the same experiments in a 50 mM sodium phosphate buffer, we could not detect any change in FRET between CyPet and YPet, even in 40% PEG1000 (Fig. 4 *B*). A possible explanation of the differences between the two buffers is the difference in their ionic strength (4 mM for HEPES and 130 mM for Phosphate), which may interfere with protein-protein interaction due to screening of electrostatic forces (27). To test this explanation, we measured the FRET signal in HEPES buffer containing 40% PEG1000 with and without NaCl (to equal the ionic strength of the Phosphate buffer). No FRET was detected when NaCl was added, validating our explanation (Fig. 4 *B*).

CyPet and YPet aggregate in crowded solutions

FCS allowed us to record intensity fluctuations and to obtain the correlation function of YPet, $G(\tau)$, in different crowded solutions, and through this to determine whether the increased FRET in the presence of PEG1000 (in 10 mM HEPES buffer) was a result of specific complexation or of aggregation. The fluorescence intensity recorded over time for 25 nM YPet with or without 500 nM CyPet showed large fluctuations in PEG1000 solutions (Fig. S4 *D*). This suggested that CyPet and YPet form aggregates in crowded solutions (in the absence of salt). To validate the aggregation, we determined the diffusion correlation time from the FCS curves (see Supporting Material). First, we used Rhodamine6G to quantify

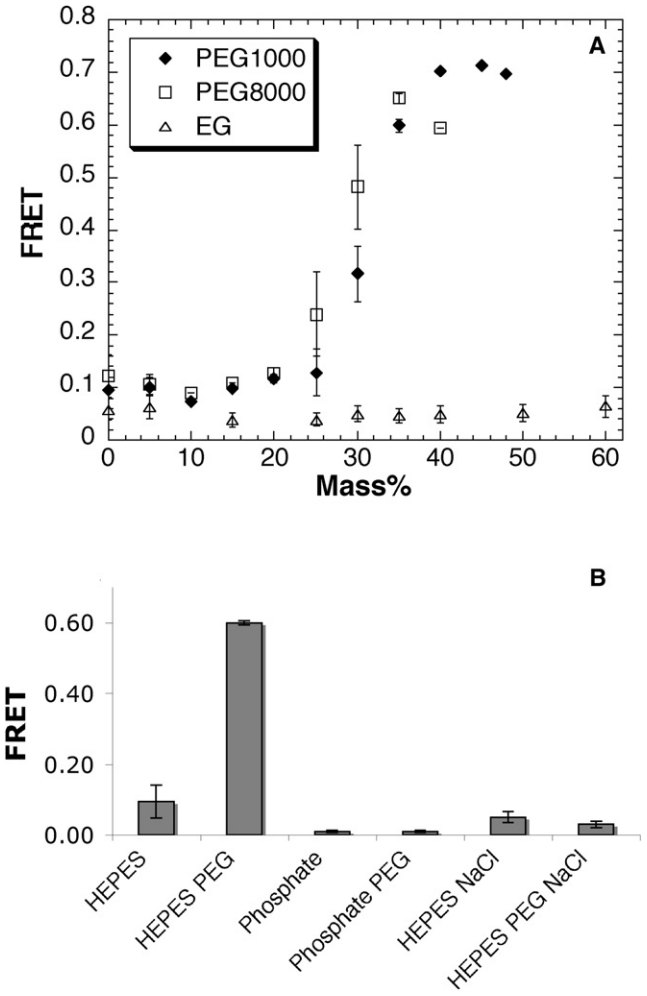

FIGURE 4 FRET measurements of CyPet binding YPet. (*A*) FRET efficiency in PEG and EG solutions of various concentrations. (*B*) Effect of ionic strength on CyPet-YPet interaction. FRET efficiency in 10 mM HEPES and 50 mM sodium phosphate buffers, with or without 40% PEG1000 and NaCl.

possible changes in the observation volume due to changes in the refraction index (similar to (37)), and found its calculated diffusion time in 30% PEG1000 to be in accordance with the SE equation. Next, we measured the diffusion time of YPet in buffer, which was ~350 μs. This value was expected based on the protein size and the FCS setup used. However, in 30 and 40% PEG1000 the estimated diffusion times of YPet were two-orders-of-magnitude longer than that in buffer, whereas the viscosities of the solutions were only 7- and 12-times larger, respectively. This analysis indicates again that YPet forms aggregates in PEG1000 solutions.

