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Stoichiometry of the α-Complementation Reaction of *Escherichia coli* β-Galactosidase As Revealed through Single-Molecule Studies

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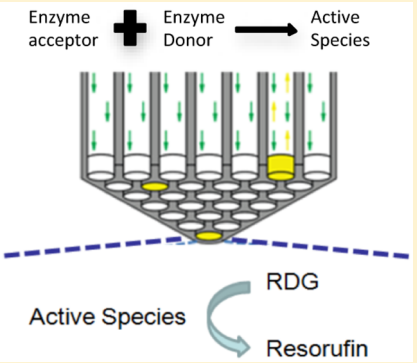
Supporting Information

ABSTRACT: The α-complementation reaction of β-galactosidase was studied at single-molecule resolution using arrays of femtoliter-sized wells. Single molecules of the complementation species were observed to be stable for long periods of time, demonstrating that the α-complementation reaction is irreversible. By directly counting the number of active molecules formed in the complementation reaction when different concentrations of enzyme acceptor (EA) and enzyme donor (ED) are used, we deduce that the EA:ED ratio in the complementation species is 4:1.



The α-complementation of β-galactosidase is a well-known reaction in which two parts of the enzyme, an inactive N-terminal deletion mutant of the enzyme [enzyme acceptor (EA)] and the inactive deleted peptide [enzyme donor (ED)], associate to generate an active enzymatic species.[1−3] This construct is commonly utilized both in the blue-white screening technique for bacteria[4] and in CEDIA immunoassays.[5] This reaction is also utilized in the detection of *in vitro* and *in vivo* protein−protein interactions[6] and in high-throughput screening assays,[7,8] among other applications.[9,10] In this paper, we examine the α-complementation reaction of β-galactosidase at the single-molecule level to characterize the active species that are generated in the complementation reaction and compare their activity to that of the native β-galactosidase enzyme.

Native β-galactosidase from *Escherichia coli* is a hydrolase that exists primarily as a tetramer.[11] The protein sequence corresponding to the α-donor forms part of the activating interface between the monomers of β-galactosidase[11] and has been shown to be necessary for both the activity and the oligomerization of β-galactosidase.[12,13] Reports are contradictory with regard to whether and to what extent oligomerization is necessary for β-galactosidase to display activity. Some studies suggest that β-galactosidase is active only as a tetramer, proposing that the α-acceptor exists primarily as dimers and is consequently inactive, and the deleted fragment therefore restores activity by forming β-galactosidase tetramers.[12,14] In other studies, active dimers of β-galactosidase have been identified and have been shown to be part of the complementation reaction, suggesting that the α-donor can replenish the activity without tetramerization.[15] The reports on the stoichiometry of the complementation reaction are also contradictory. While studies based on chromatography have determined an α-donor:monomer ratio of 1:1,[16] fluorescence fluctuation analysis of the complementation reaction using a fluorescently tagged α-donor has indicated the presence of only one donor per active species.[17]

In this work, we apply the high-density optical fiber bundle platform described previously[18,19] to isolate and monitor the activity of individual active species formed in the α-complementation reaction of β-galactosidase. Making use of the unique capability of our optical fiber system to observe multiple reactions with single-molecule resolution, we were able to directly count the number of active species generated in the complementation reaction and deduce the EA:ED ratio in the complementation species. In addition, we were able to directly compare the activity of the complementation species to that of native tetrameric β-galactosidase

MATERIALS AND METHODS

Materials. The enzyme fragment complementation (EFC) detection assay kit containing the acceptor (EA) and donors Prolabel (ED) were obtained from DiscoveRx Corp. β-Galactosidase from *E. coli* (grade VIII) was purchased from Sigma-Aldrich and purified on a Zorbax-450 HPLC column. All protein dilutions were made in the reaction buffer of 1× phosphate-buffered saline [PBS (0.14 M NaCl, 2.7 mM KCl, 8 mM NaH_2PO_4, and 2 mM K_2HPO_4, autoclaved and filtered)] and 1 mM $MgCl_2$ (pH 7.4). 10× PBS was purchased from Ambion. Resorufin β-D-galactopyranoside (RDG) and resorufin standard sodium salt were purchased from Invitrogen and stored at a stock concentration of 100 mM in dimethyl sulfoxide at −20 °C.

