Supplementary data for

Reagentless fluorescent biosensors from artificial families of antigen binding proteins

Frederico F. Miranda[a,b,1], Elodie Brient-Litzler[a,b,1], Nora Zidane[a,b], Frédéric Pecorari[c,d] and Hugues Bedouelle[a,b,*]

[a] Institut Pasteur, Department of Infection and Epidemiology, Unit of Molecular Prevention and Therapy of Human Diseases, 25 rue Docteur Roux, 75724 Paris Cedex 15, France.

[b] CNRS URA3012, 25 rue Docteur Roux, 75724 Paris Cedex 15, France.

[c] CNRS UMR6204, Biotechnology, Biocatalysis and Bioregulation, 2 rue de la Houssinière, BP 92208, 44322 Nantes Cedex 3, France.

[d] Université de Nantes, Faculté des Sciences et des Techniques, 2 rue de la Houssinière, BP 92208, 44322 Nantes Cedex 3, France.

* Corresponding author at: Institut Pasteur, CNRS URA3012, 25 rue Docteur Roux, 75724 Paris Cedex 15, France. Tel: +33 1 45688379; fax: +33 1 40613533.

Email address: hugues.bedouelle@pasteur.fr

[1] These authors contributed equally to this work.

S1. Supplementary materials and methods

Buffers

Buffer A was 500 mM NaCl, 50 mM Tris-HCl, pH 8.0; buffer B, as buffer A but pH 7.5; buffer C, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4; buffer D, 0.005% (v/v) Tween 20, 0.1 mg/mL BSA in buffer C; buffer E, 5 mM dithiothreitol (DTT) in buffer D; buffer F, 0.005 % (v/v) Tween 20 and 5 mM DTT in buffer C. Phosphate buffered saline (PBS) was purchased from Sigma-Aldrich.

Mutagenesis of the MBP3_16 gene

DARPins are formed of repeated polypeptidic modules and encoded by repeated segments of DNA. These repetitions constitute a problem for the construction of mutations by site-directed mutagenesis. We used the degeneracy of the genetic code to design a mutant allele of the *mbp3_16* gene, that was devoid of important repetitions. The mutant allele, *mbp3_16-1,* was synthesized by Genecust (Evry, France) and inserted in the same plasmid vector pQE30 (Qiagen) as the parental gene, to give the recombinant plasmid pQEMBP3_16-1. Changes of residues were introduced in the MBP3_16 protein at the genetic level, by mutagenesis of either pQEMBP3_16 for A78C and D81C, or pQEMBP3_16-1 for the other mutations.

Protein production and purification

The parental protein H4S(wt) and its mutant derivatives were produced in the cytoplasm of the recombinant strain NEB-Express-I^q(pH4S) and its mutant derivatives as follows. The producing strains were grown at 30 °C. They were streaked on plates of LB agar, supplemented with ampicillin at 100 μg/mL and chloramphenicol at 10 μg/mL. A pre-culture in 2-YT broth, supplemented with the same two antibiotics, was inoculated with an isolated colony and grown overnight. A larger culture (650 mL), supplemented with ampicillin alone, was inoculated with an aliquot of the preculture to obtain a starting absorbance A_{600nm} = 0.1, grown until A_{600nm} = 0.8, induced with 1 mM IPTG, and then grown further for 24 hours. The following purification steps were performed at 4 °C. The culture was chilled on ice,

centrifuged at 8000*g* for 20 min and the resulting pellet was frozen and kept at -20 °C. The pellet was resuspended in 30 ml of 5 mM imidazole in buffer A and the cells were disintegrated by sonication. The lysate was centrifuged at 8600*g* for 30 min and the supernatant filtered through a 0.22 μm Millex filter (Millipore). H4S(wt) and its derivatives were purified from the corresponding lysate through their hexahistidine tag by affinity chromatography on a column of Ni-NTA resin. The protein was eluted from the resin with 200 mM imidazole in buffer A.

