Quantification of the effects of ionic strength, viscosity and hydrophobicity on protein-ligand binding affinity

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Protein expression and purification

Trypsin was obtained from BDH (Dorset, UK).

The extracellular domain of TNF-a (Val 77 - Leu 233) was expressed in BL21(DE3) pLysS strain of *E. coli* as a GST-fusion protein as previously described (1). GST-TNF was purified as previously described (1) while separation of TNF from its GST fusion partner was accomplished by proteolytic cleavage with the type-14 human rhinovirus 3C protease (America Pharmacia Biotech).

Cyclin A2 (Glu 174- Leu 432) was expressed in *E. coli* BL21(DE3) as a 6xHis fusion protein, as described elsewhere (unpublished data). Briefly, human cDNA of Cyclin A2 was purchased by Geneservice (Source Bioscience UK Limited) and cloned into pet16b+. *E.coli* BL21(DE3) was used as expression host. For overexpression and purification of recombinant cyclin A_2, *E. coli* BL21transformed

with pet16b /cyclinA2 were induced with 0.5 mM IPTG at 20^{o}C for 12 h. Cells were harvested by centrifugation at 3500×g for 20 min and washed with phosphate buffer saline (PBS). Purification of 6xHis-cyclin A_2 was performed using an IMAC-Ni^{2+} column (Biorad), according to manufacturer's instructions. Purified 6xHis cyclin A2 was dialyzed against 50 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$ (the concentration of $MgCl_2$ was gradually increased from 10 to 100 mM during dialysis). After dialysis, NaN_3 and monotioglycerol were added to final concentrations of 0.01% each.

Determination of dissociation constant (K_d) from fluorescence measurements

The dissociation constant is an indicator of binding strength between two molecules. For the reaction:

$$P + L \leftrightarrow PL$$

K_d is expressed by the equation:

$$K_d = \frac{[P][L]}{[PL]} \qquad \text{(S.1)}$$

where [P] is the concentration of free Protein, [L] is the concentration of free ligand and (2) is the Ligand-bound-Protein.

Differences in fluorescence intensity between the protein/ligand complex and free protein (or free ligand) were analysed as previously described in (3) (Eq. 2) in order to determine the dissociation constant (K_d) of the various protein-ligand systems.

$$F_{obs} = F_{BG} + MF_{P_F}[P_F] + FR \cdot MF_{P_F} \cdot \frac{([L_T] + [P_T] + K_d]) \pm \sqrt{([L_T] + [P_T] + K_d])^2 - 4[P_L][L_T]}}{2} \qquad \text{(S.2)}$$

In Eq. (2) F_{obs} is the observed fluorescence intensity; F_{BG} is the fluorescence background signal; MF_{P_F} and P_F are the molar fluorescence and concentration of free protein, respectively; *FR* is the fluorescence ratio of bound protein (or bound ligand in

the case of a fluorescence ligand); L_T and $P_{T\ are}$ the total concentration of ligand and protein respectively. A detailed analysis of the development of the fluorescence ligand-binding assay is given in (3).

Fluorescence intensity was measured with a Hitachi F-2500 fluorescence spectrophotometer in 1.0 x 4.5 cm quartz cuvettes at 25^{o}C as previously described in (3) with some modification. Briefly, the following procedure was used for titration of proteins with ligands: 1.5 mL of protein solution (0.5-2μM, depending on the tested protein) was placed in a cuvette and equilibrated at the experiment temperature as indicated in the text. After equilibration, small increments (2 to 7.5 μL) of the ligand solution were injected in the cuvette. The experiments were performed in various buffers containing different additives as indicated in the text. The slits were set either at 5 and 20 nm (for TNF-α/SPD304 and cyclin A_2/RRLIF) or at 5 nm and 10 nm (for trypsin/PABA) in the excitation and emission respectively. In order to determine dilution effect of protein (due to ligand addition) and any fluorescence effect by unbound ligand, a blank sample containing Tyr,(TNF-α) or Trp (cyclin A_2) with the same fluorescence signal, was titrated with ligand additions as described above. The sample absorbance was kept below 0.1 to minimize the inner filter effect (4). Data were analysed using Prism V.5 (GraphPadSoftware, San Diego, CA).

Viscosity measurements

The solvent viscosity was measured using an Ostwald capillary viscometer, at various temperatures, and compared with values reported in literature.