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Bulk Experiments. The complementation reaction was tested in bulk format before single-molecule experiments were performed to verify the activity. The bulk experiments are described in the Supporting Information (SI 1).

Single-Molecule Experiments. Single-molecule studies were performed using the high-density array of femtoliter-sized wells generated on the optical fiber bundle platform.[18−20] The fabrication of the femtoliter-sized well array is described elsewhere.[18] Isolation of single molecules of the active species was achieved by sealing picomolar concentrations of the active enzyme species in the presence of 100 μM RDG in the 46 fL optical fiber wells (Figure 1). Using Poisson distribution

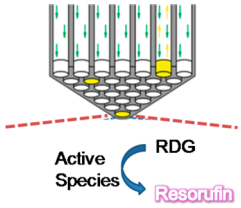

Figure 1. Isolation of single molecules of active species. The acceptor and donor molecules were preincubated for at least 30 min to allow for complementation. The incubated mixture was then diluted to an active species concentration of 3.6 pM (assuming the reaction went to completion and EA and ED associate in a 1:1 ratio) and was sealed into 46 fL optical fiber wells.

statistics $P_\mu(x) = e^{-\mu}\mu^x/x!$, where $P_\mu(x)$ gives the probability of finding x molecules per well and μ is the mean number of molecules per well, it can be determined that for a concentration of 3.6 pM, there is almost zero probability of finding more than one active species per well. Hence, every well contains either one or zero active molecules. The turnover of resorufin β-D-galactopyranoside to resorufin is used to detect the presence of the active species, as shown in Figure 1.

To isolate single molecules of the product of the complementation reaction, we must ensure that the final concentration of the product is ≤3.6 pM. First, the active species of the complementation reaction were generated by incubating 10 μL each of 200 μM EA and ED for 30 min. The maximal concentration of active species that can be formed is 100 μM (in this case, a 1:1 EA:ED ratio). Assuming that 100 μM active species were formed, serial dilutions were performed in the reaction buffer such that the final concentration of the active species was 100 pM; 3.6 μL of this solution was then diluted in 96.4 μL of a 100 μM substrate solution to give a final active species concentration of 3.6 pM. If the association between EA and ED did not go to completion or the association is not 1:1, then the final concentration of each of the molecules would be <3.6 pM. This diluted solution of complementation species (concentration of ≤3.6 pM) was used in the single-molecule experiments described herein.

Imaging. For the single-molecule experiments on the optical fiber, the optical fiber was fixed on the stage of an inverted microscope such that the nonetched end of the bundle was facing the objective. A 30 μL droplet of the substrate solution containing the diluted complementation species (concentration of ≤3.6 pM) was placed on a well-cleaned PDMS gasket on a glass slide base that was positioned on an automated z-direction stage directly beneath the fiber bundle (Figure 2). The z-direction stage was then used to mechanically seal the reaction solution within the optical fiber wells. Time-lapse imaging of the nonetched end of the optical fiber bundle was subsequently performed with a custom-built imager (Quanterix, Lexington, MA) equipped with a 200 W metal arc lamp (Prior Scientific, Rockland, MA) and a CCD camera (Infinity4-11, Lumenera, Ottawa, ON). A filter set with a λ_{ex} of 558 nm and a λ_{em} of 577 nm (Semrock, Rochester, NY) was used for imaging the resorufin product.

Data Analysis. Images were analyzed using the MATLAB image processing toolbox. An algorithm was developed to identify the wells and extract the mean intensity of each well. The percentage increase in intensity between the first and last frames (30 min) was calculated for each well, and a threshold of 20% was used to identify active wells. To convert the fluorescence intensities obtained from imaging to the number of resorufin molecules, a calibration curve was first obtained by measuring the fluorescence intensity generated by sealing different concentrations of a resorufin standard solution in the

