Supporting Information

Binding of the Gene Repressor BlaI to the *bla* Operon in Methicillin-Resistant *Staphylococcus aureus*

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Cloning of *blaI* from *S. aureus* NRS128 for expression in *E. coli*. We used the plasmid pI258 from *S. aureus* NRS128 (NCTC 8325) as the source of the *blaI* gene.(*1*) We utilized the high-fidelity *Pfu*Ultra II Fusion HS DNA Polymerase (Stratagene®) to amplify the *blaI* gene, using the following oligonucleotide primers: blaI_fw_NcoI, 5'-tacat*cc**atg**g*ccaataagcaagttg-3', and blaI_rv_HindIII, 5'-atgc*aagctt*ttacttttttactaatatcatttaaaatg-3'. These primers contain the recognition sequences for *Nco*I and *Hin*dIII restriction endonucleases, respectively (italicized in the nucleotide sequence). The amplified fragment was separated by electrophoresis on a 1% agarose gel and purified from the gel band using the QIAquick Gel Extraction Kit (QIAGEN). This fragment was cloned into the *Nco*I and *Hin*dIII sites of plasmid pET-24a(+), from Novagen. The ligation mixture was used to transform *E. coli* DH5α (Invitrogen) competent cells. Transformants were selected on LB-agar plates supplemented with 50 μg/mL kanamycin. Plasmid DNA from several transformants was isolated, and the presence of the inserted *blaI* gene was verified by digestion with *Nco*I and *Hin*dIII. Subsequently, the nucleotide sequence of the *blaI* gene was verified by sequencing of both DNA strands. The plasmid was designated as pET-blaI

Expression and purification of BlaI from *S. aureus* NRS128. *Escherichia coli* OverExpress C41(DE3) competent cells were transformed with the pET-blaI plasmid. Each one of two 250-mL Erlenmeyer flasks containing 50-mL LB broth with 50 μg/mL kanamycin were inoculated with a fresh colony, and the cultures were grown overnight at 37 °C at 250 rpm. Each 50-mL culture was divided in two sterile tubes and centrifuged for 15 min at 2,465 *g*. Each cell pellet was resuspended in 1 mL of LB, and it was used to inoculate 500 mL LB supplemented with 50 μg/mL kanamycin in a 3-L Erlenmeyer flask (four times for a total of 2 L). The culture was grown to OD_{600} of 0.9-1. BlaI expression was induced by the addition of 500 μL of 0.5 M IPTG to each 500 mL of culture (500 μM final concentration), and the cells were further grown for 4 h, at 37 °C, 250 rpm. The cultures were then centrifuged for 30 min at 4 °C at 2,465 *g* in an Eppendorf 5810R centrifuge. The cell pellet from two 500-mL cultures was resuspended in 20 mL lysis buffer (50 mM HEPES, pH 7.6, 1 mM EDTA, 0.2 M NaCl), and the combined cells were centrifuged again for 30 min at 4 °C at 2,465 *g*. The cell pellets were stored at -20 °C. The cell pellet from a 1-L culture was thawed and resuspended in 30 mL of lysis buffer supplemented with 3.33 μg/mL DNAse, 10 mM $MgSO_4$, and 1 mM PMSF. Cells were lysed by sonification and the cell extract was then centrifuged for 45 min at 9,000 *g* at 4 °C. The supernatant was loaded onto a 50-mL column with Macro-Prep High-S Support (BioRad) equilibrated in 50 mM HEPES, pH 7.6, 1 mM EDTA, 0.2 M NaCl. After washing with the same buffer (five column volumes), BlaI was eluted using a 300 mL gradient from 0.2-1 M NaCl (elution buffer was 50 mM HEPES, pH 7.6, 1 mM EDTA, 1 M NaCl). BlaI eluted at 0.4-0.5 M NaCl. The same procedure was repeated the following day with the second 1-L pellet. The BlaI protein eluted from the two cation-exchange

columns was combined, concentrated and the NaCl concentration was reduced to 0.2 M by dilution with 50 mM HEPES, pH 7.6, 1 mM EDTA. The BlaI fraction was loaded again onto a 50-mL column with Macro-Prep High-S Support (BioRad) equilibrated in 50 mM HEPES, pH 7.6, 1 mM EDTA, 0.2 M NaCl. After washing with five column volumes of the same buffer, BlaI was eluted using a 300 mL gradient from 0.2-1 M NaCl in 50 mM HEPES, pH 7.6, 1 mM EDTA. The BlaI fractions were pooled and the NaCl concentration was reduced to 0.2 M by dilution with 50 mM HEPES, pH 7.6, 1 mM EDTA. BlaI was then loaded onto a 25-mL Macro-Prep High-Q Support (BioRad) equilibrated in 50 mM HEPES, pH 7.6, 1 mM EDTA, 0.2 M NaCl. Most of BlaI was collected in the flow-through and the wash. BlaI was then loaded onto a 5-mL Heparin column (GE Healthcare) equilibrated in 50 mM HEPES, pH 7.6, 0.2 M NaCl, with Complete EDTA-free Protease Inhibitor Cocktail (Roche). After washing with five column volumes of the same buffer, the protein was eluted with a 200 mL gradient from 0.2-2 M NaCl in 50 mM HEPES, pH 7.6, with Complete EDTA-free Protease Inhibitor Cocktail. BlaI eluted at approximately 0.6 M NaCl. The fractions containing BlaI were pooled, the NaCl concentration was decreased to 0.2 M by dilution with 50 mM HEPES, pH 7.6, with Complete EDTA-free Protease Inhibitor Cocktail, and the protein was loaded again onto the Heparin column equilibrated in the same buffer as before —except that no protease inhibitor was used in the buffers in the second step—but the protein was now eluted using a longer gradient (200 mL gradient 0.2-1 M NaCl in 50 mM HEPES, pH 7.6). In the High-S and Heparin columns, several proteins co-elute with BlaI mainly at the beginning and at the end of the elution peak. After the first High-S or Heparin column, only the most pure fractions (fractions corresponding to the center of the elution

