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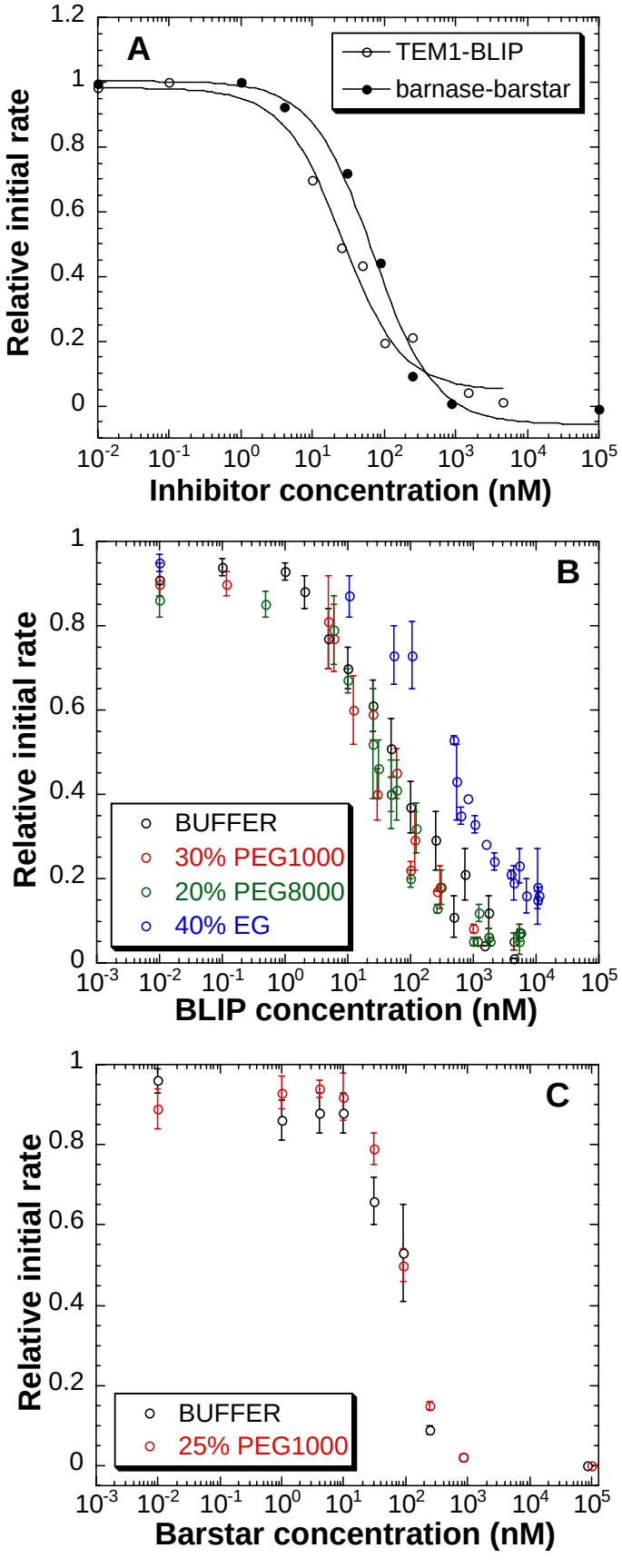
Supporting Material