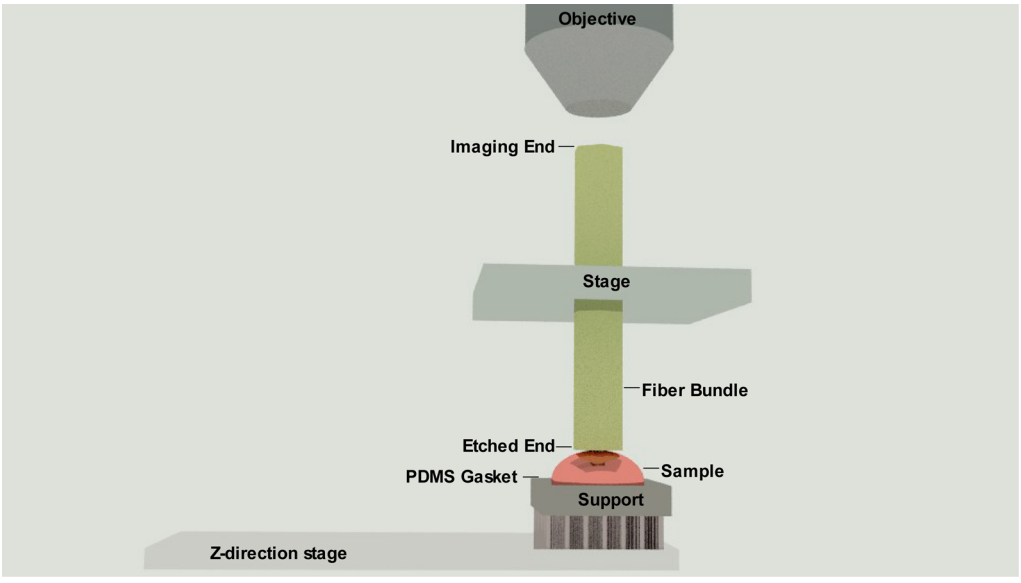

Figure 2. Schematic showing the imaging setup for the single-molecule experiments.

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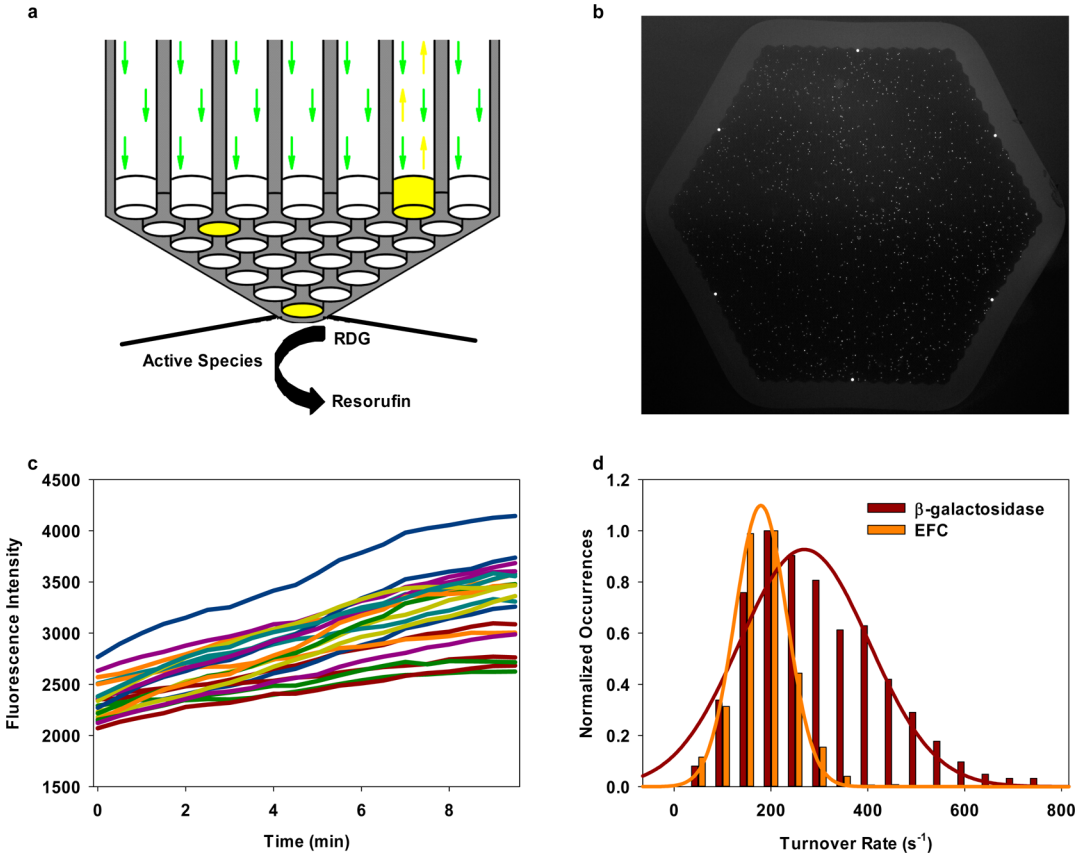