Fluorophore coupling

The conjugates between the cysteine mutants of the antigen binding protein (AgBP) and the thiol reactive fluorophore IANBD ester were prepared essentially as described (Brient-Litzler et al., 2010). Briefly, the AgBP mutants were reduced with 5 mM DTT for 30 min at 30 °C with gentle shaking and then the buffer was exchanged to PBS by size exclusion chromatography with a PD10 column (GE Healthcare). From this point on, all the experiments were done in the dark. The IANBD ester was added in a 10:1 molar excess over the AgBP mutant and the coupling reaction was carried out for 2.5 hours at 30 °C with gentle shaking. The denatured proteins were removed by centrifugation for 30 min at 13200*g*, 4 °C. The conjugate was separated from the unreacted fluorophore by chromatography on a Ni-NTA column (0.5 mL of resin) and eluted with imidazole in buffer A for the H4S derivatives or buffer B for the MBP3_16 derivatives. The conjugate between 2-mercaptoethanol and the IANBD ester was prepared by mixing the two molecules in stoechiometric amounts and then incubating the mixture for 30 min at 25 °C. The coupling yield y_c, i.e. the average number of fluorophore molecule coupled to each AgBP molecule, was calculated as described below, with ε_{280}(ANBD) = 2100 M^{-1} cm^{-1}, ε_{500}(ANBD) = 31800 M^{-1} cm^{-1}, both measured with conjugates between IANBD and 2-mercaptoethanol (Renard et al., 2002).

Let P be a protein; B, a monoconjugate between P and IANBD; Φ, the conjugated form of IANBD; A_{280} and A_{500}, the absorbances of the mixture of P and B that results from the coupling reaction and elimination of the unconjugated fluorophore. By definition,

$$y_c = [B]/([B] + [P]) \quad \text{(S1)}$$

where [B] and [P] are concentrations. Then, the reciprocal of the coupling yield is given by the following equation where ε is a molar absorbance (Brient-Litzler et al., 2010):

$$y_c^{-1} = (A_{280}/\varepsilon_{280}(P))(A_{500}/\varepsilon_{500}(\Phi))^{-1} - \varepsilon_{280}(\Phi)/\varepsilon_{280}(P) \quad \text{(S2)}$$

Interaction between AgBP and antigen

A conjugate (or biosensor) B and antigen A form a 1:1 complex B:A according to the reaction:

$$B + A \leftrightarrow B{:}A \quad \text{(S3)}$$

At equilibrium, the concentration [B:A] of the complex is given by the equation:

$$[B{:}A] = 0.5\{[B]_0 + [A]_0 + K_d - (([B]_0 + [A]_0 + K_d)^2 - 4\,[B]_0[A]_0)^{1/2}\} \quad \text{(S4)}$$

where K_d is the dissociation constant, and $[A]_0$ and $[B]_0$ are the total concentrations of A and B, respectively (Renard et al., 2003).

Quenching by potassium iodide

The experiments of fluorescence quenching by KI were performed at 25 °C in Buffer C, essentially as described for the titration experiments. The Stern-Volmer Eq. (S5) was fitted to the experimental data, where F and F^0 are the intensities of fluorescence for the AgBP conjugate in the presence or absence of quencher, respectively. The Stern-Volmer constant K_{SV} was used as a fitting parameter.

$$F^0/F = 1 + K_{SV}\,[KI] \quad \text{(S5)}$$

Affinity in solution as determined by competition Biacore

The binding reactions (250 µl) between the H4S Nanofitin and its HEL antigen were conducted by incubating 20 nM of H4S with variable concentrations of HEL for 30 min in buffer D. The concentration of free H4S was then measured by surface plasmon resonance with a Biacore 2000 instrument (Biacore Life Sciences). Lysozyme was immobilized (700 Resonance Units, RU) on the surface of a CM5 sensor chip (Biacore Life Sciences) and the reaction mixtures were injected in the sensor chip at a flow rate of 30 µL min^{-1}. The chip surface was regenerated between the runs by injecting 10 µL of a 0.05% SDS solution.

The binding reactions (100 µL) between the MBP3_16 DARPin and its MalE antigen were conducted by incubating a fixed concentration of MBP3_16 molecules with variable concentrations of MalE for > 1 h in buffer F. The wild type MBP3_16(wt) and its mutant derivatives were used at a concentration of 50 nM, except those carrying mutations T79C, D81C and W90C, which were used at 500 nM to obtain a sufficient signal. A high density (> 2000 RU) of the biotinylated form of MalE (bt-MalE) was immobilized on the surface of a streptavidin SA sensorchip (Biacore Life Sciences). Each reaction mixture was injected in the sensor chip at a flow rate of 25 µL min^{-1}. The chip surface was regenerated by injecting 10 µL of a glycine-HCl solution at pH 3.0 (Biacore Life Sciences) between each run.

