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Supplemental Data

Architecture of the 99 bp DNA-Six-Protein Regulatory Complex of the λ *att* Site

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FRET Calculations

The determinations of the efficiency of energy transfer E and R_0 and FRET calculations were performed as described (Radman-Livaja et al., 2005). The efficiency of energy transfer E was determined from the extent of donor fluorescence quenching in doubly labeled (donor and acceptor) P-arm bound with six proteins compared with donor-only-labeled P-arm bound with six proteins. Donor fluorescence quenching due to FRET is described by the following equation:

$$\frac{I_j^D - I_i^{DA}}{I_j^D} = 1 - \frac{[complex]_i^{DA}}{[complex]_j^D}(1 - rE), \qquad [1]$$

where I_i^{DA} is the fluorescence intensity of a doubly labeled P-arm-protein complex, *i.* I_j^D is the florescence intensity of a donor-only labeled P-arm- protein complex. $[complex]_i^{DA}$ and $[complex]_j^D$ are the total amounts of doubly and singly labeled P-arm-protein complexes in bands i and j, respectively, and r is the efficiency of DNA labeling with the acceptor.

Eq.1 can be written as follows:

$$I_i^{DA}\frac{[complex]_j^D}{[complex]_i^{DA}} = I_j^D(1 - rE) \qquad [2]$$

[complex] values were obtained from a Phosphorimager scan. *E* was obtained from the slope of the line formed by plotting $I_i^{DA}[complex]_j^D / [complex]_i^{DA}$ against I_j^D, the slope being equal to (1-*rE*). The value *r* was calculated from the absorbance spectrum of each acceptor-labeled oligonucleotide by using ($A^R(560)\varepsilon^{ssDNA}(260)/A^{ssDNA}(260)\varepsilon^R(560)$), with $\varepsilon^R(560\text{ nm})=91.000\ M^{-1}.cm^{-1}$.

The distance R between donor and acceptor is calculated from the measured *E* value by using the Förster equation for FRET:

$$R = R_0 \sqrt[6]{\left(\frac{1}{E} - 1\right)},$$

where R_0 is the Förster radius determined for each donor-acceptor pair.

R_0 Determination

R_0 was measured by the method of Wu and Brand (Wu and Brand, 1994) for each donor-acceptor pair by using the following equations: $R_0 = 0.211\sqrt[6]{\kappa^2 n^{-4} \phi^D J(\lambda)}$ in Å. κ^2 is the transition dipoles orientation factor estimated to be 2/3 for fluorophores that have a fast rotational diffusion rate in solution (Dale et al., 1979; Dale et al., 1980). *n* is the refractive index of the medium (1.4 for DNA aqueous solutions). ϕ^D is the emission quantum yield of the donor. $J(\lambda)$ [in $M^{-1}.cm^{-1}.(nm)^4$] is the overlap integral between the donor emission spectrum and the acceptor absorbance spectrum defined by the equation $J(\lambda) = \frac{\sum_{\Delta\lambda}\left[F_D(\lambda)\varepsilon_A(\lambda)\lambda^4\Delta\lambda\right]}{\sum_{\Delta\lambda}\left[F_D(\lambda)\Delta\lambda\right]}$, where $F_D(\lambda)$ is the donor emission spectrum and $\varepsilon_A(\lambda)$ is the spectrum for acceptor extinction coefficients.

ϕ^D for for each donor-labeled P-arm substrate was calculated as described in (Lakpwicz, 1999) by using the equation $\phi^D = \phi^R \frac{I^D}{I^R}\frac{OD^R}{OD^D}$, where ϕ^R is the fluorescence quantum yield of a fluorescence standard (Fluka, Sigma-Aldrich); I^D and I^R are the fluorescence emission intensities of Bodipy-Fl and the standard, respectively; and OD^D and OD^R are absorbance values of Bodipy-Fl at 488 nm and the standard at 496 nm, respectively. ϕ^R = 0.95± 0.03 at λex = 496 nm, when fluorescein is in 0.1 M NaOH (Brannon and Magde, 1978). The Bodipy-Fl-labeled P-arm substrates (0.8 μM) or the standard (0.4 μM) were dissolved either in buffer A (see *Methods*) or 0.1 M NaOH, respectively. A Beckman DU 520 UV/VIS spectrometer was used to collect the ODs. Fluorescence spectra of the same solutions were recorded on a Fluoromax 2 instrument (Instruments S.A.) by using a slit width of 5 nm. Excitation with Xenon lamp was at 488 or 496 nm for Bodipy-Fl or the standard, respectively. Emission spectra were collected over a wavelength range of 500-600 nm and corrected for buffer signals and lamp fluctuations. The emission maximum for Bodipy-Fl and the fluorescein standard was at 520 nm when excited at 488 and 496 nm, respectively. Fluorescence intensities were determined by integrating the curve ± 20 nm around the emission peak.

The emission spectra $F_D(\lambda)$ of Bodipy-Fl-labeled P-arm substrates for $J(\lambda)$ calculations were recorded as described in the previous paragraph. $\varepsilon_A(\lambda)$ for acceptor (tetramethylrhodamine, TAMRA) labeled P-arm substrates was obtained from absorbance spectra of 0.50 μM substrate solutions (10 mM Tris-HCl pH 7.5, 50 nM NaCl, 1 mM EDTA) collected in the wavelength range of 190-800 nm on a HP8452A UV/VIS spectrophotometer. Absorbance spectra $A(\lambda)$ were then converted to $\varepsilon_A(\lambda)$

spectra by using the "50 μg/ml rule" (i.e., $\varepsilon_A(\lambda) = \frac{A(\lambda)}{A(260nm)}\varepsilon_{DNA}(260nm)$, for λ = 500 to 600 nm and $\varepsilon_{DNA}(260nm) = \frac{50 * 10^{-3} g/l}{Mr(DNA) * 1cm}$, where *Mr(DNA)* is the molecular weight of the DNA substrate in g/mol. *J(λ)* calculations and spectral data manipulations were done by using Microsoft Excel spreadsheets.