peak) were collected and passed through a second identical column. The impurities present in the initial sample show a similar pattern of elution in the second step, with a higher concentration at the beginning and at the end of the BlaI peak. Again, only the most pure fractions (center of the peak) were collected. This protocol (together with a less steep elution gradient in the second Heparin column) considerably improved the purity of the protein. All purification steps were carried out at 4 °C. The BlaI chromatography peak in the last purification step was not symmetrical, and upon resolution of the protein in the different fractions in a 15 % SDS-PAGE gel, a small contamination by a 30-kDa entity could be detected in the fractions corresponding to the end of the peak. BlaI absorption spectrum was recorded and BlaI was quantified using the theoretical absorption coefficient ε_{280} of 19,940 $M^{-1}cm^{-1}$. After the five purification steps, BlaI was pooled in two fractions: approx. 7.5 mg of homogeneous BlaI (first half of the peak, referred to as BlaI-1, Figure S1), and approx. 11 mg of less pure BlaI (second half of the peak with the minor 30-kDa contamination, Figure S1, referred to as BlaI-2). BlaI was concentrated to 5-7 mL and dialyzed against four 6-hour changes of 1 L of 50 mM HEPES, pH 7.6, 0.2 M NaCl, 20 mM $MgCl_2$. An aliquot of the same buffer used in the dialysis was saved as reference for equilibrium sedimentation assays. Finally, the concentrated sample was passed through Protein Desalting Spin columns (Pierce-Thermo Scientific) equilibrated with 50 mM HEPES, pH 7.6, 0.2 M NaCl, 20 mM $MgCl_2$. The homogeneous BlaI-1 sample was then used for equilibrium sedimentation and fluorescence anisotropy measurements.

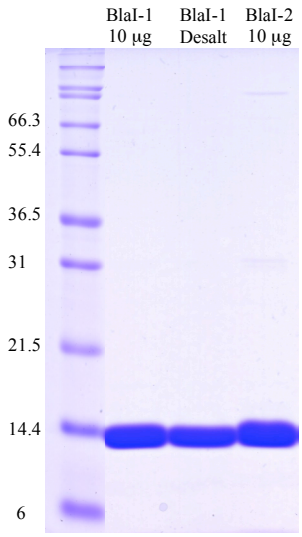

Figure S1. Purified BlaI (fractions BlaI-1 before and after desalting column, and BlaI-2) resolved in a 15% SDS-PAGE Gel stained with Coomasie.

Analytical ultracentrifugation. Sedimentation equilibrium experiments were performed using an Optima XL-I analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA) in an An-60 Ti rotor equipped with a standard two-channel cell. BlaI samples (4.37, 23, and 72.3 µM) in 50 mM HEPES, pH 7.6, 0.2 M NaCl, 20 mM $MgCl_2$, were independently rotated at 22,000 rpm at 20 °C until equilibrium was attained. Absorbance monitoring was performed at 280 nm (23 and 72.3 µM samples) and 235 nm (4.37 µM sample). A self-association model was used for global fitting of multiple equilibrated scans for each protein concentration, using the Optima™ XL-A/XL-I Data Analysis Software (Beckman Coulter). The BlaI partial specific volume was 0.749 mL/g, as calculated from the mass average of the partial specific volumes of the individual amino acids.(*2*)

Determination of the *in vivo* concentration of BlaI. The concentration of BlaI was determined in *S. aureus* NRS128 (NCTC 8325) and NRS70 (N315) in the exponential

and stationary phases. For each strain, 100 mL of LB in a 500-mL Erlenmeyer flask was inoculated with 100 μL of an overnight culture. The cultures were grown at 37 °C, 220 rpm, until OD_{625} reached 0.8 (4-5 h). An aliquote was used to prepare serial dilutions for cell count. A 30-mL portion of each culture was centrifuged at 4 °C, and the resulting cell pellets were frozen at -20 °C. The remaining cultures were grown until OD_{625} reached 2 (approximately 2 additional hours). An aliquote was used to prepare serial dilutions for cell count. A 15-mL portion of each culture was centrifuged at 4 °C, and the resulting cell pellets were frozen at -20 °C. For each culture, the number of bacterial cells was determined in five individual determinations by counting of colonies subsequent to serial dilution and growth on LB-agar plates. The BlaI protein was quantified by Western blotting. The cell pellets were thawed and resuspended in Lysis Buffer (100 mM sodium phosphate, pH 7.5, 50 mM $NaHCO_3$, 200 μg/mL lysostaphin, 20 mM $MgCl_2$, 15 μg/mL DNase I, 15 μg/mL RNase A, 1 mM EDTA, 1X Complete EDTA-free Protease Inhibitor Cocktail), and incubated for 30 min at 37 °C. Total protein was quantified using the BCA Protein Assay Kit (Pierce®, Thermo Scientific); for each sample, an 80-μg portion of the total protein was developed in 15% SDS-PAGE gels. Different known amounts of the purified BlaI protein were loaded onto each gel and used to generate an internal calibration curve. After electrophoresis, the proteins were transferred to 0.45 μm nitrocellulose membranes (Trans-Blot® Transfer Medium, Bio-Rad), using Tris-glycine-methanol buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol) at 4 °C, 2 h, 200 mA. The membranes were blocked at RT overnight in Blotto (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3% BSA, 3% non-fat milk, 0.02% sodium azide). The BlaI protein was detected by Western Blot, using a 1/1000 dilution of a custom BlaI antibody (1.86

mg/mL, antibodies generated by immunization of a rabbit with purified BlaI, $(NH_4)_2SO_4$-precipitated, and purified by Protein A-chromatography, by EZBiolab) as the primary antibody, and a 1/15,000 dilution of Goat-anti-rabbit Horse Radish-Peroxidase-Conjugated secondary antibody (Pierce®, Thermo Scientific). The antibodies were diluted in Tween-Tris Buffered Saline (T-TBS; 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) with 1.1% non-fat milk. The membranes were incubated with the primary and secondary antibodies for 1 h at RT. Four 15-min washes at RT with approximately 30 mL of T-TBS were done after blocking, in between antibodies incubations, and after the incubation with secondary antibody. The membranes were developed by incubation for 5 min at RT with the chemioluminiscent substrate SuperSignal® West Dura Extended Duration Substrate (Pierce®, Thermo Scientific), and the membranes were then exposed to X-Ray Films (CL-XPosure™ Film, Thermo Scientific) for 15-30 min. The BlaI bands were quantified using the software ImageJ (rsb.info.nih.gov/ij/). The intensity of the purified BlaI samples was plotted as a function of the protein quantity (ng), and the equation resulting from a linear fit of the data was used to calculate the amount of BlaI in *S. aureus* extracts (Figure S2). The protein ranges that we determined correspond to the concentration of protein calculated based on two cell radii reported in the literature: 500 nm (*3*) and 700 nm.(*4*) The volume of *S. aureus* was calculated for a sphere within the range of 5.24×10^{-16} L and 1.44×10^{-15} L for the respective radii.