Figures

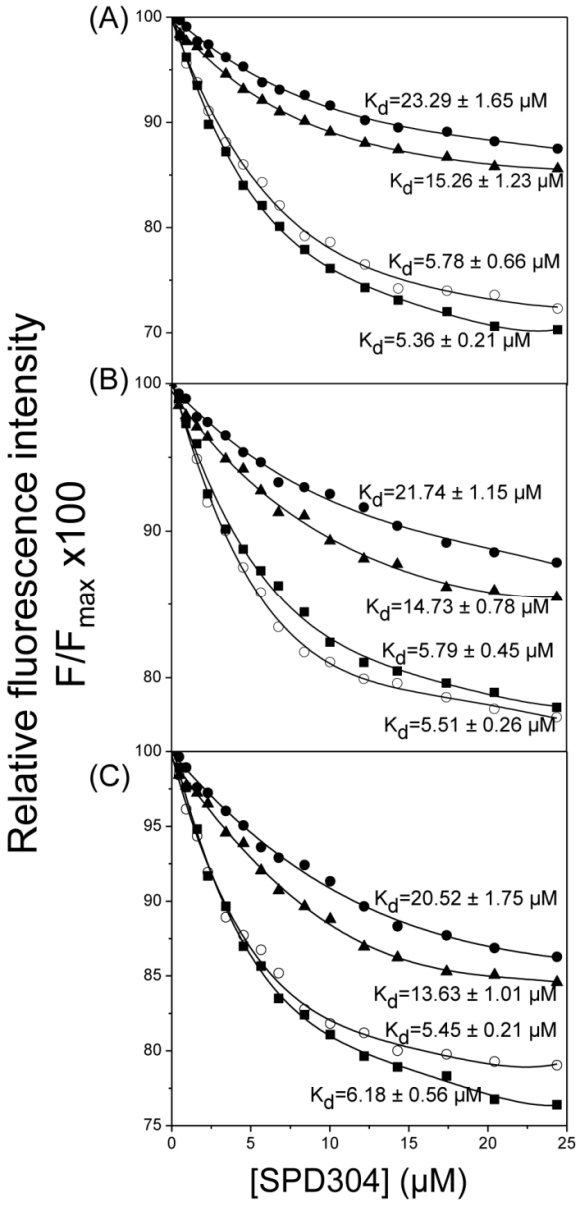

Figure S.1. Effect of temperature and glycerol concentration on TNF-α/SPD304 interaction. Changes of TNF-α fluorescence intensity (λ_{ex}=274nm / λ_{em}=302nm) were measured after titration of TNF-α with SPD304 at 25°C (A) or 30°C (B) or 37°C (C). Experiments were performed in 10 mM citrate-phosphate buffer containing % DMSO and a different amount of glycerol. The following concentrations of glycerol were tested: 0% (■); 10%(○); 25%(▲) and 50% (●). The mean values of three independent values are presented.

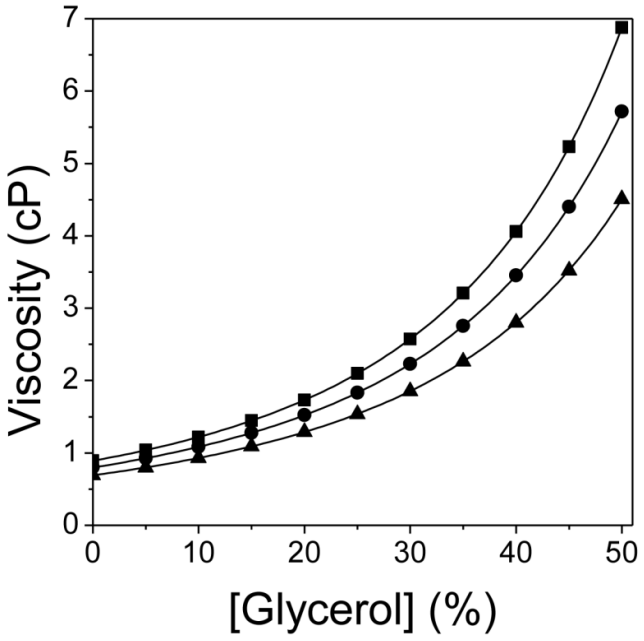

Figure S.2. Viscosity of glycerol solutions at 25 (■), 30 (●) and 37°C (▲).