Anisotropy Measurements

Steady-state anisotropies (*r*) were measured for all Bodipy-Fl-labeled or acceptor (TAMRA)-labeled P-arms both unbound and in the context of P-arm-six protein complex. Samples contained the labeled substrates (50 nM) in binding buffer (see above) with or without six proteins. *r* was calculated from $r = \frac{I_{VV} - GI_{VH}}{I_{vv} + 2GI_{vH}}$. *I* is the fluorescence intensity of the sample. The first and second letters in the subscript describe the positions of the excitation and emission polarizers, respectively (*V*, vertical; *H*, horizontal). *G* is the correction factor for differences in sensitivities of the detection system for vertically and horizontally polarized light and is equal to I_{HV}/I_{HH} (Lakowicz, 1999). Fluorescence intensities were measured on a Fluoromax 2 fluoimeter with autopolarizers (Instrument S.A.) using excitation wavelengths of 488 and 560 nm for Bodipy-Fl and TAMRA, respectively. Emission was recorded over 500-540 nm and 570-610 nm ranges for Bodipy-Fl and TAMRA, respectively. *r* values ranged 0.16-0.20 for unbound Bodipy-Fl P-arms. Proteins addition increased *r* only slightly, in the range of 0.20-0.26. *r* values of acceptor-labeled P-arms were 0.18-0.24 and 0.22-0.25 with and without proteins, respectively. While the measured anisotropies suggest somewhat constrained rotational

motion of the dyes, we assumed they were low enough to justify the use of 2/3 for κ^2, as this value has been widely accepted for fluorophores with our anisotropies ranges (Lorenz et al., 1999; Mekler et al., 2002; Rasnik et al., 2004) for review see(Lakowicz, 1999).

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Table S1. Oligonucleotide Sequences

oligonucleotide name	sequence (5' to 3') (top strand)	Length (bp)
Parm	ATACATAGTGACTGCATATGTTGTGTTTTACAATATTATGTAGTCTG TTTTTTATGCAAAATATAATTTAATATATTGATATTTATATAATTTTACATA	99
X1/X1.5/X2[#]	GGTGCA***TATGTTGTGTTTT***ACAGTAT***TATGTAGTCTGTTTT***TTATGG	47
X1/X1.5/~~X2~~	ACTGCATATGTTGTGTTTTACAGTATCGCCGCACGCACCCCTTATGC	47
~~X1~~/X1.5/X2	ACTGCACGCAGCACTGGGGACAGTATTATGTAGTCTGTTTTTTATGC	47
X1.5	GGTTTTACAGTATTATGTGG	19
X2F	ACAGTATTATGTAGTCTGTTTTTTATGCAAAATCTAATT	39
P2X1	TAGGATACATAGTGACTGCATATGTTGTGTTTTCGAATAG	40

[#] X1 and X2 are underlined.

Table S2. R_0 Measurements

Donor	Φ^{D} [*]	Acceptor	$J(\lambda)$ [#] ($M^{-1}\cdot cm\text{-}1.nm^4$)/$10^{15}$	R_0 [%] (Å)
fP2	0.81	fX1	2.20	54.5
fP2	0.81	fX1.5	2.41	55.9
fP2	0.81	fH2A	2.97	57.9
fP2	0.81	fH2B	2.79	57.3
fP2	0.81	fH2C	3.08	58.3
fP2	0.81	fH2D	2.65	56.8
fX1	0.77	fX1.5	2.49	55.6
fX1	0.77	fH2A	3.48	58.9
fX1	0.77	fH2B	2.54	55.9
fX1	0.77	fH2C	2.34	55.2
fX1	0.77	fH2D	2.74	56.7
fF	0.82	fX1	2.20	55.2
fF	0.82	fX1.5	1.69	52.8
fF	0.82	fH2A	1.95	54.1
fF	0.82	fH2B	1.63	52.5
fF	0.82	fH2C	2.40	56.0
fF	0.82	fH2D	2.66	57.0
fH2A	0.86	fX1.5	2.05	55.0
fH2A	0.86	fH2B	2.28	56.0
fH2A	0.86	fH2C	1.68	53.2
fH2A	0.86	fH2D	1.82	53.9
fH2B	0.74	fX1.5	1.82	52.5
fH2B	0.74	fH2C	2.62	55.8
fH2B	0.74	fH2D	2.28	54.6
fH2C	0.70	fX1.5	1.76	51.8
fH2C	0.70	fH2D	2.13	53.5
fH2D	0.88	fX1.5	2.28	56.2

[*] Φ^{D} is the fluorescence quantum yield of the donor.

[#] $J(\lambda)=\frac{\sum_{\Delta\lambda}\left[F_D(\lambda)\varepsilon_A(\lambda)\lambda^4\Delta\lambda\right]}{\sum_{\Delta\lambda}\left[F_D(\lambda)\Delta\lambda\right]}$ Where $F_D(\lambda)$ is the fluorescence intensity of the donor and $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor at wavelength λ.

[%] $R_0 = 0.211\sqrt[6]{\kappa^2 n^{-4}\phi^D J(\lambda)}$, R_0 is the Förster distance between donor and acceptor. κ^2 (transition dipoles orientation factor) =2/3 and n (refractive index of the medium) = 1.4.