The following equation results from the laws of mass action and conservation:

$$[P] = 0.5\{[P]_0 - [A]_0 - K_d + (([P]_0 - [A]_0 - K_d)^2 + 4\,K_d[P]_0)^{1/2}\} \quad \text{(S6)}$$

where $[A]_0$ is the total concentration of antigen in the reaction mixture; $[P]_0$, the total concentration of AgBP; and [P], the concentration of free AgBP (Lisova et al., 2007). The association between the reaction mixture at equilibrium and immobilized antigen was monitored as described (Brient-Litzler et al., 2010). In these conditions, the initial slope r of the association curve follows the equation:

$$r = r_0[P]/[P]_0 \quad \text{(S7)}$$

where r_0 is the value of r for $[A]_0 = 0$. The values of K_d and r_0 were determined by fitting Eq. (S7), in which [P] is given by Eq. (S6), to the experimental values of r.

Kinetic measurements by Biacore

The kinetics were measured in buffer E at a flow rate of 30 µL min^{-1} with CM5 sensor chips. A first cell of the sensor chip was used as a reference, i.e. no ligand was immobilized on the corresponding surface. A second cell was loaded with 500-1000 RU of HEL. Solutions (200 µL) of the H4S derivatives at 15 different concentrations (1 nM to 6 µM) were injected to monitor association and then buffer alone (150 µL) for dissociation. The chip surface was regenerated between the runs by injecting 10 µL of a 0.05% SDS solution. The signal of the buffer alone was subtracted from the raw signals to obtain the protein signals, and then the protein signal on cell 1 was subtracted from the protein signal on cell 2 to obtain the specific

signal of interaction. The kinetic data were cleaned up with the Scrubber program (Biologic Software) and then the kinetic parameters were calculated by applying a simple model of Langmuir binding and a procedure of global fitting, as implemented in the Bia-evaluation 4.1 software (Biacore Life Sciences).

Data analysis

The fittings of equations to experimental data were performed with the Kaleidagraph software (Synergy Software). The standard error (SE) on the free energy of dissociation $\Delta G = -RT\ln K_d$ was deduced from the SE value on K_d by the equation:

$$SE(\Delta G) = RT SE(K_d)/K_d \quad \text{(S8)}$$

The SE value on the variation of interaction energy resulting from a mutation $\Delta\Delta G = \Delta G(\text{wt}) - \Delta G(\text{mut})$ was deduced from the SE values on ΔG by the equation:

$$[SE(\Delta\Delta G)]^2 = [SE(\Delta G(\text{wt}))]^2 + [SE(\Delta G(\text{mut}))]^2 \quad \text{(S9)}$$

S2. Supplementary Results

Binding parameters of H4S derivatives

The K_d values of the H4S conjugates varied with the position of the fluorophore (Table 1). To determine whether these variations in affinity were due to the mutation of the parental side chain into Cys or to the coupling of the fluorescent group, we measured the kinetic parameters of interaction between H4S(wt) and five of its cysteine mutants on the one hand, and hen egg white lysozyme (HEL) on the other hand by Biacore (Section S1). The kinetics were measured in the presence of dithiothreitol to prevent dimerization of the cysteine mutants. We analyzed the kinetic data with a 1:1 model, calculated the corresponding dissociation constant from the rate constants, i.e. $K_d' = k_{off}/k_{on}$, and also derived the dissociation constant at steady state K_d'' from these kinetic experiments (Table S1). We found that the values of K_d, K_d' and K_d'' were close for H4S(wt), 40 ± 2 nM, 31 nM and 18 nM respectively. The K_d value, determined in solution, was slightly higher than the K_d' and K_d'' values, determined at the interface between a solid and a liquid phase. Comparison of the K_d, K_d' and K_d'' values between H4S(wt) and either the Cys mutants or the conjugates indicated that the lower affinity of the conjugates relative to H4S(wt) was mainly due to the mutation into Cys at positions Val21 and Lys24, whereas it was mainly due to the coupling of the fluorophore at positions Trp8, Ala26 and Lys39.