Common crowding agents have only a small effect on protein-protein interactions

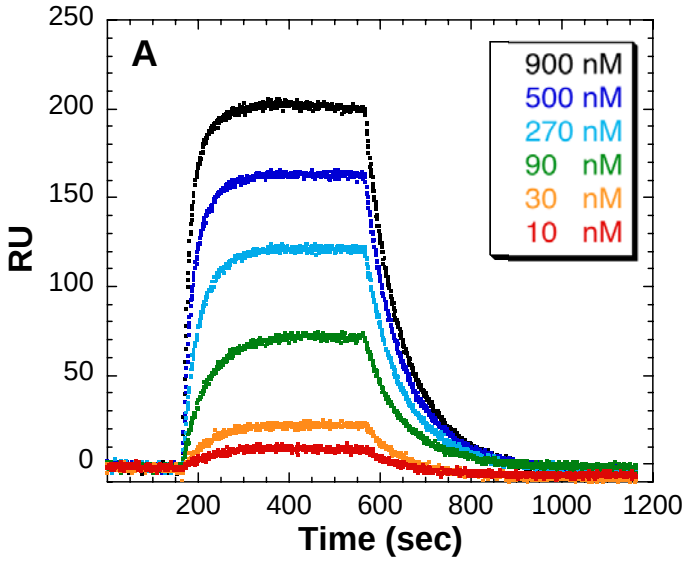
Yael Phillip, Eilon Sherman, Gilad Haran, and Gideon Schreiber

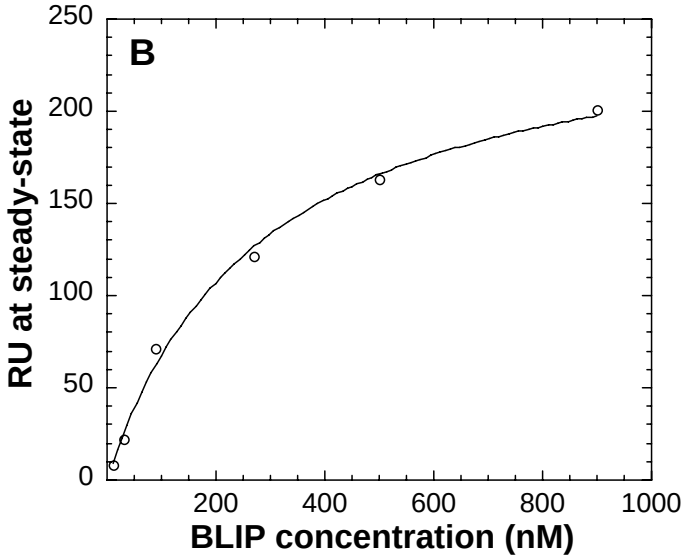
SUPPLEMENTARY FIGURES AND TABLES

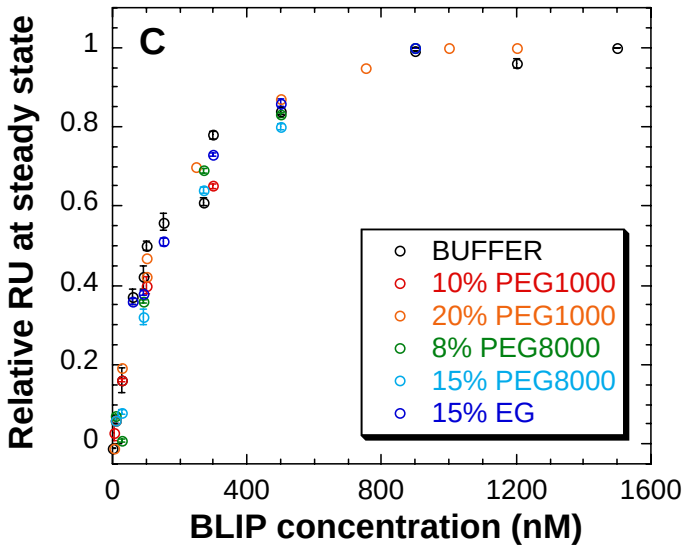


SUPPLEMENTARY FIGURE 1.

Enzyme inhibition assay **A,** Relative initial rates of substrate degradation upon addition of increasing concentrations of a protein inhibitor. The dots were fitted to Eq. 3, which describes a simple 1:1 binding process. TEM1-wt binding BLIP F142A and barnase K27A binding barstar Y29A **B,** Relative initial rates of Nitrocefin degradation by TEM1-wt upon addition of increasing concentrations of BLIP F142A. **C,** Relative initial rates of RNAse substrate degradation by barnase K27A upon addition of increasing concentrations of barstar Y29A.

