ONLINE SUPPLEMENT FOR:

PROTEIN PEGYLATION DECREASES OBSERVED TARGET ASSOCIATION RATES VIA A DUAL BLOCKING MECHANISM

Susanne Kubetzko[1], Casim A. Sarkar[1] and Andreas Plückthun

Department of Biochemistry, University of Zürich, CH-8057 Zürich, Switzerland (S.K., C.A.S., A.P.) and Division of Oncology, Department of Internal Medicine, University Hospital, University of Zürich, CH-8044 Zürich, Switzerland (S.K.)

MATERIALS AND METHODS

Construction of the cysteine mutants. To enable site-specific PEGylation of the monomeric scFv 4D5 (Knappik and Plückthun, 1995; Waibel et al., 1999; Willuda et al., 2001; Wörn and Plückthun, 1998) and the dimeric antibody fragment 4D5-dhlx (Willuda et al., 2001) with maleimide-PEG20, we added a hinge cysteine at the C-terminus of the peptide chains. For the monomeric scFv 4D5 we introduced the cysteine by PCR mutagenesis, using the primer sk-cyst.rev (GCATAAGCTT TCATTAACAA CCACCGTGAT GGTGATGGTG GTGGTTCAGG TCTTCTTCAG), which encodes a Gly_2-linker, followed by a single unpaired cysteine, 2 stop codons and a HindIII restriction site. The cysteine mutant of the dimeric miniantibody 4D5-dhlx was generated by ligation of the EcoRI/HindIII fragment of the plasmid pAK300-B2 into the vector pIG6-4D5 (Willuda et al., 2001; Wörn and Plückthun, 1998). This insert was thereby placed downstream of the variable domains of the scFv 4D5 and contained the dimerization domain dhlx, followed by a His_6-tag, a Gly_4-spacer and a cysteine. Both constructs were cloned in the expression vector pIG6 (Ge et al., 1995), like their unmodified counterparts.

Expression and purification. All constructs, with and without the additional C-terminal cysteine residue, were expressed in the periplasm of the *E. coli* strain SB536, which is devoid of the periplasmic proteases HhoA and HhoB (Bass et al., 1996). Freshly transformed cells were grown overnight, and 25 ml of this starter culture were then inoculated in 1 l 2YT medium containing 50 μg/ml ampicillin. The culture was grown in a 5 l baffled shake flask at 25°C. Expression of the antibody fragments was under the control of a *lac* promoter (Ge et al., 1995) and induced with 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG, Eurogentec) at an OD_{600nm} of 0.5 – 0.7. After induction, the expression was allowed to continue for 4 to

6 h until the culture reached a final OD_{600nm} of 4 – 6. Cells were harvested by centrifugation at 4000*g* for 15 min at 4°C and stored as a pellet at -80°C.

Purification of the antibody fragments was carried out by two subsequent affinity chromatography steps on a GradiFrac system (Pharmacia) at 4°C. The harvested *E. coli* cells were resuspended in 20 mM Tris (pH 7.4) buffer, containing 150 mM NaCl, 4 mM $MgCl_2$ and DNase (1 mg/100 ml), and then passed through a French Press (SLS Instrument Inc., Urbana Illinois, USA) at 10,000 p.s.i. for cell lysis. The lysate was immediately centrifuged in a SS-34 rotor at 48,000*g* for 30 min at 4°C to pellet cell debris. The filtered (0.22 μm) lysate was then loaded onto a Ni-NTA superflow column (Qiagen) and washed with running buffer (20 mM Tris, pH 7.4, 150 mM NaCl) until the absorption (280 nm) reached the baseline. After two additional washing steps, first with 1 M NaCl and then with 40 mM imidazole, the bound protein was eluted with 200 mM imidazole (pH 7.4). The eluted sample was applied to a Protein A Sepharose column (Pharmacia), equilibrated with the same running buffer as used for the Ni-NTA column. After the absorption (280 nm) of the column flow-through had reached the baseline again, the bound miniantibody was eluted with 100 mM citric acid, pH 3.5. The collected sample was immediately neutralized by titration with a 1 M Tris solution (200 μl/1 ml eluate), which was performed on each fraction upon elution. The quality of the purified protein was analyzed by UV-spectrometry and SDS-PAGE (12% gel, Coomassie staining).

MODEL DERIVATIONS

Diffusion-limited binding model. To calculate the translational diffusion rate constant on a spherical cell, we solve the following steady-state diffusion equation for the ligand concentration as a function of radial distance, $L(r)$, with a radiation boundary condition at the cell surface and the ligand concentration far from the cell equal to the bulk concentration L_0 (Collins and Kimball, 1949; Lauffenburger and Linderman, 1993; Shoup and Szabo, 1982):

$$\frac{D_t}{r^2}\frac{d}{dr}\left(r^2\frac{dL}{dr}\right)=0;\quad D_t\frac{dL_s}{dr}=k_aL_sR;\quad L(r\to\infty)=L_0 \qquad \text{Eq. A1}$$

where D_t is the translational diffusion coefficient, $L_s = L(r_c)$ is the concentration of ligand at the surface of a cell with radius r_c, R is the molar density of free receptors on the cell surface, k_a is the intrinsic association rate constant, and k_{on} is the apparent association rate constant. Integrating with respect to r and applying the radiation boundary condition at the cell surface ($r = r_c$) to solve for the constant of integration, A_1:

$$\frac{dL}{dr}=\frac{A_1}{r^2};\quad D_t\frac{A_1}{r_c^2}=k_aL_sR\Rightarrow A_1=\frac{k_aL_sRr_c^2}{D_t} \qquad \text{Eq. A2}$$

