Supporting Information

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SI Text

SI Methods. ***Protein purification and labeling.*** The preparation, purification and labeling of the SR1C and SR1WC mutants were done as described (1). *Escherichia coli* BL21 cells bearing a plasmid containing the gene for SR1 modified with a His_5-tag at the C terminus and the mutation K327C or the mutations F44W/K327C were grown overnight on a Luria broth agar plate containing 100 μg/ml ampicillin. Cells were washed off the plate with 10 ml of 2 × TY medium and added to 500 ml of 2 × TY medium containing 100 μg/ml ampicillin. The cells were grown at 37 °C until an O.D. of 0.5 at 600 nm was reached. Protein expression was then induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were then grown for another 6 hours and centrifuged at 5000 × *g* for 10 min. The pellet was resuspended in 50 mM Tris·HCl buffer (pH 7.5) containing 10% (w/v) sucrose and then centrifuged as before. The pellet was stored at −80 °C until further use. The pellet was then resuspended in 50 mM Tris·HCl buffer (pH 7.5) containing 0.5 M NaCl, 10 mM β-mercaptoethanol (buffer A) and 10 mM imidazole. The cells were disrupted by sonication in the presence of 1 mM phenylmethanesulphonylfluoride. The clear lysate obtained after centrifugation at 30,000 g for 30 min was loaded on a 1 ml HisTrap HP column (Amersham Pharmacia, Uppsala, Sweden) and eluted with a 60 ml gradient of 10–500 mM imidazole in buffer A. Fractions containing SR1 were combined and concentrated using a Vivaspin device (Sartorius, Goettingen, Germany) with a 100 kDa cut-off. The concentrated protein was transferred into a Tris·HCl buffer solution (pH 7.5) containing 10 mM KCl and 10 mM $MgCl_2$ (G10K buffer), using a PD-10 desalting column (Amersham Pharmacia, Uppsala, Sweden). In order to remove residual bound polypeptide contaminants, the protein was incubated in 20% (v/v) dimethylsulfoxide for 1 hr at room temperature followed by centrifugation at 21,000 g for 30 min and exchange of the supernatant into G10K buffer as above. Gel electrophoresis confirmed that the amount of free monomers in the SR1 solution was lower than 10%. In addition, sedimentation equilibrium analysis was used to determine that the fraction of rings with bound monomer is less than 10%.

Protein modification with Atto 655 maleimide (Atto-tec, Siegen, Germany) was carried out at room temperature in the dark. The protein oligomer concentration during labeling was 80 μM (assessed by absorbance at 280 nm and verified by gel densitometry). Labeling was carried out in G10K buffer containing ∼20% (v/v) dimethylsulfoxide and 65 μM of the fluorescent dye chosen to achieve a labeling stoichiometry of ∼0.8 fluorophores per ring. The labeling reaction was quenched after 2 hours by addition of β-mercaptoethanol to a final concentration of 650 μM. A small amount of protein that precipitated was removed by centrifugation at 21,000 g for 30 min. The unreacted fluorophore was removed by exchange into G10K buffer using a PD-10 column followed by repeated concentration and dilution using the Vivaspin device. The labeled protein was divided into aliquots, snap-frozen and stored at −80 °C. The final labeling ratio was ∼0.7 fluorophore per ring, indicating a very efficient labeling reaction.

Sample preparation. SR1 samples for FCS measurements were prepared (except for samples with GroES) by addition of 2.5 μL of 100 nM SR1 into 250 μL of ATP at the desired concentration in G10K buffer. Samples with GroES were prepared by addition of 5 μL of 10 μM GroES to 2.5 μM SR1 and 10 μL of 500 μM ATP, and incubation for 30 sec followed by addition of 500 μM ATP to a final volume of 250 μL. Under these conditions, more than 99% of SR1 molecules are expected to form a stable complex with GroES.

Flow cells were produced by forming a gap between two hydrogen fluoride (HF)-treated cover slips. HF treatment of the cover slips was conducted as previously described (1). The gap was formed by placing two strips of parafilm on the bottom coverslip and layering the top coverslip over them. After forming the gap, the flow-cell components were glued together by heating this construct for 10 min at 110 °C. The glass surface was passivated before loading the sample by incubating the flow cell for ∼30 min with 200 μL of 2 mg/ml BSA (A-7030, Sigma) in G10K buffer. Prior to loading the sample, the cell was washed with 400 μL of G10K. Finally, 250 μL of 1 nM SR1 solution at the desired ATP concentration (with or without 200 nM GroES) were loaded into the flow cell.