To validate that CyPet-YPet indeed aggregate at high PEG concentrations, we repeated these experiments using DLS, which has the advantage that a fluorescence probe is not needed for the measurements. In buffer, the protein peak appears at a size of 3.5 nm, which is in accordance with its known size (Table 3). PEG alone gave a peak at 60–100 nm. No change in protein-peak location was observed for

TABLE 3 Size of protein complexes of CyPet-YPet and TEM1-BLIP as measured by DLS in buffer and in PEG1000 solutions

PEG1000 (w/w)	Position of protein peak (nm)	
	CyPet-YPet*	TEM1-BLIP†
0	3.3	3.2
10	3.5	3.3
20	3.7	ND
25	‡	3.0
30	‡	ND

A peak at 60–100 nm was observed for PEG alone.
*0.4 g/L from each protein were mixed together in the indicated solution.
†0.5 g/L from TEM1 and 0.3 g/L from BLIP were mixed together in the indicated solution.
‡Only a peak corresponding to ~200 nm size was resolved.

solutions of 10 and 20% PEG1000. However, at PEG1000 concentrations of 25 and 30%, the protein peak disappeared. Conversely, for TEM1-BLIP a protein peak at ~3.2 nm was present at all PEG1000 concentrations. Although these are only qualitative measures, their value lies in the ability to compare PEG alone to the solution containing also TEM1/BLIP or CyPet/YPet. The DLS measurements indicate that CyPet and YPet indeed aggregate in concentrated PEG1000 solutions, while TEM1 and BLIP do not. FCS, FRET, and DLS measurements of CyPet/YPet were done using protein concentrations of 25 nM, 1 μM, and 15 μM, respectively, yet the PEG1000 concentration driving aggregation was constant (~25%). This suggests that PEG and not protein concentration drives the observed aggregation.

DISCUSSION

In this study we wished to complete our knowledge about the effects of crowding agents on association and dissociation rate constants of protein heterodimers and on their binding affinity. The results are discussed with respect to the theoretically predicted effects of crowding on protein-protein interaction.

Based on the scaled particle theory, Minton showed that in the case of spherical molecules in a solution of inert spherical background molecules, the association reaction would always be favored (12). The extent of the enhancement depends on the volume occupancy of the background molecules (higher volume occupancy leads to higher enhancement in association) and on the reaction stoichiometry (oligomerization is enhanced more than dimerization). Even in the case of two spherical proteins with identical volumes, which undergo dimerization in solution containing background molecules with the same volume, the theory predicts that the enhancement in affinity can reach two orders of magnitude at physiological volume occupancy. Berg proposed a refinement of the theory (41), by including the water as a separate component to the hard-sphere mixture (instead of treating it as an inert structureless solvent, as in Minton's approach). Using the refined approach, Berg found that the excluded volume effect

enhances association in the case where two spherical monomers form a dimer, which is also spherical. In the more likely scenario, in which the complex would be shaped roughly as a dumbbell, the excluded volume effect would be much smaller and can even lead to destabilization of dimerization. Calculations based on scaled particle theory do not take into account the polymeric nature of the crowding agents. The concentrations of PEG used in this article were above their c^* values (Table 1). In the semidilute regime the polymer chains overlap and entangle, and protein molecules are embedded within the polymer mesh. Work by Chatterjee and Schweizer on depletion in polymer solutions attempted to correct this (16). As shown in a previous publication from our lab (1), application of their theory leads to a rather modest effect, more in line with the current findings. For example, the affinity of TEM1-BLIP in 10% PEG1000 is predicted to be enhanced by a factor <2.