Integrating one more time and applying the second boundary condition to solve for the second constant of integration, A_2:

$$L=A_2-\frac{k_aL_sRr_c^2}{D_t}\left(\frac{1}{r}\right);\quad L(r\to\infty)=L_0\Rightarrow L=L_0-\frac{k_aL_sRr_c^2}{D_t}\left(\frac{1}{r}\right) \qquad \text{Eq. A3}$$

To eliminate the L_s term, which is unknown and cannot be measured, we evaluate the entire expression at r_c and solve for L_s (= $L(r_c)$) in terms of known or measurable quantities:

$$L_s=L_0-\frac{k_aL_sRr_c}{D_t}\Rightarrow L_s=\frac{L_0}{1+\dfrac{k_aR}{D_t/r_c}}=\frac{L_0}{1+Da} \qquad \text{Eq. A4}$$

where *Da* is the Damköhler number. Note that this Damköhler number is identical to that given in Eq. 9 in the main text. The full ligand profile with respect to radial distance can be obtained by inserting Eq. A4 into Eq. A3:

$$L(r) = L_0\left(1 - \frac{k_a R}{D_t / r_c + k_a R}\left(\frac{r_c}{r}\right)\right) \qquad \text{Eq. A5}$$

The diffusion-limited transport velocity k_t can be calculated from a flux balance at the cell surface (at steady state, the diffusive flux from the bulk to the surface must equal the diffusive flux at the cell surface):

$$k_t\left(L_0 - L_s\right) = D_t \frac{dL_s}{dr} \qquad \text{Eq. A6}$$

Evaluating dL/dr at $r = r_c$ from Eq. A5 and substituting this expression into Eq. A6:

$$k_t = \left(\frac{1}{L_0 - L_s}\right)\left(D_t\right)\left(\frac{k_a R L_0}{D_t / r_c + k_a R}\left(\frac{1}{r_c}\right)\right) \qquad \text{Eq. A7}$$

Substituting L_s with $L_0/(1 + Da)$ from Eq. A4, and simplifying terms:

$$k_t = \left(\frac{1 + Da}{L_0\left(Da\right)}\right)\left(D_t\right)\left(\frac{L_0\left(Da\right)}{1 + Da}\right)\left(\frac{1}{r_c}\right) = \frac{D_t}{r_c} \qquad \text{Eq. A8}$$

This is identical to the expression of k_t given in the main text (prior to Eq. 9).

Kinetic intramolecular- and intermolecular-blocking model. The mass-action kinetic model for complex formation given in Eq. 12 can be multiplied on both sides by an exponential term and then rearranged:

$$\frac{dC}{dt} + \left(\left(\frac{1+\varepsilon}{1+\alpha}k_a\right)L_0 + k_d\right)C = \frac{k_a L_0 R_0}{1+\alpha} \Rightarrow \frac{d}{dt}\left(Ce^{\left(\left(\frac{1+\varepsilon}{1+\alpha}k_a\right)L_0 + k_d\right)t}\right) = \frac{k_a L_0 R_0}{1+\alpha} e^{\left(\left(\frac{1+\varepsilon}{1+\alpha}k_a\right)L_0 + k_d\right)t} \qquad \text{Eq. A9}$$

Integrating once and applying the initial boundary condition ($C(0) = 0$):

$$Ce^{\left(\left(\frac{1+\varepsilon}{1+\alpha}k_a\right)L_0 + k_d\right)t} = \frac{k_a L_0 R_0}{1+\alpha} \cdot \frac{1}{\left(\frac{1+\varepsilon}{1+\alpha}\right)k_a L_0 + k_d} \cdot \left(e^{\left(\left(\frac{1+\varepsilon}{1+\alpha}k_a\right)L_0 + k_d\right)t} - 1\right) \qquad \text{Eq. A10}$$

This expression can be rearranged to give Eq. 13.

Equilibrium intermolecular- and intramolecular-blocking model. Using Eqs. 10 and 16 to express *L* and *R* in terms of *C*, we obtain the following relation at equilibrium:

$$\frac{dC}{dt} = k_a LR - k_d C = k_a \left(\frac{L_0 - C}{1+\alpha} \right) \left(R_0 - (1+\varepsilon) C \right) - k_d C = 0 \qquad \text{Eq. A11}$$

This expression can be rearranged to give Eq. 17. The physically meaningful solution to this quadratic equation is:

$$C = \frac{1}{2} \left[\left(L_0 + \frac{R_0}{1+\varepsilon} + \frac{1+\alpha}{1+\varepsilon} K_D \right) - \sqrt{\left(\left(L_0 + \frac{R_0}{1+\varepsilon} + \frac{1+\alpha}{1+\varepsilon} K_D \right) - 4 L_0 \frac{R_0}{1+\varepsilon} \right)} \right] \qquad \text{Eq. A12}$$

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