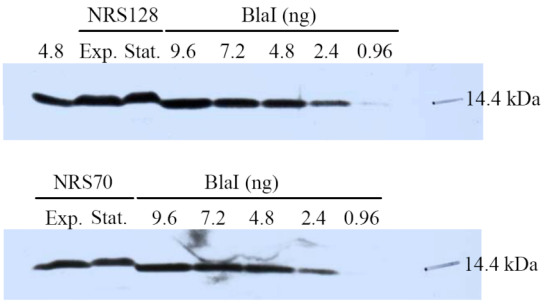

Figure S2. BlaI quantification in MRSA cells by Western blot.

BlaI in *S. aureus* NRS128 in the presence of β-lactam antibiotics. A 30-μL aliquot of an overnight *S. aureus* NRS128 culture was used to inoculate 30 mL of LB media in a 250-mL Erlenmeyer flask. The culture was grown at 37 °C, 220 rpm, until OD_{625} of 0.7. The culture was divided into two equal portions and the β-lactam antibiotic CBAP (2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid) was added to one of the cultures to a final concentration of 10 μg/mL (MIC for the antibiotic with the strain is 64 μg/mL). The cultures were allowed to grow at 37 °C, 220 rpm for 3 h, until OD_{625} of approximately 2 was reached. The cells were harvested by centrifugation at 4 °C. The cell pellets were resuspended in 1 mL of Lysis Buffer (100 mM sodium phosphate, pH 7.5, 50 mM $NaHCO_3$, 200 μg/mL lysostaphin, 10 mM $MgCl_2$, 15 μg/mL DNase I, 15 μg/mL RNase A), and were allowed to incubate for 30 min at 37 °C. After lysis, the total protein was quantified using the BCA Protein Assay Kit (Pierce®, Thermo Scientific). The purified BlaI was used as an internal calibration curve (Figure S3). BlaI was detected by Western blot, as described in the previous procedure.

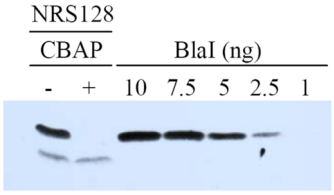

Figure S3. BlaI in *S. aureus* NRS128 grown in the presence and in the absence of sub-MIC concentration of CBAP, detected by Western blot.

Binding of BlaI to *bla* Operator Sequences Measured by Fluorescence Anisotropy. The dissociation constants for BlaI binding to DNA were determined by measuring the changes in fluorescence anisotropy of fluorescein-labeled dsDNA upon titration with increasing concentrations of BlaI. Three DNA sequences were prepared and used. These

were two 30-bp fragments containing the R1 or Z dyads of the *bla* operator, and a 61-bp DNA fragment containing both the R1 and Z dyads. In each case, the dsDNA fragment was obtained by annealing a 5'-fluorescein-labeled oligonucleotide with an unlabeled reverse complementary oligonucleotide; all oligonucleotides were purified on HPLC. Oligonucleotides were prepared by custom synthesis at Eurofins MWG-Operon. The oligonucleotides used to generate dsDNA corresponding to the R1 dyad were 5'fluorescein-tgacaccg**atattacaattgtaatat**tatt and 5'aataatattacaattgtaatatcggtgtca; the ones used to generate dsDNA corresponding to the Z dyad were 5'fluorescein-atttataa**aaattacaactgtaatat**cgga and 5'tccgatattacagttgtaatttttataaat; and the ones used to generate dsDNA corresponding to the R1-Z dyads were 5'fluorescein-tgacaccg**atattacaattgtaatat**tattgatttataa**aaattacaactgtaatat**cgga and 5'tccgatattacagttgtaatttttataaatcaataatattacaattgtaatatcggtgtca. Each pair of complementary oligonucleotides was annealed at a 1:1 ratio. Annealing was carried out by heating the reaction mixture to 95 °C for 5 min, followed by slow cooling (16 h) to room temperature in 10 mM Tris-HCl, pH 7.5. The annealing sample was run on an 18% acrylamide gel in 0.5 x TBE to verify the efficiency of the annealing reaction. The fluorescein-labeled DNA fragments were detected using a Storm Scanner. The gel was then stained with ethidium bromide to verify the complete disappearance of the unlabeled oligonucleotide upon annealing. dsDNA was quantified by absorbance at 260 nm. Fluorescence anisotropy measurements were performed on a Beacon 2000 (PanVera, Madison, WI) at 25 °C using the standard fluorescein filters supplied with the instrument. Different dilutions of BlaI were prepared in 50 mM HEPES, pH 7.6, 200 mM NaCl and 20 mM $MgCl_2$ (0-60 μM for the R1-dyad and Z-dyad dsDNA, or 0-6 μM for the R1-Z-

dyads dsDNA), and an appropriate amount of the concentrated fluorescein-labeled dsDNA was added, to achieve a final concentration of 1 nM dsDNA in a final volume of 200 μL. After mixing, the mixture was incubated in the dark at room temperature for 30 min to reach the equilibrium. The anisotropy was measured at 25 °C, after 60 s incubation of the mixture at the same temperature in the Beacon equipment. The anisotropy of each sample was the average of three measurements. Each complete assay was done in duplicate. The total fluorescence intensity showed no significant change upon addition of different concentrations of BlaI to the fluorescein-labeled dsDNA samples.