The effect of crowding on the equilibrium constant of the reaction can also be investigated from a kinetic point of view:

$$K_A = \frac{k_a}{k_d}. \quad (4)$$

Protein association is a multistep process, where randomly colliding proteins may form an encounter complex, which transforms to the final complex through a transition state. The effects of crowded solutions on reaction rates will depend on the sum of the effects on each of those steps located before the transition state. The first step in protein association is the collision of two proteins, which rate is linearly proportional to their diffusion coefficients and therefore inversely proportional to the solution viscosity (see Eqs. 2 and 3 and (31)). As crowded solutions have higher viscosity, the rate of collision is decreasing (32). Next the transition from collision to binding occurs. This step is dictated by rotational diffusion, structural rearrangement, and desolvation. According to the SE relation, rotational diffusion is also inversely proportional to solution viscosity, so the effect of crowding should be the same as for translational diffusion. However, experimental measurements have shown that the influence of macromolecular crowding agents (but not of small viscogens) on rotation is much less than that expected from SE (1).

The transition state represents the free energy barrier between reactants (protein monomers) and products (protein complex). Geometrically, the transition state and the final complex were shown to be quite similar (27). In other words, the transition state and the final complex have roughly the same excluded volumes, so the stabilization due to overlap of excluded volumes should be similar in both, which would reduce the transition state energy (28). As a similar excluded volume effect should already appear at the encounter complex, the increase in rate of association should be due to the stabilization of both the encounter and transition states.

The overall effect of macromolecular crowding on the association rate depends upon the nature of the association reaction. If the association is diffusion-limited, then the binding rate should decrease, and if the association is transition-state-limited (also called reaction-limited), the binding rate should increase (28). Zhou suggested that for most binding reactions there is no clear division into diffusion-limited and transition-state-limited, and the overall effect would be a combination of the two opposing effects, manifested only in a moderate effect on the binding rate over a wide range of volume occupancy (42). This notion is supported by a recent Brownian dynamics simulation, which took into account the probability of an encounter complex to form a final complex (43). Experimentally, the association rate constants of several dimerization reactions were reported to be indifferent to macromolecular crowding (29,30). In the case of TEM1-BLIP association in up to 35% mass of PEG, a small decrease in the association rate constant was reported (32). Here we showed that the same is true for dextran solutions and for the association rate constant of barnase with barstar. When presenting the relative association rate constants in terms of α (Fig. 1, *C* and *D*), which essentially eliminates the effect of viscosity, the depletion force is detected as a positive deviation from the line of unity in polymer solutions, but not in small viscogens' solutions. The total rate of association is therefore a combination of the two opposing effects, manifested in a net slowdown that is less than that expected from viscosity alone.

So far, the effect of crowding on the dissociation process has gained very little interest compared to the association process. Since both the transition state and the final complex have similar excluded volumes, no change is expected in the dissociation rate from final complex back to the encounter complex (usually termed k_{-2}) (12). As k_{-2} is the rate-limiting step for dissociation (27), no significant change should also be observed for k_d. Although there is a consensus regarding the lack of influence of crowding on dissociation rates, no direct measurement of them have been made. Our measurements of k_d of TEM1-BLIP in polymer solutions largely confirmed the above prediction. The slightly decreased dissociation rates, notable mostly in dextran solutions, may be viewed as an increase in rebinding from the encounter complex, which results from its prolonged lifetime (1,43).

The results for reaction rates coincide well with the affinity measurements as shown in Fig. 3 *A*; a slight decrease in association and dissociation rates leads to no detectable changes in affinity in polymer solutions, whereas decreased association and increased dissociation rates leads to lower affinity in EG solutions. The dissociation behavior in EG could be explained by the same mechanism proposed before to explain the association behavior; namely, that preferential hydration of the proteins in EG solutions cause a repulsion between the proteins (31,32), which is realized both by faster dissociation and by slower association rates.

Fig. 3, *B* and *C*, show the relative affinity of TEM1-BLIP and of barnase-barstar in PEG1000 and in EG solution. The effect of both additives is the same for both complexes,

suggesting a pure nonspecific interaction with the proteins. Ethylene glycol and glucose can serve as a control for the chemical effect of PEG and dextran solutions, as the only difference between them is the polymeric nature that dictates steric repulsion. Throughout this article, we related the rates and affinities of binding in various crowding solutions to that in buffer. However, one can relate the measured rates in polymer solutions to those in monomer solutions, thus keeping the chemistry of the solution the same. In 15–20% PEG or dextran solutions, the affinity was two- to threefold higher than in their respective monomer solutions, demonstrating the additional effect polymers exert.