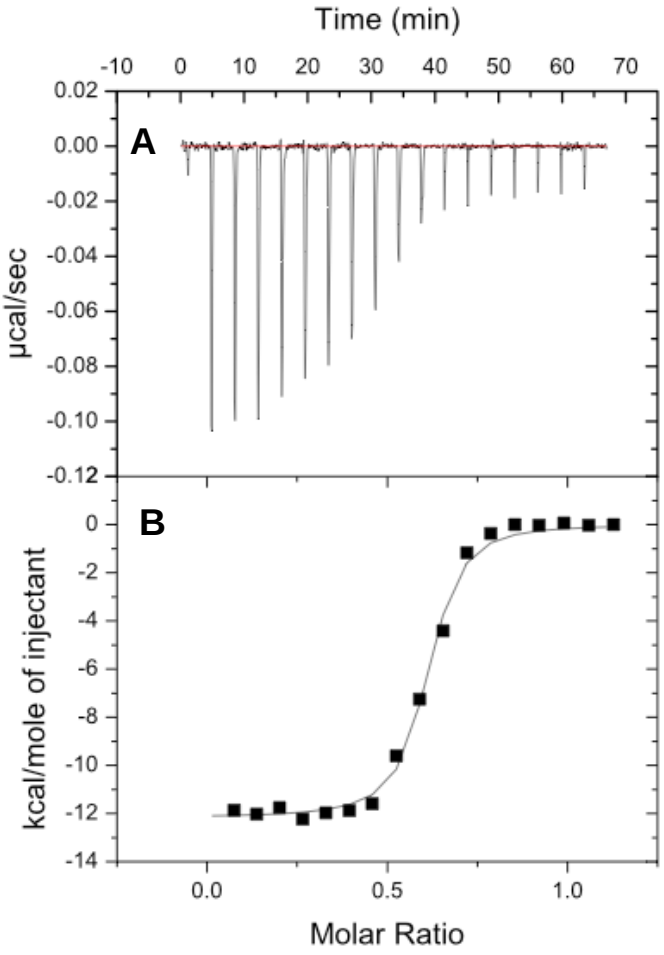






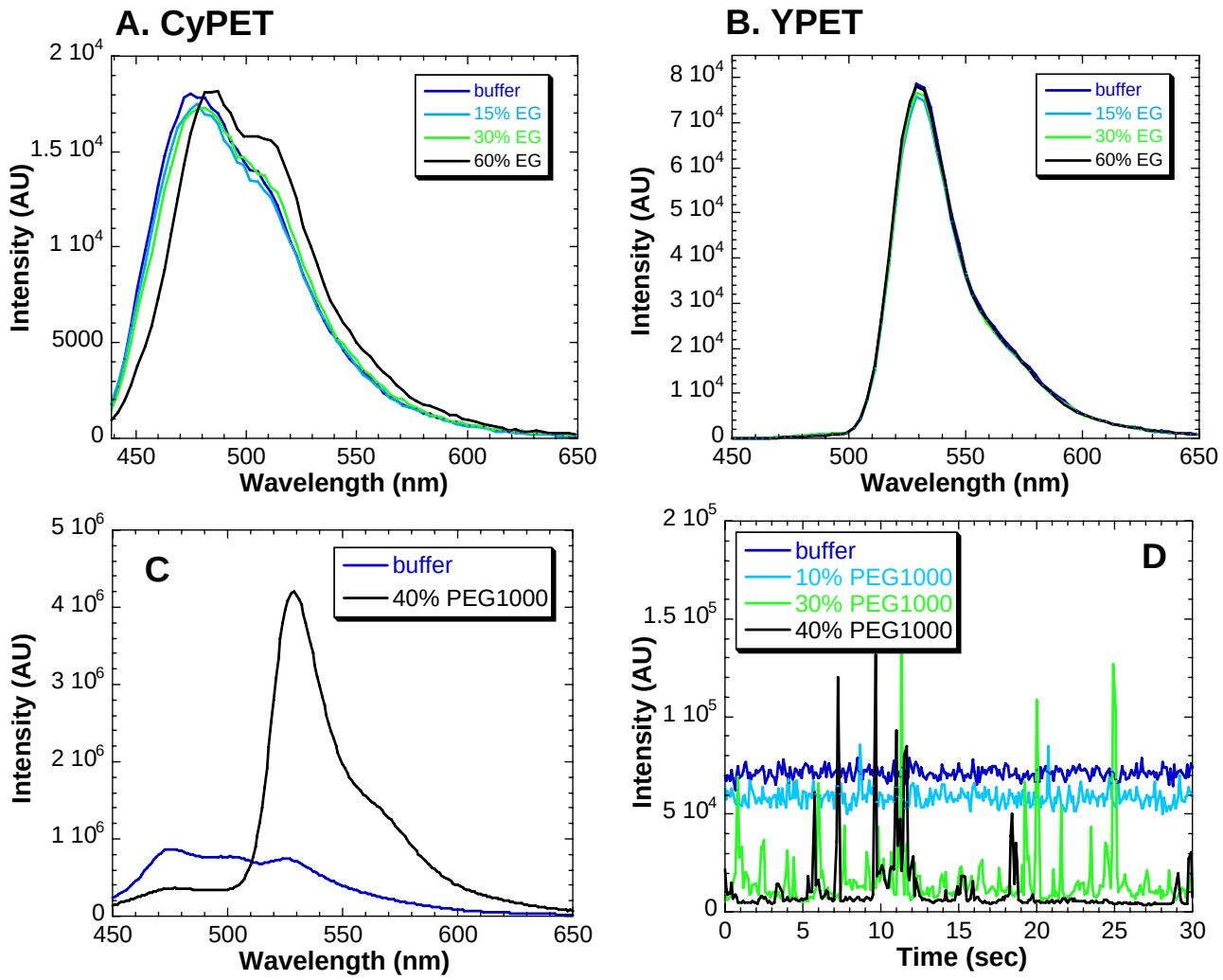
SUPPLEMENTARY FIGURE 2.

Binding measurements in real time using SPR. **A,** Real time SPR of TEM1-wt binding to six different concentrations of BLIP F142A. RU stands for refractive units (1 RU corresponds to 1 pg protein/mm^2**). B,** Measured values at equilibrium were plotted as a function of BLIP concentration. The dots were fitted to Eq. 2, which describes a simple 1:1 binding process. **C,** Steady-state measurements of TEM1-wt binding to BLIP F142A in different viscogenes. Error bars represent standard errors of multiple experiments.



SUPPLEMENTARY FIGURE 3. ITC measurement of the interaction between barnase K27A and barstar Y29A in 50% EG. **A,** Incremental heat upon titration of barstar (9µM) by barnase (50µM). **B,** Data points were obtained by integrating the above peaks and normalized to the amount of barnase injected. These were fitted using a 1:1 binding model with Origin 7.0.

SUPPLEMENTARY FIGURE 4. FRET and FCS studies of CyPET-YPET interaction. **A,** CyPET emission spectrum (excitation at 420nm) at different EG concentrations, showing independence on solution conditions. Similar results were obtained in PEG1000 and PEG8000. **B,** YPET emission spectrum (excitation at 480nm) at different EG concentrations, showing independence on solution conditions. Similar results were obtained in PEG1000 and PEG8000. **C,** A typical emission spectrum of CyPET and YPET (1μM each) with (black) and without (blue) 40% PEG1000. **D**, YPET intensity over time as recorded by the FCS setup. YPET (25 nM) was mixed with CyPET (500 nM) and with increasing concentrations of PEG1000. Large intensity fluctuations in the crowded solutions indicate the appearance of aggregates.



Supplementary table 1. Dissociation rates in buffer and in 25% mass EG of different TEM1-BLIP wt and mutant complexes.