The anisotropy values were plotted as a function of total BlaI concentration. Different equations were derived to account for different models. The data were fit using user-defined equations in SigmaPlot (Systat Software Inc.). The experimental data could not be fit with equations that account for a model of binding of a single protein species to DNA (DNA + Protein ⇌ DNA.Protein). For the individual Z and R1 dyads, the data did not give a sigmoidal behavior and hence the attempts to fit with equations that account for simultaneous binding of two BlaI monomers (M) to DNA failed (DNA + 2 M ⇌ DNA.D; where D stands for the dimer of BlaI). The data for BlaI binding to the DNA fragment containing both the R1 and Z dyads did not produce a sigmoidal behavior either, and could not be fit to models that considered simultaneous binding of two BlaI dimers to DNA. The best fit of the data was obtained with the following model, involving three distinct types of interactions, with M and D denoting monomeric and dimeric species of BlaI. The parameter K_d is the dissociation constant for the dimer to monomer equilibration of BlaI.

$$\text{DNA} + \text{M} \overset{K_{d1}}{\rightleftharpoons} \text{DNA.M}$$

$$\text{DNA.M} + \text{M} \overset{K_{d2}}{\rightleftharpoons} \text{DNA.D} \qquad K_d = \frac{[M]^2}{[D]}$$

$$\text{DNA} + \text{D} \overset{K_{d3}}{\rightleftharpoons} \text{DNA.D}$$

At each total protein concentration, the change in fluorescence anisotropy of fluorescein-labelled DNA (A) is the sum of the fractional contributions of the anisotropy of each DNA species present at the equilibrium. If A_f is the anisotropy of free DNA, A_{b1} is the anisotropy of DNA in DNA.M, A_{b2} is the anisotropy of DNA in DNA.D, F_f is the fraction of free DNA, F_{b1} is the fraction of bound DNA present in complex DNA.M, and F_{b2} is the fraction of bound DNA present in complex DNA.D, the measured anisotropy A can be described by the following equations:

$$A = F_f \cdot A_f + F_{b1} \cdot A_{b1} + F_{b2} \cdot A_{b2}$$
$$F_f + F_{b1} + F_{b2} = 1$$

The equation below relates the changes in fluorescence anisotropy of the fluorescein-labelled DNA with total protein concentration for the equilibria described above, and was deduced — following the analysis of Lundblad *et al.* (*5*) — for a [DNA] << K_{d1}, K_{d2}, K_{d3}, and a constant quantum yield of the fluorophore throughout the titration. The best fit of the fluorescence anisotropy data was obtained using this equation (Figure 2).

$$A = Af + \frac{[M]}{K_{d1} \cdot \left(1 + \frac{[M]}{K_{d1}} + \frac{K_d \cdot ([protein]_T - [M])}{2 \cdot K_{d1} \cdot K_{d2}}\right)} \cdot (A_{b1} - A_f) +$$

$$+ \frac{K_d \cdot ([protein]_T - [M])}{2 \cdot K_{d1} \cdot K_{d2} \cdot \left(1 + \frac{[M]}{K_{d1}} + \frac{K_d \cdot ([protein]_T - [M])}{2 \cdot K_{d1} \cdot K_{d2}}\right)} \cdot (A_{b2} - A_f) \qquad \text{Eq. E1}$$

where: $[M] = \frac{-K_d \cdot \sqrt{K_d^2 + 8 \cdot K_d \cdot [protein]_T}}{4}$ and $K_{d3} = \frac{K_{d1} \cdot K_{d2}}{K_d}$

Based on the concentration of BlaI in *S. aureus* NRS128, and on the concentration of the pI258 plasmid (~ 5 copies/cell, which corresponds to 5-15 nM plasmid for a cell radius of 500-700 nm), we used the dissociation constants obtained for BlaI binding to DNA to estimate the concentration of protein-DNA complexes expected in the cell. The program DynaFit(*6*) was used to simulate the accumulation of the different species in equilibrium, for different DNA concentrations.

BlaI has a tendency to oligomerize, and the formation of higher order oligomers was more evident at high protein concentrations and with protein that had been flash frozen and stored at - 80 °C. The oligomerization with frozen BlaI was evident at high BlaI concentration in the DNA binding assays by fluorescence anisotropy. Hence, the sedimentation equilibrium and fluorescence anisotropy assays were carried out with protein that had been recently purified and kept at 4°C. The higher order oligomerization dynamics are significant at high BlaI concentrations (>10 μM BlaI in the fluorescence anisotropy assays), and given the *in vivo* BlaI concentration, the only relevant oligomerization equilibrium *in vivo* is the monomer-dimer equilibrium.

We considered a total of seven different models for DNA binding by BlaI. The equations for each are described below, and we show the fits and the residuals, compared to the cases of the fit with equation E1. The fits with equation E1 gave the best residual distributions, the higher r^2 value (closer to 1) and the lower errors in the parameters fit.

Model 2: BlaI as a single monomer population (Figure S4).

$$\text{DNA} + \text{P} \overset{K_{d1}}{\rightleftharpoons} \text{DNA.P}$$

$$A = Af + \frac{K_{a1} \cdot [protein]_T}{1 + K_{a1} \cdot [protein]_T} \cdot (A_b - A_f) \qquad K_{a1} = 1/K_{d1};\ \text{Eq. E2}$$

Model 3: BlaI as a single monomer population, with binding of two protein molecules simultaneously to DNA (Figure S5).

$$\text{DNA} + 2\ \text{P} \overset{K_{d1}}{\rightleftharpoons} \text{DNA.P}_2$$

$$A = Af + \frac{K_{a1} \cdot [protein]_T^{\,2}}{1 + K_{a1} \cdot [protein]_T^{\,2}} \cdot (A_b - A_f) \qquad K_{a1} = 1/K_{d1};\ \text{Eq. E3}$$

Model 4: Taking into account the concentrations of monomer and dimer, but only BlaI dimer binds to DNA (Figure S6).