The question of whether PEG specifically interacts with proteins is still debated. A recent study proposed that PEG could bind to the hydrophobic surface of cytochrome *c* (44), suggesting that PEG is not an inert crowding agent. In contrast, Spelzini et al. (45) suggest that small proteins with hydrophilic nature, such as lysozyme, have no tendency to interact with PEG molecules, and that they remain in the PEG-rich phase under partitioning due to excluded volume effects. The heat associated with the PEG-lysozyme and PEG-ovalbumin interactions (two hydrophilic proteins) has been determined to be ~1–2 kcal/mol, again suggesting poor (if any) interaction between these molecules (46). Kulkarni et al. (17) noted that the second virial coefficient obtained for lysozyme and BSA in PEG solutions of >1000 MW at concentrations of up to c^* can be fully understood using the Polymer Reference Interaction Site Model theory (16). However, at higher PEG concentrations, a weak attractive force between PEG and the protein may play a role (17). The interaction between PEG and Pepsin was suggested to be dependent on the molecular weight of the PEG (45). As all the proteins used in our study are both small and hydrophilic, one may suggest that they do not specifically interact with PEG, and if they do, this interaction is weak. This conclusion is strengthened by previous studies on TEM1-BLIP in PEG solutions (1,32), where we have shown a simple linear relation between D_t (the translational diffusion rate) and η (as expected from SED). Moreover, we showed that the effect of PEG and Ficoll on k_a is similar, suggesting no specific role for PEG. However, because of the uncertainty concerning PEG-protein interactions and their effect on binding (depletion and specific interactions may be additive to one another), we validated our experimental results using dextran as a crowding agent. Dextran is widely considered as a chemically inert crowding agent (8,21,47). In addition to measuring TEM1-BLIP association, we repeated the experiments with the barnase-barstar pair as well. The similar results obtained with PEG and dextran for both protein complexes indicate that the fact that crowding agents have only a minor effect on the kinetics and equilibrium constants of protein interaction is not due to specific interaction of the crowding agents with the proteins.

In the CyPet-YPet system, which was used as a model for a low-affinity complex, aggregation, and not enhanced dimerization, was observed in high-mass-percent PEG solutions. Still, even in very high concentration of PEG solutions, the fluorescent proteins retained their native structure inside the aggregates. This fact suggests that aggregate formation was driven by excluded volume effects, not protein denaturation. It also emphasizes the fact that most of the reported association enhancements in the literature were for oligomerization, polymerization, and aggregation, an effect not observed by us for heterodimerization.

CONCLUSION

The effects of crowding on protein-protein association were extensively investigated. However, most measurements were done for oligomerization and polymerization reactions with little focus on dimerization. At least for the latter, the separate analysis of association and dissociation rates proved to be much more successful in understanding the influence of crowding on binding. For association, crowding will enhance the development of the final complex from the encounter, thus acting to reverse the effect of slowed diffusion. However, for dissociation, the increase in rebinding from the encounter complex will have only a small effect on the overall dissociation rate, as the encounter complex tends to dissociate rather than reforming the complex. A notable difference between heterodimerization and either oligomerization or polymerization is the single-versus-multiple binding interfaces, which result, for oligomerization/polymerization reactions, in a higher probability of a dissociated encounter complex to rebind. This will stabilize oligomerization and polymerization in crowded solutions, compared to heterodimerization. Interestingly, the effects of crowding agents on association and dissociation rates were similar to the effects observed for favorable electrostatic forces, which also act through the stabilization of the encounter complex. Increasing electrostatic forces did not change the rate of transmission from the encounter complex to the final complex and vice versa. Our results imply that at least for protein dimerization, the effects of crowding agents are relatively mild, and thus one may speculate that in vitro measurements in aqueous solutions are applicable to reactions occurring in cells, keeping in mind that, within the cell, proteins and other biological macromolecules constitute the crowd. Further investigation of the behavior of dimerization as well as higher-order association model systems in crowded solutions is critical for gaining comprehensive understanding of the crowding phenomena in general.

SUPPORTING MATERIAL

Four figures and one table are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)01032-7.

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