Complex		k_d *(buffer)** ($10^{-5}s^{-1}$)	k_d *(EG)** ($10^{-5}s^{-1}$)	Relative k_d† $\frac{k_d(buffer)}{k_d(EG)}$
TEM1	BLIP			
wt	N89K, D163K, V165K	7.8	19.8	0.39
R243A	N89K, D163K, V165K	11.1	34.5	0.32
wt	wt	13.8	30.6	0.45
E104A	N89K, D163K, V165K	54.4	276	0.20
E104A	wt	516	1130	0.46
wt	F142A	1160	2840	0.41
R243A	F142A	1440	3470	0.41
E104A	F142A	1650	4210	0.39

*Measurements were done using the ProteOn XPR36 instrument.

†The standard error of the mean for the relative values is ± 16%.

SUPPLEMENTARY METHODS

Viscogens. Viscogen concentrations are expressed as weight percentage of the viscogenic agent from the total weight of the solution. More accurate and reproducible results are obtained when preparing viscous solutions by weight, and not by volume. The polydispersity of the PEG preparations is expected to be small. For example, analytical grade PEG1000 from Sigma has a polydispersity index of 1.044 (1).

Association rate measurements. Equal concentration (250 nM) of wt TEM1 with BLIP +4 (D163K, N89K, V165K) and wt barnase with wt barstar were mixed in the presence of the indicated viscogenic agents using a stopped-flow fluorescence spectrometer (Applied PhotoPhysics, Leatherhead, UK). For barnase-barstar, the buffer contained 50 mM NaCl. k_a values in buffer relative to k_a values in a solution containing the viscogenic agent are reported in the paper. The standard error of the mean for the relative k_a values is ± 18%. We have previously

compared the rates of association determined from pseudo-first order and second-order conditions, and found them to be the same (within experimental error) (2). In this study, we used only second-order conditions as they provide a much better signal to noise ratio.

Binding measurements using SPR. Measurements were done using *ProteOn XPR36 Protein Interaction Array System* (BioRad, Hercules, CA, USA), in PBS buffer with 0.005% surfactant P20 and the indicated viscogenic agent. The viscogenic agent was present in the solution throughout the experiment. Each measurement consisted of 6 analyte concentrations (in the range of 3-1800 nM) injected simultaneously. Flow rates in the range of 30-100 µl/min were applied with no significant difference between them; hence experiments were done at 30 µl/min flow. Data were analyzed using the *BIAevaluation* software version 4.0 after subtraction of a blank channel. To extract the dissociation rate constants (k_d), the recorded signals were fitted to a single exponential equation:

$$R = R_0 e^{-k_d (t - t_0)} + offset \qquad (1)$$

k_d values in buffer relative to k_d values in a solution containing the viscogenic agent are reported in the paper. The standard error of the mean for the relative k_d values is ± 10%.

To extract the association constant (K_A), the refractive index at steady state was plotted against the analyte concentration (C) and fitted to the mass action equation:

$$R = \frac{C \cdot R_{max}}{C + 1/K_A} \qquad (2)$$

Association constants are reported in the paper as the ratio between K_A in buffer and K_A in a solution containing the viscogenic agent. The standard error of the mean for the relative values is ± 11%.

Enzyme inhibition assay. wt TEM1 solutions at a constant final concentration of 1 or 1.5 nM were mixed with increasing concentrations of BLIP F142A inhibitor and viscogenic agent as indicated. The mixtures, placed in disposable multiwell plates, were left on a rocking table at room temperature for 1 hr. An aliquot of Nitrocefin (Calbiochem) substrate solution at a final concentration of 30 µM was added to each well, and the initial rate of substrate hydrolysis was followed by absorption at 486 nm on a microplate autoreader. For barnase-barster interaction, 5