Figure 3. (a) Schematic for isolation of single molecules of the active complementation species in optical fiber wells. After single molecules of active species had been sealed in the presence of excess substrate within the optical fiber wells, time-lapse imaging was performed for 30 min from the other end of the fiber. (b) Snapshot of the optical fiber bundle consisting of 50000 fibers at the end of the time-lapse imaging. The wells that contain the active species light up because of the turnover of the fluorogenic substrate into the product resorufin. (c) Fluorescence time traces of some of the active wells in panel b. The imaging was performed with 30 s between each captured image. (d) Turnover rate histograms of the active species generated from the complementation reaction and a typical turnover rate histogram of single molecules of β-galactosidase. The histograms are fit to Gaussian distributions $y = a \times \exp\{-[(x - b)^2/(2c^2)]\}$ (a = 0.9, b = 269, and c = 134 for β-galactosidase; a = 1.1, b = 179, and c = 53.7 for EFC). To obtain the turnover rates, the fluorescence intensities in panel c were first converted into numbers of resorufin molecules using a calibration factor of 120 molecules of resorufin/unit of fluorescence intensity. The slope at each point of the trajectory gives the instantaneous turnover rate, which was then corrected for photobleaching and averaged over the reaction time to obtain the average turnover rate.

fiber wells. The fluorescence intensity $[F(t)]$ was then converted into the number of resorufin molecules $[P(t)]$ by multiplying by the calibration factor. The slope at each point of the trajectories was computed to obtain the instantaneous turnover rate $[P'(t)]$. To correct for photobleaching, 10 μM resorufin was sealed in optical fiber wells and time-lapse imaging was performed. The fluorescence trajectories from the photobleaching experiment were fit to a monoexponential decay curve to obtain the photobleaching rate, k_{ph}. The photobleaching-corrected instantaneous turnover rates were computed using the equation $P_{corr}(t) = P'(t) + K_{ph}xP(t)$. The instantaneous turnover rates of a given trajectory were then averaged to obtain the average turnover rate corresponding to that trajectory.

RESULTS AND DISCUSSION

The active species was formed in a bulk solution by incubating EA and ED for 1 h. Single molecules of the active species were then isolated in the optical fiber wells along with excess substrate.

Isolation of Single Molecules of the Active Species. The results for single-molecule complementation experiments are shown in Figure 3. Figure 3b shows a snapshot of the signal generated in the optical fiber wells after 30 min. Figure 3c shows the fluorescence intensity profiles of the active wells during the time course of the reaction. Fluorescence intensity steadily increased because of the activity of single molecules in the fiber wells during the observation time without any abrupt pauses in activity. This result suggests that the active species is stable and does not dissociate during the time course of observation. In the event that the complemented enzyme dissociates, the probability of reassociation of a single donor with four acceptor molecules in an individual microwell during the time of observation is very low (assuming the apparent reaction association rate of ~200 M^{-1} s^{-1}).[17] The formation of the active species is therefore an irreversible reaction, at least during the time course of the experiment. The substrate turnover rates of the active species were calculated from the fluorescence intensity trajectories (see Data Analysis). Histograms of the turnover rates of the active species generated in the complementation reactions were plotted (Figure 3d) along with a typical turnover rate histogram of β-galactosidase. As can be seen in the figure, the activity distribution is much narrower than that of the native tetrameric enzyme and is skewed toward the lower activities. The activity of the native enzyme is clearly not recovered completely in the complementation reaction.