Fluorescence correlation spectroscopy (FCS). FCS measurements were carried out using a homemade confocal setup. Excitation light emitted from a 641 nm diode laser (LDH-D-C-640 PicoQuant, Berlin, Germany) was passed through a single mode fiber and through a 2x telescope. A dichroic mirror (Z633 RDC, Chroma, Rockingham, VT, USA) directed the excitation beam into an infinity-corrected water immersion objective (Plan-Apo 60x/1.20 w Olympus, Japan). The objective focused the light into a flow cell loaded with the sample. The emission was collected by the same objective and transmitted as a collimated beam through the dichroic mirror and a set of emission filters (HQ685/70, Chroma and 57889, Oriel, Stradford, CT). After the emission filters, the fluorescent signal was passed through a spatial filter consisting of a 200 mm achromatic lens, a 50 μm pinhole (Thorlabs, Newton, NJ) and a 50 mm achromatic lens. A nonpolarizing beam splitter (CBS0403, Casix, Fuzhou, Fujian, China) split the light exiting the spatial filter into two channels. The light in each channel was focused on an avalanche photodiode (SPCM-AQR-15, PerkinElmer Optoelectronics, Fremont, CA) by a 50 mm achromatic lens. Cross-correlation functions between the signals arriving from the avalanche photodiodes were calculated by a hardware digital correlator (Flex02-12D/C, Correlator.com, Bridgewater, NJ).

All FCS measurements were conducted at 25 °C. At least 30 curves of 60 sec were collected and averaged for each ATP concentration and this procedure was repeated four times, thus yielding four independent datasets for each experimental condition. Errors were estimated by comparing results from separate analyses of the four datasets. A small number of curves in each dataset was found to be aberrant and filtered out before averaging.

SI Discussion. ***Relation between SVD weights and population fractions.*** Consider a solution of two uncorrelated species, with N_1 and N_2 molecules in the sampling volume, and quantum efficiencies Q_1 and Q_2, respectively. The correlation function of the solution can be written in terms of the correlation functions of the two pure species in the following way:

$$G(t) = \frac{Q_1^2 N_1^2}{(Q_1 N_1 + Q_2 N_2)^2} G_1(t) + \frac{Q_2^2 N_2^2}{(Q_1 N_1 + Q_2 N_2)^2} G_2(t). \quad \textbf{[S1]}$$

$G_1(t)$ and $G_2(t)$ are proportional to $1/N_1$ and $1/N_2$, respectively. We define new correlation functions for the two pure species as: $g_i(t) = N_i G_i(t)$. The new functions can be readily calculated after fitting the original functions to a standard functional form (2),

$G_i(t) = \frac{1}{N_i}[1 + K(t)]D(t)$, in which $D(t)$ is the term due to diffusion and $K(t)$ is the term due to all the kinetic processes that modulate the fluorescence, which is written as a sum of exponential functions (see Eq. **S3** below). The N_is are obtained as fit parameters. We normalize all other correlation functions so that their long-time parts, which are only due to diffusion, match those of the two pure correlation functions. This normalization effectively leads to a new set of correlation functions, which have the following form:

$$g(t) = \frac{Q_1^2 N_1}{Q_1^2 N_1 + Q_2^2 N_2} g_1(t) + \frac{Q_2^2 N_2}{Q_1^2 N_1 + Q_2^2 N_2} g_2(t). \qquad \textbf{[S2]}$$

The SVD analysis provides the weights of the functions $g_i(t)$. Population fractions $f_i = \frac{N_i}{N_1 + N_2}$ can be readily calculated from the above weights if the ratio of quantum efficiencies, Q_1/Q_2, is known.

Nonlinear fit of FCS curves. A global nonlinear least squares analysis was conducted on the FCS curve of SR1WC in the absence of ATP and the FCS curve of SR1WC in the presence of 500 μM ATP and 250 nM GroES. The following functional form was used for the fit:

$$g(t) = \left(1 + \sum_i A_i e^{-t/\tau_i}\right) D(t) \qquad \textbf{[S3]}$$

In this equation, $D(t)$ is the term due to diffusion as above. The A_is are the amplitudes of exponential processes responsible for correlation loss, which can be related to equilibrium constants for these processes (2), while the τ_i's are their lifetimes. In the global nonlinear fit, all the parameters in $D(t)$ as well as the τ_i's are shared between the two curves. The parameters obtained from the fit are given in the Table S1 below and the fitted functions are shown in Figure 2*B* of the main text. In the absence of ATP, four exponential functions are required to fit the short-time part of the FCS curve. On the other hand, FCS curves of SR1WC taken in the presence of saturating ATP concentrations and GroES require only two of these exponentials to fit their short-time parts.