$$\text{DNA} + \text{D} \overset{K_{d1}}{\rightleftharpoons} \text{DNA.D}$$

$$A = Af + \frac{[M]^2}{K_d \cdot K_{d1} \cdot \left(1 + \frac{[M]^2}{K_d \cdot K_{d1}}\right)} \cdot (A_b - A_f) \qquad \text{Eq. E4}$$

$$[M] = \frac{-K_d \cdot \sqrt{K_d^{\,2} + 8 \cdot K_d \cdot [protein]_T}}{4} \quad \text{and} \quad K_d = \frac{[M]^2}{[D]}$$

Model 5: Taking into account the concentrations of monomer and dimer; both BlaI monomer and dimer bind to DNA; two monomer molecules bind simultaneously (Figure S7).

$$\text{DNA} + 2\,\text{M} \overset{K_{d1}}{\rightleftharpoons} \text{DNA.D}$$

$$\text{DNA} + \text{D} \overset{K_{d2}}{\rightleftharpoons} \text{DNA.D}$$

$$A = Af + \frac{\left(\left[protein\right]_T - [M]\right)}{2 \cdot K_{d2} \cdot \left(1 + \frac{\left[protein\right]_T - [M]}{2 \cdot K_{d2}}\right)} \cdot (A_b - A_f) \qquad \text{Eq. E5}$$

$$[M] = \frac{-K_d \cdot \sqrt{K_d{}^2 + 8 \cdot K_d \cdot [protein]_T}}{4} \quad and \quad K_d = \frac{[M]^2}{[D]} = \frac{K_{d1}}{K_{d2}}$$

Model 6: For DNA with two protein-binding regions; taking into account the concentrations of monomer and dimer; two dimer molecules bind simultaneously (Figure S8).

$$\text{DNA} + 2\,\text{D} \overset{K_{d1}}{\rightleftharpoons} \text{DNA.D}_2$$

$$A = Af + \frac{\left(\left[protein\right]_T - [M]\right)^2}{4 \cdot K_{d1} \cdot \left(1 + \frac{\left(\left[protein\right]_T - [M]\right)^2}{4 \cdot K_d}\right)} \cdot (A_b - A_f) \qquad \text{Eq. E6}$$

$$[M] = \frac{-K_d \cdot \sqrt{K_d{}^2 + 8 \cdot K_d \cdot [protein]_T}}{4} \quad \text{and} \quad K_d = \frac{[M]^2}{[D]}$$

Model 7: For DNA with two protein-binding regions; taking into account the concentrations of monomer and dimer, with sequential binding of two dimer molecules (Figure S9).

$$\mathrm{DNA} + \mathrm{D} \overset{K_{d1}}{\rightleftharpoons} \mathrm{DNA.D}$$

$$\mathrm{DNA.D} + \mathrm{D} \overset{K_{d2}}{\rightleftharpoons} \mathrm{DNA.D_2}$$

$$A = Af + \frac{[protein]_T - [M]}{2 \cdot K_{d1} \cdot \left(1 + \frac{[protein]_T - [M]}{2 \cdot K_{d1}} + \frac{[protein]_T - [M]}{2 \cdot K_{d2}}\right)} \cdot (A_{b1} - A_f) + \quad \text{Eq. E7}$$

$$+ \frac{[protein]_T - [M]}{2 \cdot K_{d2} \cdot \left(1 + \frac{[protein]_T - [M]}{2 \cdot K_{d1}} + \frac{[protein]_T - [M]}{2 \cdot K_{d2}}\right)} \cdot (A_{b2} - A_f)$$

$$[M] = \frac{-K_d \cdot \sqrt{K_d{}^2 + 8 \cdot K_d \cdot [protein]_T}}{4} \quad \text{and} \quad K_d = \frac{[M]^2}{[D]}$$