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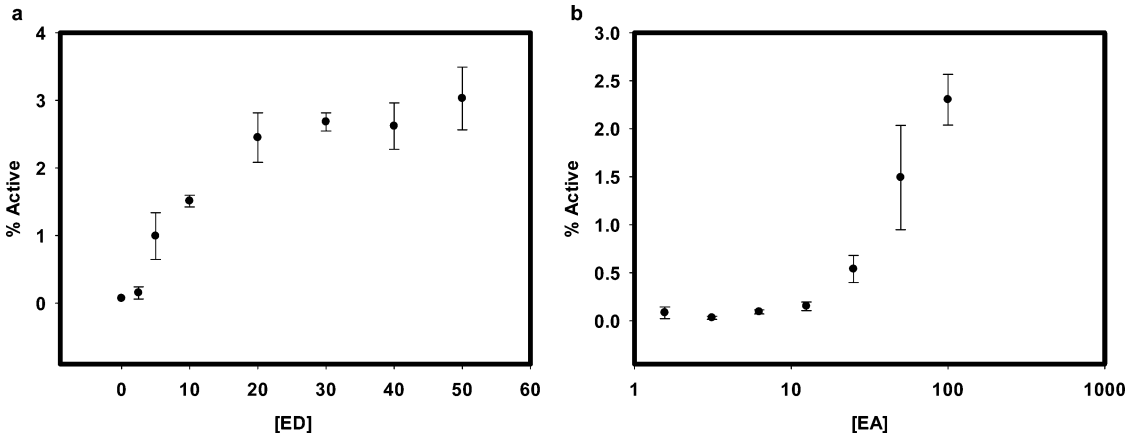

Figure 4. (a) Varying concentrations of ED were incubated with 100 μM EA for 30 min followed by a 10000-fold dilution. The diluted solution was then mixed with substrate in a ratio of 3.6:96.4, sealed in the optical fiber bundle wells, and imaged. The number of active wells observed is plotted as a percentage of total wells (% Active). (b) The experiment was repeated for varying concentrations of EA. The % Active wells observed when the concentration of EA is varied while the concentration of ED is held constant at 100 μM are plotted.

Table 1. Expected Percent Active Wells for the Experiment in Figure 4a for Different EA:ED Ratios[a]

	50 μM ED	40 μM ED	30 μM ED	20 μM ED	10 μM ED	5 μM ED	2.5 μM ED	0 μM ED
experimental	3.02 ± 0.46	2.62 ± 0.34	2.68 ± 0.13	2.45 ± 0.37	1.51 ± 0.09	0.99 ± 0.35	0.15 ± 0.09	0.07
for 1:1 EA:ED	5	4	3	2	1	0.5	0.25	0
for 1:2 EA:ED	2.5	2	1.5	1	0.5	0.25	0.125	0
for 2:1 EA:ED	5	4	3	2	1	0.5	0.25	0
for 2:2 EA:ED	2.5	2	1.5	1	0.5	0.25	0.125	0
for 4:2 EA:ED	2.5	2	1.5	1	0.5	0.25	0.125	0
for 4:1 EA:ED	2.5	2.5	2.5	2	1	0.5	0.25	0

[a]The 4:1 ratio best matches the experimentally determined values.

Table 2. Expected Percent Active Wells for the Experiment in Figure 4b for Different EA:ED Ratios[a]

	100 μM EA	50 μM EA	25 μM EA	12.5 μM EA	6.25 μM EA	3.125 μM EA	1.5625 μM EA	0 μM EA
experimental	2.30 ± 0.26	1.49 ± 0.54	0.54 ± 0.14	0.15 ± 0.04	0.09 ± 0.02	0.03 ± 0.02	0.08 ± 0.06	0.035
for 1:1 EA:ED	10	5	2.5	1.25	0.6	0.31	0.15	0
for 1:2 EA:ED	5	5	2.5	1.25	0.6	0.31	0.15	0
for 2:1 EA:ED	5	2.5	1.25	0.625	0.3	0.15	0.07	0
for 2:2 EA:ED	5	2.5	1.25	0.625	0.3	0.15	0.07	0
for 4:4 EA:ED	2.5	1.25	0.625	0.3	0.15	0.078	0.037	0
for 4:2 EA:ED	2.5	1.25	0.625	0.3	0.15	0.078	0.037	0
for 4:1 EA:ED	2.5	1.25	0.625	0.3	0.15	0.078	0.037	0

[a]The 4:4, 4:2, and 4:1 ratios best match the experimentally determined values. The EA:ED ratio of 4:1 therefore matches the experimental data from both panels a and b of Figure 4.