nM of barnase K27A and increasing concentrations of barstar Y29A inhibitor were used. A final concentration of 6.4 nM RNase substrate solution (5´ 6-FAM™ -ArGAA-3´ TAMRA™ from IDT) was added at time zero. Because of the fast rates of hydrolysis of the substrate, these measurements were done in a stopped-flow apparatus. The initial rate of substrate hydrolysis was followed by excitation at 489 nm and fluorescence was monitored using a cutoff filter of 515 nm. The initial rates of hydrolysis were plotted as a function of the inhibitor concentration. The curves were fitted to Eq. 3 which expresses the initial rate of hydrolysis by the free enzyme, [R], as a function of the association constant (K_A) and the total molar concentrations of the enzyme [E_T] and its inhibitor [I_T] (3):

$$[R] = \frac{A}{[E_T]}\left(\frac{[E_T]-[I_T]-1/K_A}{2} + \left(\frac{\left([E_T]+[I_T]+1/K_A\right)^2}{4} - [E_T][I_T] \right)^{1/2} \right) \qquad (3)$$

A is the initial rate measured for the non-inhibited sample.

Association constants are reported in the paper as the ratio between K_A in buffer and K_A in a solution containing the viscogenic agent. The standard error of the mean for the relative values is ± 34% for TEM1-BLIP interaction and ± 16% for barnase-barstar interaction.

Isothermal titration calorimetry. Measurements were done using an iTC_{200} instrument (Microcal, Northampton, MA, USA). Proteins were centrifuged at 13,000 rpm in a microcentrifuge for ten minutes and then diluted with buffer containing viscogenic agent to the required final concentration. Seventeen 2.3 µl injections (4.6 seconds per injection) were performed at four-minute intervals, with continues mixing at 1000 rpm by the injection syringe. Heats of dilution and mixing were determined in control experiments in which barnase was titrated against buffer or viscogenic agent, buffer or viscogenic agent were titrated against barstar and separately prepared viscogenic solutions were titrated against each other. The heats of dilution and mixing were of essentially the same magnitude as the heats obtained at the end of the titration (at saturation). The binding data were analyzed using OriginTM software (Microcal, Northampton, MA, USA) and were fitted with no parameters fixed to obtain K_A. Association constants are reported in the paper as the ratio between K_A in buffer and K_A in a solution containing the viscogenic agent. The standard error of the mean for the relative values is ± 30%.

Fluorescence resonance energy transfer. Normalized emission spectra, S_{CyPET} and S_{YPET}, were measured separately for CyPET and for YPET by excitation at 420 and 480 nm, respectively. In the FRET experiments, the two proteins were mixed together with the viscogenic agent as indicated, and incubated at room temperature over night. Using the normalized spectra of the separate proteins, we determined the best fit of the function $F_{CyPET} \bullet S_{CyPET} + F_{YPET} \bullet S_{YPET}$ (where F_{CyPET} and F_{YPET} are weights) to the measured FRET spectrum. The FRET efficiency, E, was then calculated according to:

$$E = \frac{F_{YPET}}{3 \cdot F_{CyPET} + F_{YPET}} \qquad (4)$$

where the numerical factor 3 corrects for the difference in the quantum yield of YPET relative to that of CyPET (4). This calculation assumes that one donor and one acceptor contribute to each FRET event, an assumption that is probably only approximate in an aggregate.

Fluorescence correlation spectroscopy. Measurements were performed straight after mixing with the viscogenic agent, without any substantial incubation time. While the correlation curve in buffer could be readily fitted to a standard single-species FCS function (5), this could not be done in solutions of crowding agents, where apparently multiple species existed. To circumvent complex fitting, we used the time at the mid-point of the correlation curve as a rough estimate for the diffusion correlation time (this estimate is exact for a single diffusing species).

Dynamic light scattering. DLS experiments were performed on a Viscotek 802 DLS instrument. Proteins and buffers were centrifuged at 12,000 rpm in a microcentrifuge for 30 minutes and then diluted to the required final concentration. CyPET and YPET were mixed together to a final concentration of 0.4 g/l each (~15 μM). TEM1-wt and BLIP F142A were mixed together to a final concentration of 0.5 g/l and 0.3 g/l respectively (~17 μM each). Data was analyzed using OmniSIZE software version 3.0.

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