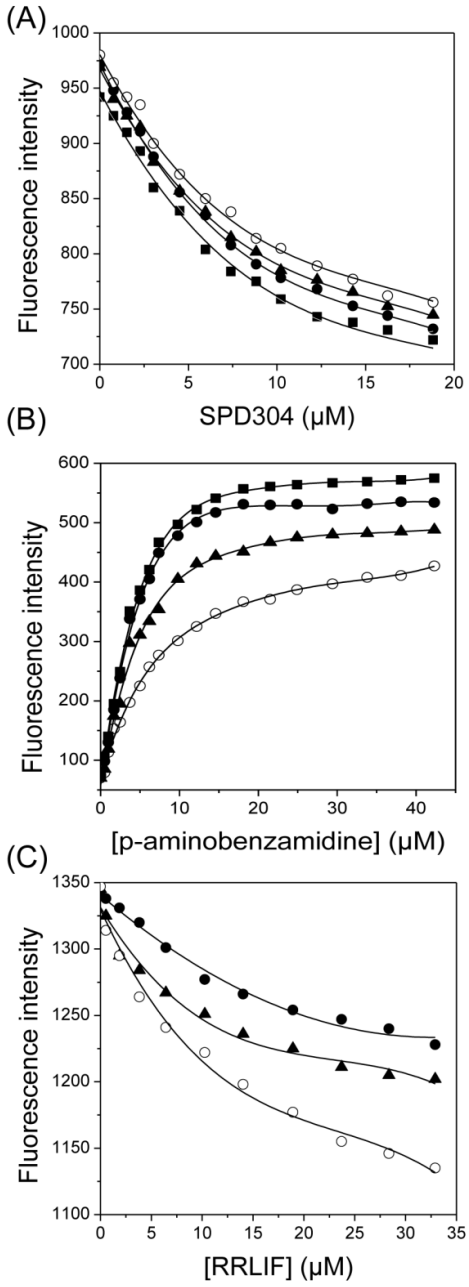

Figure S.3 Effect of NaCl concentration on binding affinity of protein/ligand systems. (A) Titration of TNF-α with SPD304. The ligand was titrated in TNF-α solution in 10 mM citrate-phosphate buffer (pH 6.5) containing 5% DMSO and various NaCl concentrations and the fluorescence intensity was measured at 304nm. (B) Titration of tryspin with PABA. The ligand was titrated in protein solution in 20 mM phosphate buffer (pH 7.4) containing 0.1% PEG6000 and the changes of fluorescence intensity at 370 nm were monitored. (C) Titration of cyclin A2 with RRLIF. The ligand was titrated in protein solution in 50 mM Tris-HCl pH 8.0, containing 0.01 % monothioglycerol and various NaCl concentrations and the fluorescence intensity was measured at 345nm. The following concentrations of NaCl were tested: 50 mM (■); 100 mM (●); 250 mM (▲); 500 mM (○). The mean values of three independent measurements are presented.

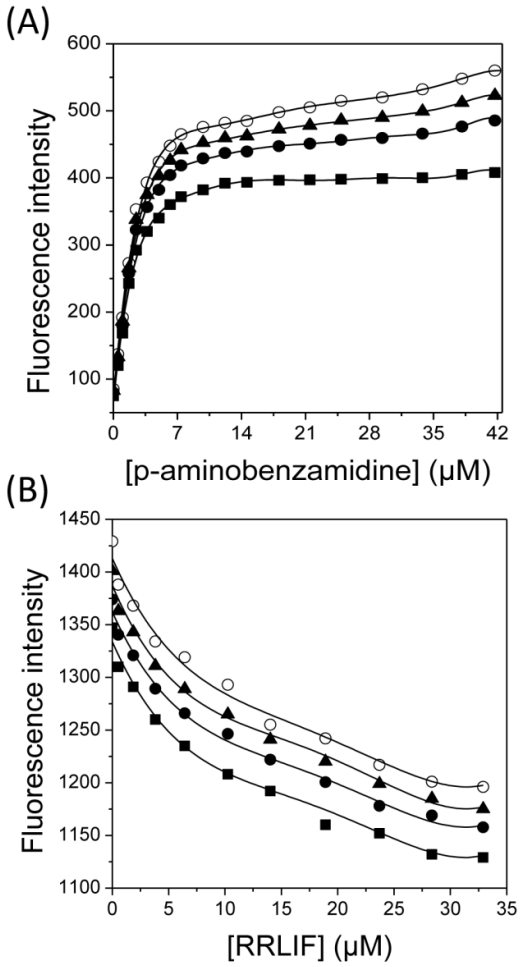

Figure S.4 Effect of DMSO concentration on binding affinity of protein/ligand systems. (A) Titration of tryspin with PABA. The ligand was titrated in protein solution in 20 mM phosphate buffer (pH 7.4) containing 100 mM NaCl plus 0.1% PEG6000 and the changes of fluorescence intensity at 370 nm were monitored. (B) Titration of cyclin A2 with RRLIF. The ligand was titrated in protein solution in 50 mM Tris-HCl pH 8.0, containing 0.01% NaN_3, 0.01 % monothioglycerol and various NaCl concentrations and the fluorescence intensity was measured at 345nm. The following concentrations of DMSO were tested: 0% (■); 2.5% (●) 5 %(▲); 10 % (○).The mean values of three independent measurements are presented.

References

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