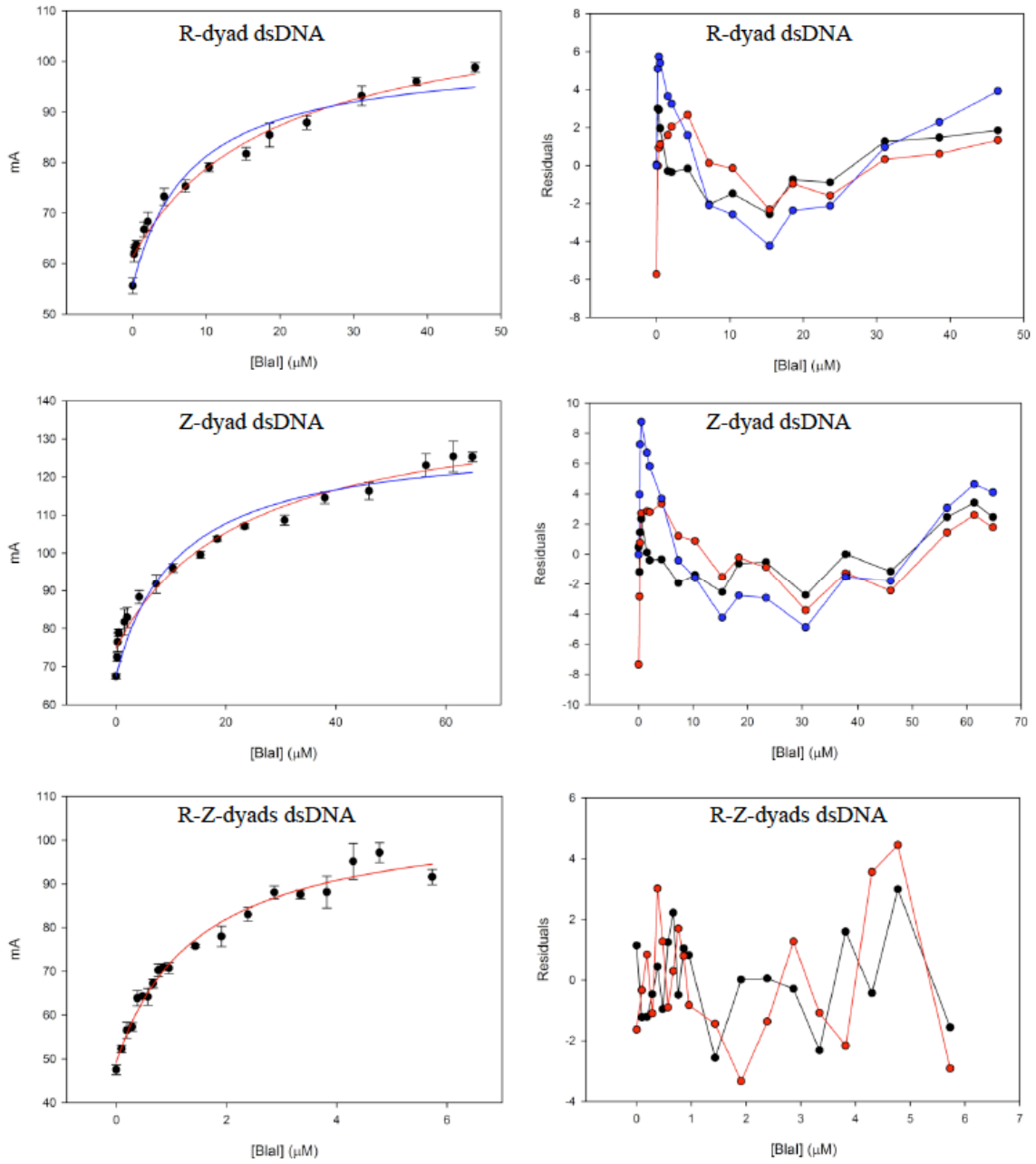

Figure S4. Left panels: Fits of the anisotropy data to Model 2 (red: A_f allowed to float; blue: A_f fixed to experimental value in the absence of protein). Right panels: Comparison of the residuals obtained from the fits with equation E1 (black), and the fits to equation E2 (Model 2; red: A_f allowed to float; blue: A_f fixed to experimental value in the absence of protein).

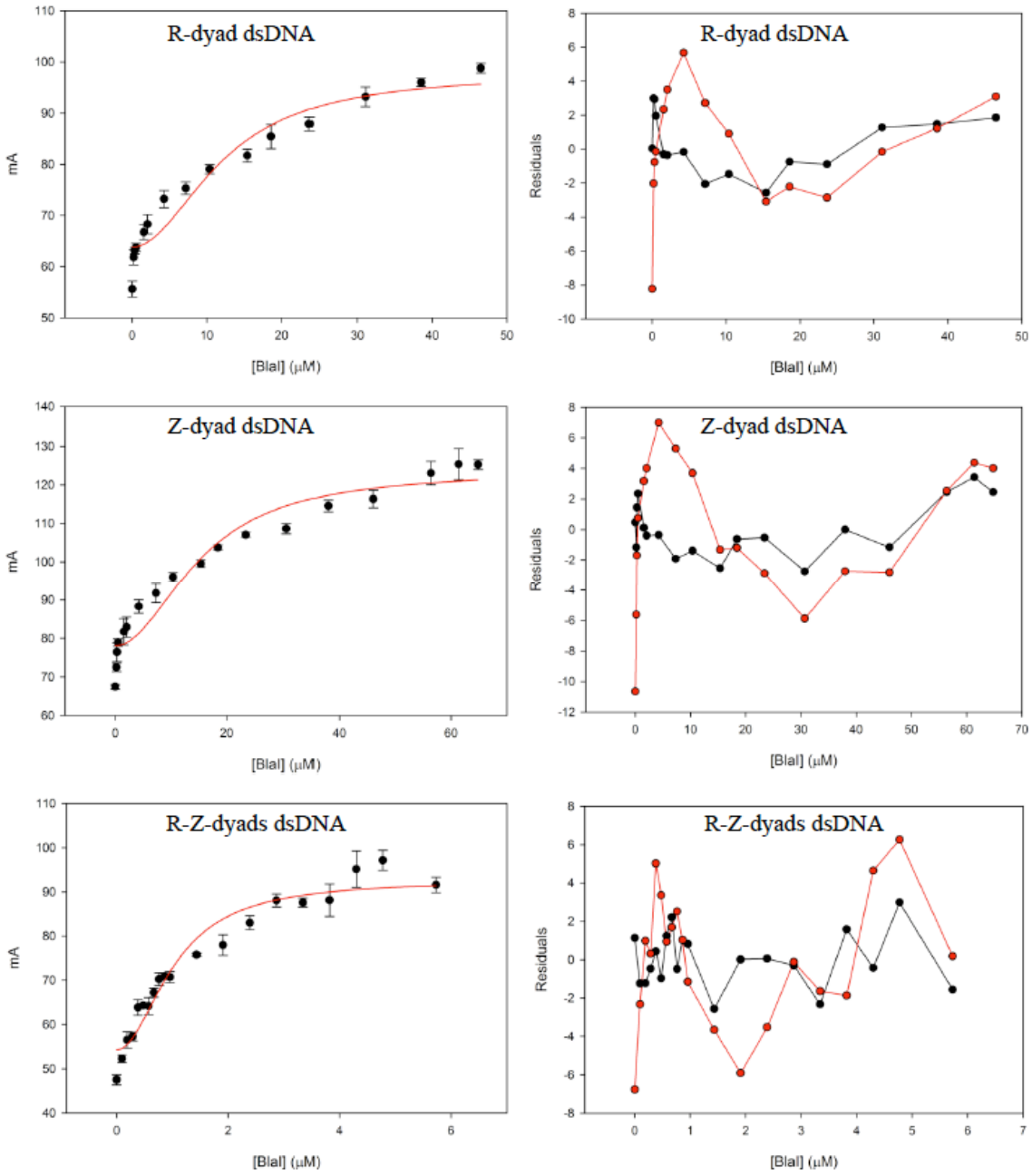

Figure S5. Left panels: Fits of the anisotropy data to Model 3 (A_f allowed to float). Right panels: Comparison of the residuals obtained from the fits with equation E1 (black), and the fits to equation E3 (Model 3; red). In all cases, the r^2 values and residuals of the fits with equation E3 were significantly worse, if the A_f value was fixed to experimental value in the absence of protein (data not shown).