Mechanism of fluorescence variation

Eleven of the H4S conjugates were sensitive to the binding of HEL, with $\Delta F_\infty/F_0$ between 0.5 and 8.8. We used potassium iodide (KI) to explore the physico-chemical mechanism by which the fluorescence intensity of the conjugates varied on antigen binding. We found that the fluorescence of the H4S(K24ANBD) conjugate was quenched by KI, both in its free and HEL-bound states. The quenching varied linearly with the concentration of KI (Fig. S4). This law of variation indicated that the molecules of fluorophore were identically exposed to KI and constituted a homogeneous population in either case (Lakowicz, 1999). It confirmed that the fluorescent group was specifically coupled to the mutant cysteine. The Stern-Volmer

constant was higher for the free conjugate than for its complex with the target antigen: K_{SV} = 6.7 ± 0.1 M^{-1} versus 2.5 ± 0.1 M^{-1} (SE in the curve fits of Fig. S4). These values indicated a lower accessibility of the fluorophore to KI in the bound state of the conjugate than in its free state. They showed that the fluorescence increase was due to a shielding of the fluorescent group from the solvent by the binding of the antigen, as previously observed for other conjugates with IANBD (Renard et al., 2003; Brient-Litzler et al., 2010). Thus the mechanism of fluorescence variation was consistent with our rules of design.

Fluorescence variation in serum

We observed that the fluorescence response of the H4S(K24ANBD) conjugate was lower in serum than in buffer, as reported previously for conjugates between IANBD and other proteins (Renard et al., 2003; Brient-Litzler et al., 2010). To better understand this difference, we measured the variations of the F_0 and F_∞ parameters as functions of the concentration in serum. We used a conjugate between IANBD and 2-mercaptoethanol as a control (Fig. S3). The absorbance of the serum alone increased linearly with its concentration, in agreement with the Beer-Lambert law, at both 485 nm and 536 nm, which were the wavelengths of fluorescence excitation and emission in our experiments. The value of F_∞ for H4S(K24ANBD) decreased linearly with the concentration in serum. Therefore, the absorption of the excitation and emission lights by serum could account for the variation of F_∞. The value of F_0 for H4S(K24ANBD) increased with the concentration in serum, up to 35% (v/v) of serum, and then decreased slowly. We observed the same variations for the conjugate between IANBD and 2-mercaptoethanol. Therefore, the initial increase of F_0 could result from the interaction between the fluorescent group and molecules of the serum, until saturation, and its subsequent decrease from the absorbance of light by the serum.

Supplementary references

Binz, H.K., Amstutz, P., Kohl, A., Stumpp, M.T., Briand, C., Forrer, P., Grutter, M.G., Pluckthun, A., 2004. High-affinity binders selected from designed ankyrin repeat protein libraries. Nat. Biotechnol. 22, 575-582.

Brient-Litzler, E., Pluckthun, A., Bedouelle, H., 2010. Knowledge-based design of reagentless fluorescent biosensors from a designed ankyrin repeat protein. Protein Eng. Des. Sel. 23, 229-241.

Lakowicz, J.R., 1999. Principles of fluorescent spectroscopy, 2nd ed. Kluwer Academic/Plenum, New York.

Lisova, O., Hardy, F., Petit, V., Bedouelle, H., 2007. Mapping to completeness and transplantation of a group-specific, discontinuous, neutralizing epitope in the envelope protein of dengue virus. J. Gen. Virol. 88, 2387-2397.

Renard, M., Belkadi, L., Bedouelle, H., 2003. Deriving topological constraints from functional data for the design of reagentless fluorescent immunosensors. J. Mol. Biol. 326, 167-175.

Renard, M., Belkadi, L., Hugo, N., England, P., Altschuh, D., Bedouelle, H., 2002. Knowledge-based design of reagentless fluorescent biosensors from recombinant antibodies. J. Mol. Biol. 318, 429-442.

Legends to the supplementary figures

Fig. S1. Sequence of the parental H4S Nanofitin. The numbering does not take the first 11 residues into account. The randomized positions are colored red; residues Lys28 and Lys39 are colored green.