The average activity of the β-galactosidase computed from the histogram in Figure 3d is 268 ± 9 s^{-1}, which is about 1.5 times the average activity of EFC active species (172 ± 6 s^{-1}). The time constant of the acceptor and donor association was determined to be 10.6 ± 2.8 min using the single-molecule platform (SI 2 of the Supporting Information) and matches up well with the time constant reported in the literature[17] (8.8 ± 1.7 min).

To obtain the kinetics of the irreversible formation of the active species, the single-molecule experiments were performed with varying concentrations of the donor, keeping the acceptor concentration constant at 100 μM (Figure 4a) and vice versa (Figure 4b). For each experiment, first a 10000-fold dilution of the complementation mixture was made in the reaction buffer followed by a 3.6:96.4 dilution into a substrate solution. The number of active species generated was counted by counting the number of active wells (data analysis) and plotting them as a percentage of the total wells in the fiber bundle. The activity distribution was plotted whenever single-molecule activity was observed and was always identical to that in Figure 3. The average activity of the single molecule species generated in all these experiments is 200 s^{-1}, showing very little variability.

The data plotted in Figure 4 are also summarized in Tables 1 and 2 (experimental). Table 1 shows the percentage of active wells expected assuming different EA:ED ratios in the complementation species. For example at 100 μM EA and 50 μM ED, at a 1:1 EA:ED ratio, the expected concentration of active species is 50 μM; 10000-fold dilution results in an active species concentration of 50 pM, followed by 3.6:96.4 dilution into substrate, resulting in a final concentration of 1.8 pM. At this concentration, 5% of the wells are expected to show activity. Similar calculations were made for the rest of the concentrations at different EA:ED ratios. From Tables 1 and 2, it can be deduced that the EA:ED ratio of 4:1 best matches the

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experimental data from both experiments shown in Figure 4. To be more accurate, the number of ED molecules per complementation species was determined for each experiment by dividing the expected percent active in the case of one ED molecule with the experimentally determined percent active. A weighted average of the number of ED molecules calculated in this manner (the square inverses of standard deviations were used as weights) results in 0.8 ± 0.2 molecule per complex. Similar analysis for Table 2 results in 4.4 ± 0.6 molecules of EA per complex. Therefore, the EA:ED ratio is 4.4 ± 0.6:0.8 ± 0.2. The ratio of 4:1 best matches the experimental results as well as the structure of β-galactosidase known from the literature.

Therefore, we conclude the complementation reaction results in a pentamer consisting of four acceptors and one donor molecule. This result confirms the EA:ED ratio determined using fluorescence fluctuation analysis.[17]

CONCLUSIONS

The α-complementation reaction of β-galactosidase results in stable, irreversible active species that can be observed for long periods of time at the single-molecule level. The complementation species is a tetramer consisting of four acceptors and one donor. The acceptor dimers are themselves inactive because a loop from a different dimer is required to complete the active site. The α-donor mediates dimer−dimer binding, which allows the active site to form on the adjoining dimer. The crystal structure of *E. coli* β-galactosidase predicts four donors per tetramer such that each donor is anchored to one monomer.[12,21] The donors promote dimer−dimer binding by stabilizing a four-helix bundle that forms part of the dimer−dimer binding interface, also called the activation interface.[12] In addition, contact between two donor molecules directly contributes to the activation interface.[21] From our results, we can deduce that a single donor is sufficient to recover the activation interface. Therefore, the direct contribution of the donors to the contacts that make up the activation interface is not critical.[12] The donor potentially stabilizes the dimer conformation required for dimer−dimer binding. Consequently, this new conformation readily associates with other dimers to form a stable tetramer. Activity histograms show that only 66% of the activity of native β-galactosidase is restored in the complementation species. The lack of complete recovery of activity could be an effect of reconstitution of the tetramer with only one dimer. Direct comparison of the activities of wild-type β-galactosidase and the complementation species has only been studied at the bulk level and suggests that the activities are similar.[12,16] This result contrasts with the results from the experiments reported in this paper, thus demonstrating how single-molecule studies can elucidate mechanistic details that cannot be accessed via bulk studies.

ASSOCIATED CONTENT

Supporting Information

Results of bulk experiments with varying concentrations of EA and ED (SI 1) and single-molecule experiments performed to determine the association time constant of the complementation reaction (SI 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

EFC, enzyme fragment complementation; EA, enzyme acceptor; ED, enzyme donor.

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