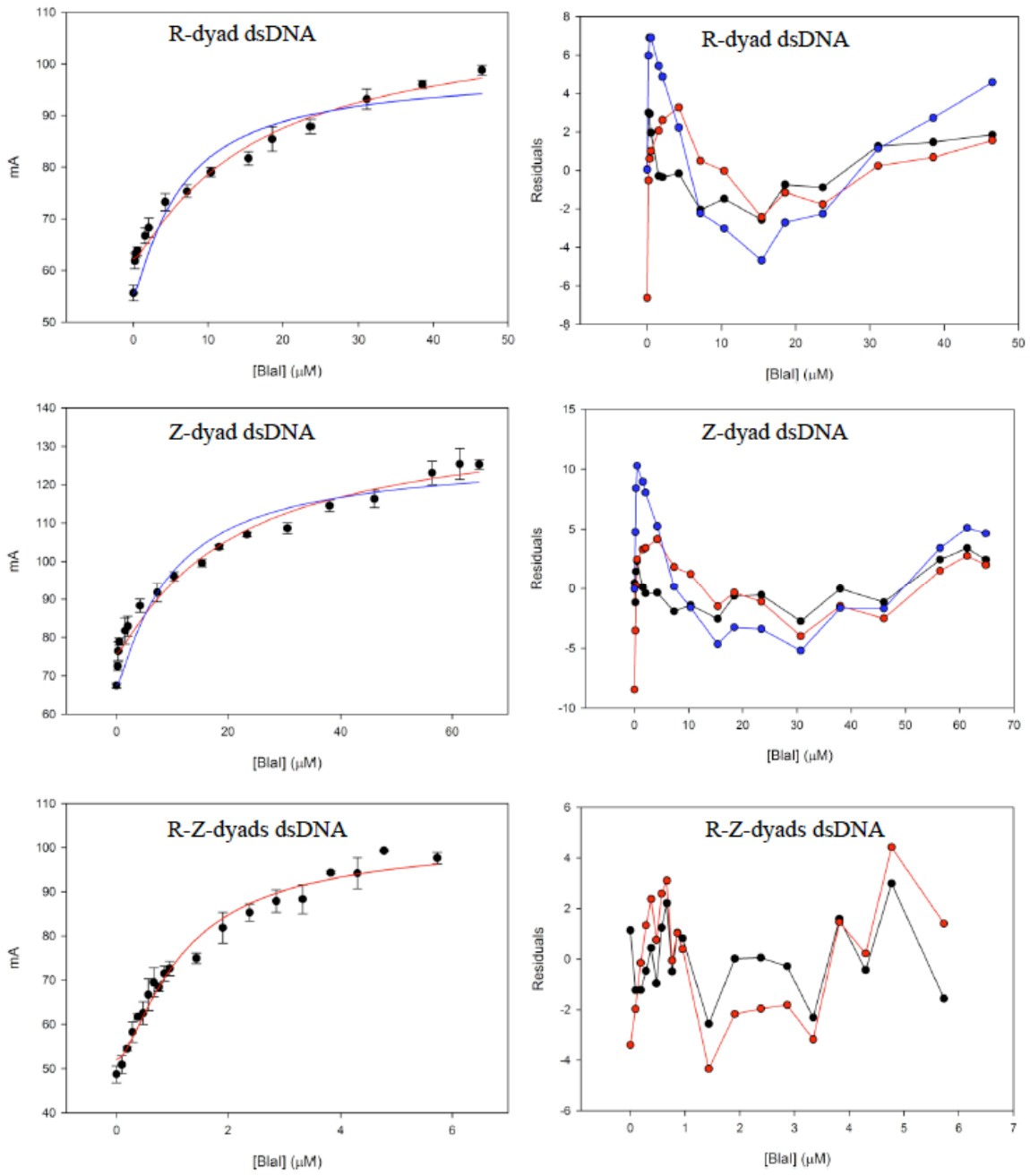

Figure S6. Left panels: Fits of the anisotropy data to Model 4 (red: A_f allowed to float; blue: A_f fixed to experimental value in the absence of protein). Right panels: Comparison of the residuals obtained from the fits with equation E1 (black), and the fits to equation E4 (Model 4; red: A_f allowed to float; blue: A_f fixed to experimental value in the absence of protein).