Fig. S2. Sequence of the parental MBP3_16 DARPin. AR1 and AR2, ankyrin repeats 1 and 2 respectively. Positions 2, 3, 5, 13, 14, and 33 in each ankyrin repeat are fully randomized and colored red. Position 26 in each repeat is partially randomized and colored green. Position 43 in the N-cap module is fully randomized and position 109 in AR2 is not randomized (Binz et al., 2004).

Fig. S3. Effect of the concentration in serum on the fluorescence signal for conjugates between IANBD and either H4S(K24C) or 2-mercaptoethanol. The experiments were performed in a mixture (v:1-v) of serum and buffer C at 25 °C. The total concentration of conjugate was equal to 0.3 μM. The total concentration of HEL was equal to 10 μM and thus saturating (see Table 1). Open squares, 2-mercaptoethanol-ANBD (the results were identical in the presence or absence of HEL); open circles, H4S(K24ANBD) without HEL; closed circles, H4S(K24ANBD) with HEL. *F*, intensity of fluorescence in arbitrary units. The continuous curves were drawn only for clarity.

Fig. S4. Quenching of the H4S(K24ANBD) fluorescence by KI. *F* and F^0, fluorescence of the conjugate with and without quencher respectively. The experiments were performed in buffer C at 25 °C. The concentration of conjugate was equal to 0.3 μM. The continuous curves were obtained by fitting Eq. (S5) to the experimental data. Closed circles, conjugate in the absence of HEL; open circle, conjugate in the presence of a saturating concentration of HEL (10 μM). The corresponding Stern-Volmer constants K_{SV} were equal to 6.7 ± 0.1 M^{-1} and 2.5 ± 0.1 M^{-1} respectively (value ± SE in the fitting).

Fig. S5. Determination of the dissociation constant K_d between MBP3_16(wt) and MalE in solution at 25 °C in buffer F, by competition Biacore. The total concentration of MalE in the binding reaction is given along the *x* axis. The *r* signal, which is proportional to the concentration of free MBP3_16(wt) in the binding reaction, is given along the *y* axis. Fifteen concentrations of MalE were used. The curve was obtained by fitting Eq. (S7) to the experimental data, with K_d and r_0 as floating parameters.

Fig. S6. Relation between R_{eq} and K_d for the interaction between MalE and the Cys mutants of MBP3_16. The values of R_{eq} and K_d were determined by Biacore (Table 2). The curve was obtained by fitting Eq. (8) to the experimental values of R_{eq} and K_d, with C = [MBP3_16] = 50 nM and R_{max} as a floating parameter. The values of R_{max} and Pearson coefficient R in the fitting were equal to 595 ± 27 RU and 0.96524 respectively.

Fig. S7. Titration of MBP3_16 conjugates by MalE, monitored by fluorescence. The experiments were performed at 25 °C in buffer C. The total concentration in MBP3_16, as measured by A_{280nm}, was equal to 1.0 μM. The total concentration in MalE protein is given along the *x* axis; a data point at 10 μM is not shown on the figure. The continuous curves correspond to the fitting of Eq. (2) to the experimental values of $\Delta F/F_0$ (Section 2.3). Open triangle, position Met43; open circle, Asn45; closed triangle, Ala78; closed circle, Lys89.

Fig. S8. Relative sensitivities s_r of MBP3_16 conjugates at 25 °C in buffer C as a function of their concentration. This figure is a plot of Eq. (5), using the parameters listed in Table 3. Open triangle, position Met43; open circle, Asn45; closed triangle, Ala78; closed circle, Lys89. The curve for Ser76 has not been represented for clarity; it is located between those for Met43 and Ala78.

Table S1. Binding parameters for the Cys mutants of H4S, as determined by Biacore experiments.

Mutation	Monomer (%)	k_{on} (10^5 M^{-1} s^{-1})	k_{off} (10^{-2} s^{-1})	K_d' (nM)	K_d" (nM)
WT	100	4.9	1.5	31	18
W8C	86	4.5	3.0	68	66
V21C	88	0.4	1.1	332	389
K24C	100	1.6	2.3	117	104
A26C	80	3.8	2.3	60	54
K39C	85	3.1	1.2	38	24

WT, parental H4S protein. The percentage of monomers was quantified with the Un-scan-it software (Silk Scientific), as described in Section 2.2. HEL was immobilized on a CM5 sensorchip. The association and dissociation rate constants, k_{on} and k_{off}, were determined at 25 °C in buffer E and used to calculate $K_d' = k_{off}/k_{on}$. K_d" is the dissociation constant at steady state. We applied a simple kinetic model of Langmuir binding (Section S1).