Kinetic model. Consider the following simple enzymatic reaction:

$$E + S \leftrightarrow ES \overset{k_{cat}}{\rightarrow} EP \overset{k_r}{\rightarrow} E + P$$

where E, S and P stand for the enzyme, substrate, and product, respectively, and k_{cat} and k_r stand for the respective catalytic and product release rate constants. The fraction of substrate-bound sites is, therefore, given by:

$$\bar{Y} = \frac{[ES]}{[E] + [ES] + [EP]} \qquad \textbf{[S5]}$$

where the concentration of the enzyme-product complex is not neglected as is common practice. Assuming that the concentrations of $[EP]$ and $[ES]$ are in steady state, we can rewrite Eq. **S5**, as follows:

$$\bar{Y} = \frac{[S]/K}{1 + [S]/K(1 + k_{cat}/k_r)} \qquad \textbf{[S6]}$$

where K is the Michaelis constant. Inspection of Eq. **S6** shows that the saturation function reaches $1/(1 + k_{cat}/k_r)$ instead of 1 at saturating substrate concentrations. A value close to 1 is reached at saturating substrate concentrations when the rate of product release is much faster than the rate of catalysis.

The above type of analysis can also be carried out for an allosteric enzyme with N catalytic sites, each of which is in equilibrium between low (T) and high (R) affinity states for a substrate. The fraction of molecules in the R state, f_R, is given by:

$$f_R = \frac{\sum_{i=0}^{N} [RS_i]}{\sum_{i=0}^{N} [TS_i] + \sum_{i=0}^{N} [RS_i] + \sum_{i=1}^{N} [R''P_i]} \qquad \textbf{[S7]}$$

where the sums run over all N sites. It is assumed that both the T and R states bind the substrate but only the R state can hydrolyze it and that the product-bound enzyme is in a state (R″) that is different from R state with respect to our observable. Eq. **7** can be rewritten, as follows:

$$\begin{aligned} f_R &= \{[R](1 + K_R[S])^N\} / \{[T](1 + K_T[S])^N + [R](1 + K_R[S])^N \\ &\quad + [R](k_{cat}/k_r)K_R[S](1 + K_R[S])^{N-1}\} \\ &= \{(1 + K_R[S])^N\} / \{L(1 + K_T[S])^N + (1 + K_R[S])^N \\ &\quad + (k_{cat}/k_r)K_R[S](1 + K_R[S])^{N-1}\} \end{aligned} \qquad \textbf{[S8]}$$

where L is the T to R equilibrium constant in the absence of ATP, K_T and K_R are the binding constants of ATP to the T and R states, respectively, and k_{cat} and k_r are now the rates of formation of ADP from ATP and of its dissociation from GroEL, respectively. Inspection of Eq. **8** shows that $f_R \approx 1/(L(K_T/K_R)^7 + 1 + k_{cat}/k_r)$ at high substrate concentrations, which is the result used in the main text.

In the case of a chemical reaction that involves the release of two products (such as ADP and P_i) upon ATP hydrolysis, the expression for f_R at high substrate concentrations is given by: $f_R \approx 1/(L(K_T/K_R)^7 + 1 + k_{cat}/k_{r1} + k_{cat}/k_{r2})$, where k_{r1} and k_{r2} are the rate constants of release of the two products. Hence, f_R is more likely to deviate from 1 at high substrate concentrations if there are multiple intermediates that are not in the R state.

1. Frank, G.A. et al. (2008) Design of an optical switch for studying conformational dynamics in individual molecules of GroEL. *Bioconjugate Chem* 19:1339–41.

2. Krichevsky, O. & Bonnet, G. (2002) Fluorescence correlation spectroscopy: The technique and its applications. *Rep Prog Phys* 65:251–297.

Table S1. Parameters from nonlinear fit of FCS curves

	1		2		3		4	
	A	τ (μs)	A	τ (μs)	A	τ (μs)	A	τ (μs)
SR1WC	0.14 ± 0.01	130±20	0.27 ± 0.02	13 ± 2	0.29 ± 0.03	2.4 ± 0.6	1.0 ± 0.1	0.34 ± 0.01
SR1WC +500 μM ATP +250 nM GroES	——	——	0.059 ± 0.005	13 ± 2	——	——	0.43 ± 0.02	0.34 ± 0.01