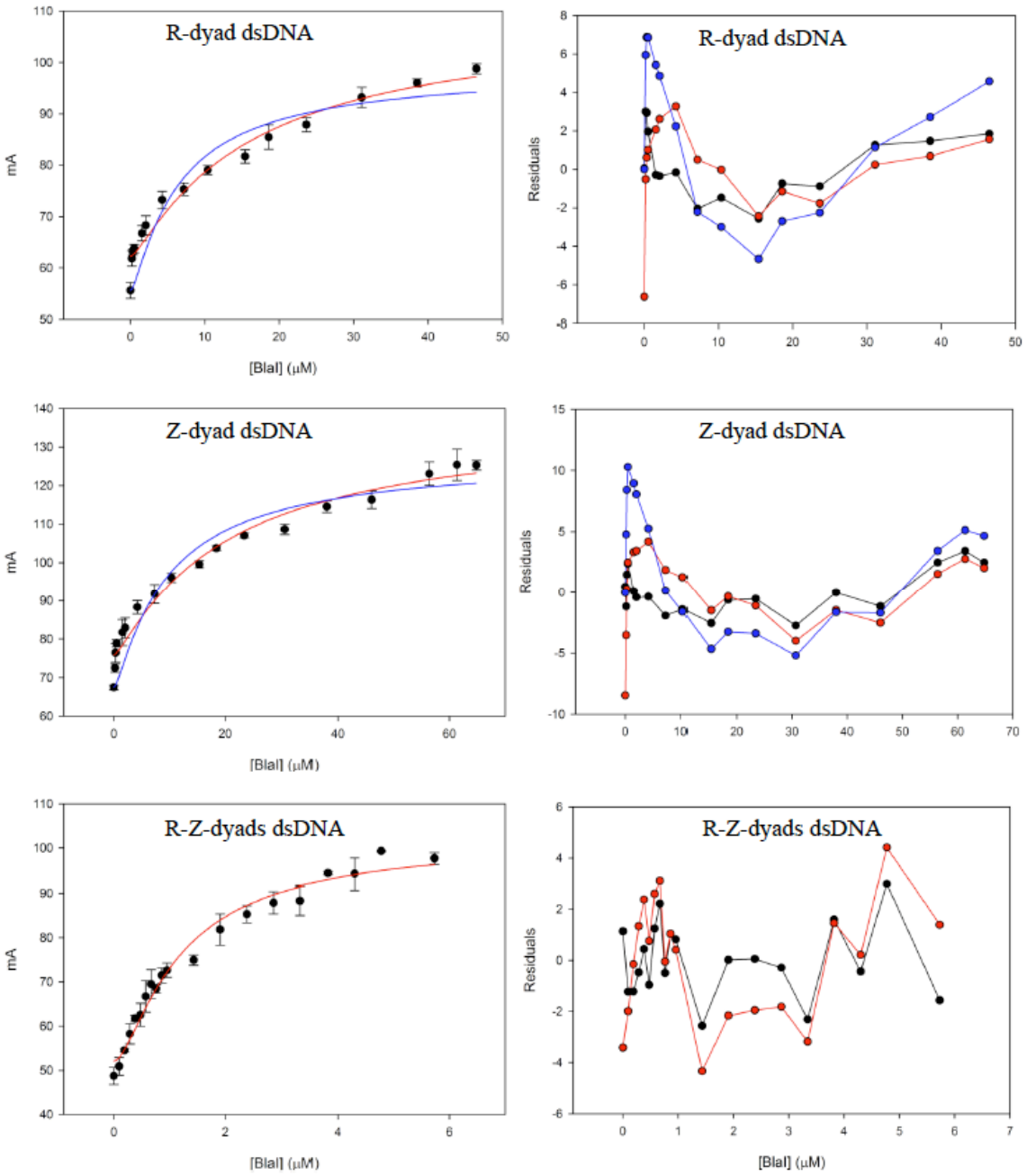

Figure S7. Left panels: Fits of the anisotropy data to Model 5 (red: A_f allowed to float; blue: A_f fixed to experimental value in the absence of protein). Right panels: Comparison of the residuals obtained from the fits with equation E1 (black), and the fits to equation E5 (Model 5; red: A_f allowed to float; blue: A_f fixed to experimental value in the absence of protein).

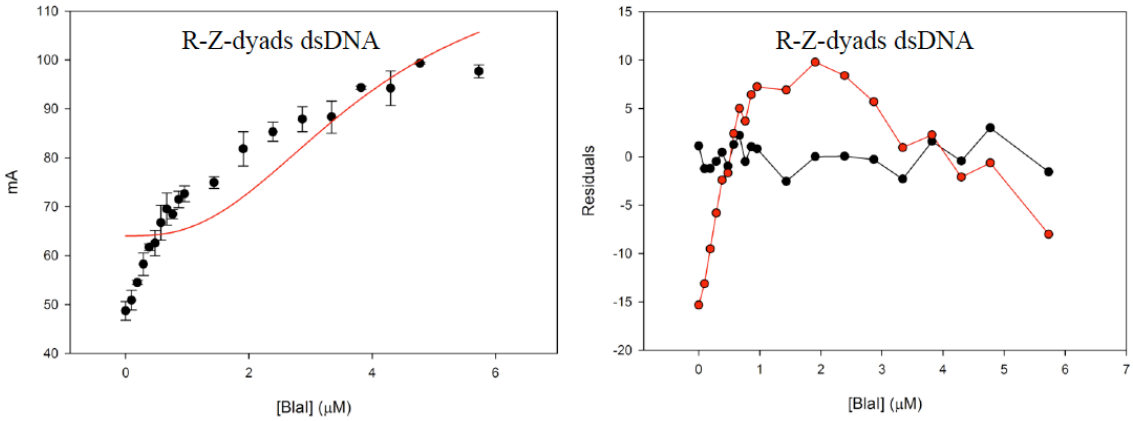

Figure S8. Left panels: Fit of the anisotropy data to Model 6 (A_f allowed to float). Right panels: Comparison of the residuals obtained from the fits with equation E1 (black), and the fit to equation E6 (Model 6; red). In all cases, the r^2 values and residuals of the fits with equation E6 were significantly worse if the A_f value was fixed to experimental value in the absence of protein (data not shown).

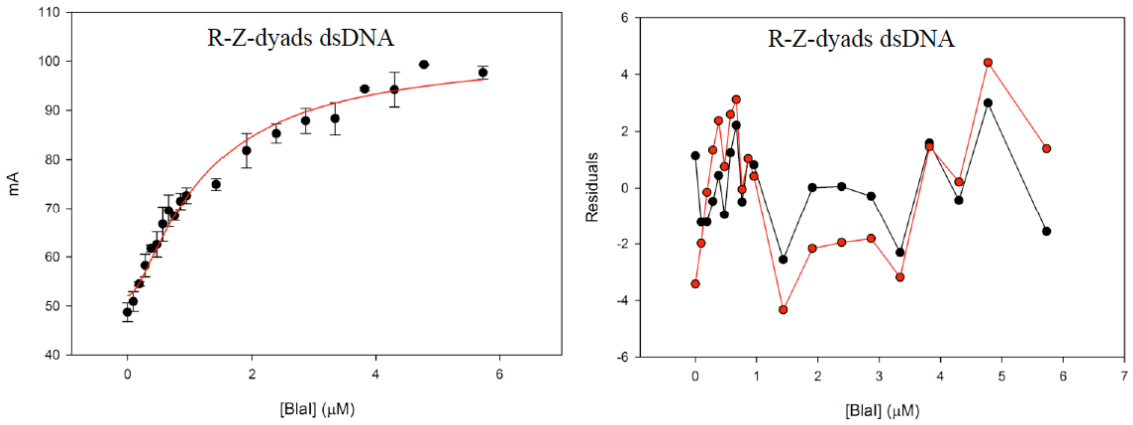

Figure S9. Left panels: Fit of the anisotropy data to Model 7 (A_f allowed to float). Right panels: Comparison of the residuals obtained from the fit with equation E1 (black), and the fit to equation E7 (Model 7; red). In all cases, the r^2 values and residuals of the fit with equation E7 were significantly worse if the A_f value was fixed to experimental value in the absence of protein (data not shown).

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