H4S

```
-10          1         10         20         30
 MRGSHHHHHHGSVKVKFFWNGEEKEVDTSKIVWVKRAGKSV
           40         50         60
  LFIYDDNGKNGYGDVTEKDAPKELLDMLARAEREKKLN
```

Figure S1

MBP3_16

```
       1        10         20         30         40
N-cap: MRGSHHHHHHGSDLGKKLLEAAHAGQDDEVRILMANGADVNAM
                      50         60         70
AR1   :             DNFGVTPLHLAAYWGHFEIVEVLLKYGADVNAS
                      80         90        100
AR2   :             DATGDTPLHLAAKWGYLGIVEVLLKYGADVNAQ
                   110        120        130
C-cap:              DKFGKTAFDISIDNGNEDLAEILQKLN
```

Figure S2

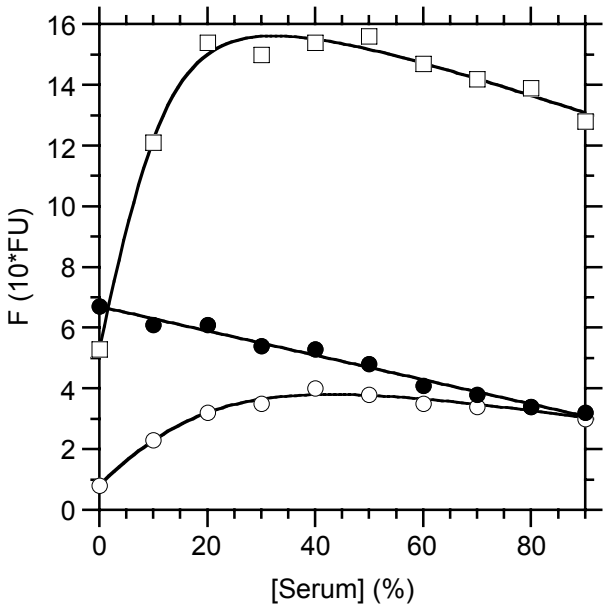

Figure S3

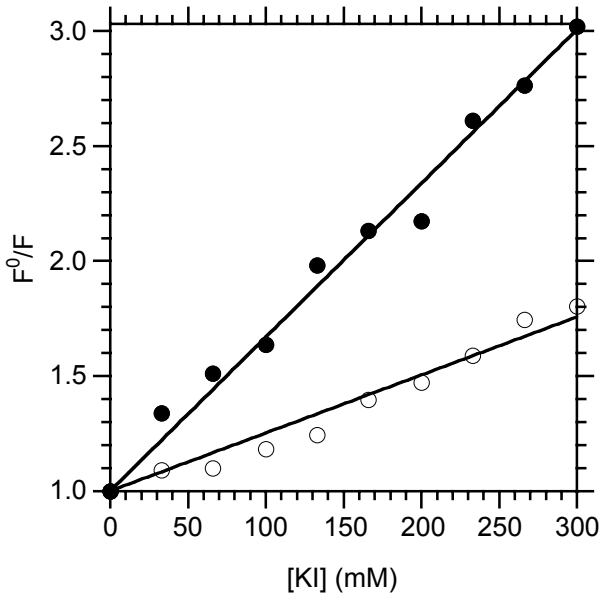

Figure S4

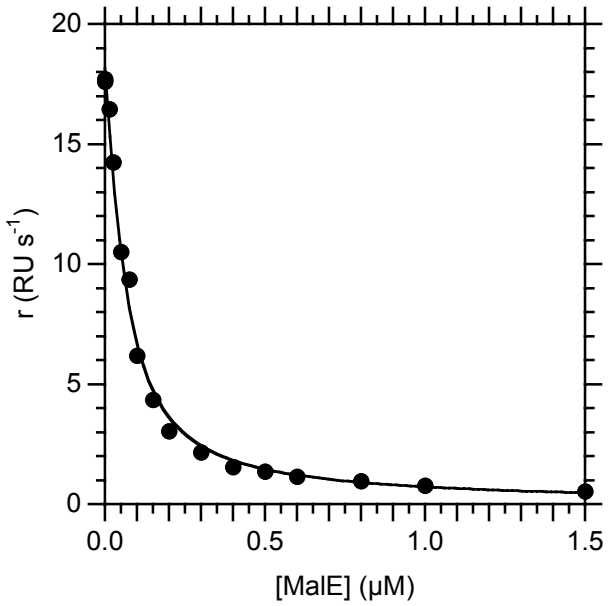

Figure S5

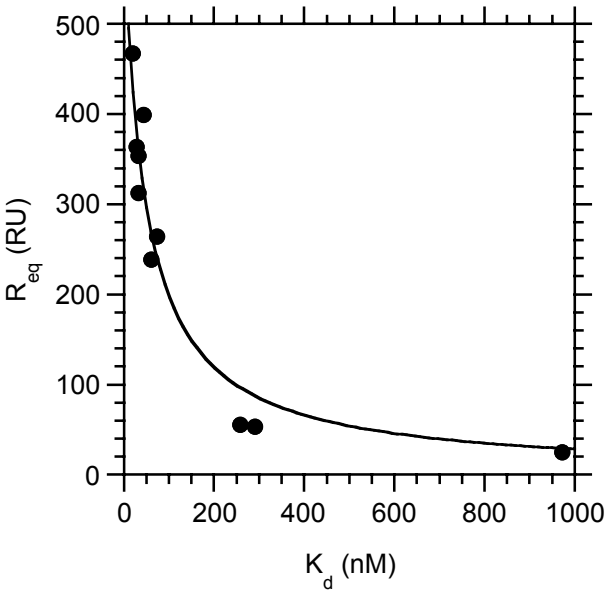

Figure S6

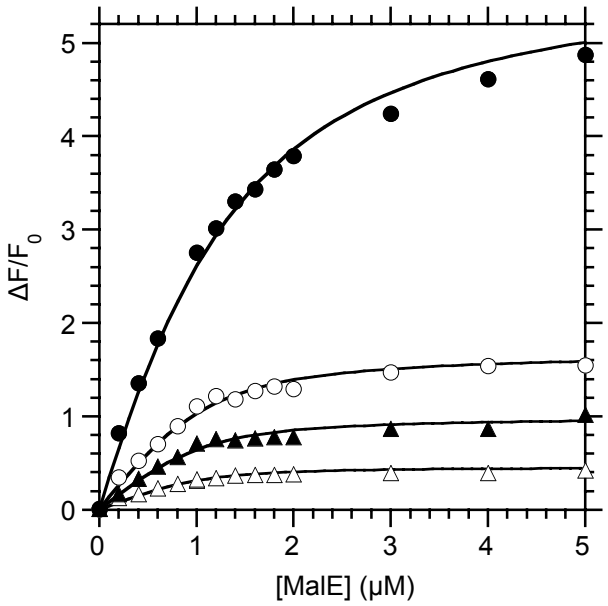

Figure S7

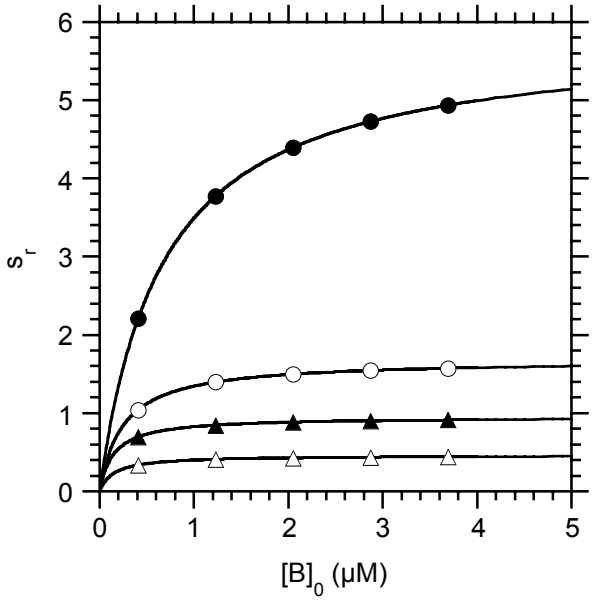

Figure S8