The run-on oligomer filament enzyme mechanism of SgrAI: Part 2. Kinetic modeling of the full DNA cleavage pathway

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Edited by Karin Musier-Forsyth

Filament or run-on oligomer formation by enzymes is now recognized as a widespread phenomenon with potentially unique enzyme regulatory properties and biological roles. SgrAI is an allosteric type II restriction endonuclease that forms run-on oligomeric filaments with activated DNA cleavage activity and altered DNA sequence specificity. In this two-part work, we measure individual steps in the run-on oligomer filament formation mechanism to address specific questions of cooperativity, trapping, filament growth mechanisms, and sequestration of activity using fluorophore-labeled DNA, kinetic FRET measurements, and reaction modeling with global data fitting. The final models and rate constants show that the assembly step involving association of SgrAI–DNA complexes into the run-on oligomer filament is relatively slow (3–4 orders of magnitude slower than diffusion limited) and rate-limiting at low to moderate concentrations of SgrAI–DNA. The disassembly step involving dissociation of complexes of SgrAI–DNA from each other in the run-on oligomer filament is the next slowest step but is fast enough to limit the residence time of any one copy of SgrAI or DNA within the dynamic filament. Further, the rate constant for DNA cleavage is found to be 4 orders of magnitude faster in the run-on oligomer filament than in isolated SgrAI–DNA complexes and faster than dissociation of SgrAI–DNA complexes from the run-on oligomer filament, making the reaction efficient in that each association into the filament likely leads to DNA cleavage before filament dissociation.

Enzyme regulation via filament formation has only recently been appreciated as a widespread phenomenon and is implicated in the regulation of metabolism, signaling, and translation (1–7). This mechanism appears to have evolved independently in multiple systems and may provide advantages such as rapid activation, storage of inactive enzymes, and buffering or sequestering of enzyme activity (2, 6, 8). Dysfunction in the control of such pathways is implicated in human diseases including cancer, diabetes, and developmental problems (2). Being a new enzyme mechanism, several important questions regarding the mechanism remain unknown and are addressed in this two-part work using the SgrAI system.

SgrAI is an allosteric, type II restriction endonuclease (RE)2 that in its activated state forms a filamentous structure we call a run-on oligomer (ROO). SgrAI derives from the bacterium Streptomyces griseus and is part of bacterial immunity against bacterial viruses (i.e. bacteriophage). Bacteria and their corresponding phage are among the oldest and most numerous organisms on Earth, evolving and coevolving strategies of attack and defense in what is known as the “phage-host arms race” (9). In this arms race, properties such as speed likely matter, such that SgrAI is able to cleave the invading phage DNA prior to viral transcription or replication. At the same time, damaging dsDNA cleavage of the host genome must be minimized and therefore the potentially destructive activity of SgrAI must be sequestered to the phage DNA.

The unusual mechanism evolved by SgrAI involving ROO filament formation may be a result of the particular biological niche found in S. griseus. SgrAI cleaves two types of recognition sites, primary (CRCCGGYG, 3 unique sequences) and secondary (CRCCGGY(A or C or T) and CRCCGGGG, 14 unique sequences), but will cleave the secondary sites only when on the same DNA as a primary site, or alternatively in cis when concentrations of SgrAI bound to primary site DNA are high (10, 11). Primary sites are protected from DNA cleavage by the cognate SgrAI methyltransferase in the host organism. However, invading phage DNA will not be methylated at the primary site sequences, and hence SgrAI enzymes will bind to those sites, assemble into ROO filaments, and rapidly cleave the DNA. The ROO filament will also incorporate SgrAI bound to secondary sites within the phage DNA, activating SgrAI and resulting in rapid cleavage of those sequences as well. This expansion of specificity to include secondary site sequences may have evolved to increase the number of cleavage sites in invading phage DNA, because the primary recognition site is relatively long (8 bp versus 4–6 bp of many REs). The longer recognition

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2 The abbreviations used are: RE, restriction endonuclease; 18M-1, 18-bp DNA containing the primary SgrAI recognition sequence; ES, SgrAI bound to uncleaved 18M-1; EP, SgrAI bound to self-annealed PC DNA; EP, SgrAI bound to 18M-1 cleaved in the SgrAI pattern; Flo, 6-carboxyfluorescein; Kintek GKE, Kintek Global Kinetic Explorer software; PC or PC DNA, pre-cleaved primary site DNA with 16 flanking bp DNA; ROO, run-on oligomer(s) formed from SgrAI bound to DNA; Rox, rhodamine-X or S(5-carboxy)-rhodamine.

This work was supported by National Science Foundation Grant MCB-1410355, by the Office of the Director of the National Institutes of Health under Grant S10OD013237, and by the NIGMS, National Institutes of Health under Grant T32GM008659 (to J. L. S.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
site may have evolved to protect the host genome, which is also relatively large. More host DNA means more recognition sites, which must be protected by the cognate methyltransferase or be cleaved by SgrAI. This may also explain the unusually slow cleavage rate of SgrAI in the absence of ROO filament formation (rate constant of 0.1 min⁻¹, compared with 20 min⁻¹ of the RE EcoRI (12)). However, the slow DNA-cleavage rate and rare recognition sequence make for very poor anti-phage activity. However, the allosteric activation by binding and ROO filament assembly with multiple unmethylated primary sites increases the DNA-cleavage rate by 200–1000-fold (8, 13). In addition, the expansion of DNA sequence specificity from 3 primary sites to also include the 14 secondary site sequences results in very rapid cleavage of many sites on the phage DNA, making for an effective anti-phage enzyme.

Structural studies of SgrAI in its low activity form show a canonical dimeric RE fold enzyme bound to one recognition site (primary or secondary) in duplex DNA (14, 15). These SgrAI–DNA complexes are then the building blocks for the high activity state of SgrAI, which forms a left-handed helix with approximately four SgrAI–DNA complexes per turn, which we call a run-on oligomer or ROO filament (Fig. 1A) (16). The SgrAI–DNA complexes associate using protein–protein and protein–DNA interactions between neighboring SgrAI–DNA complexes (Fig. 1A), and the DNA (when contiguous sites are bound by SgrAI) is predicted to weave in and out of the filament (17). The ROO filament can also theoretically extend indefinitely from either end, and ROO filaments of 30 or more SgrAI–DNA complexes have been visualized via EM (16). The conformation of SgrAI is altered in the ROO filamentous activated state compared with the unoligomerized low activity state, as expected for an allosteric enzyme (16).

Because SgrAI bound to primary site DNA forms the ROO filament, which also binds and activates SgrAI bound to secondary site DNA, the primary site DNA is an allosteric effector of both the activity and substrate specificity of SgrAI. To explain this allosteric behavior, a model has been proposed wherein SgrAI exists in equilibrium between an active and an inactive conformation (16). The active conformation readily forms the ROO filament, and in turn, the ROO filament stabl-
lizes the active conformation. The active conformation forms more readily (is more energetically stable) when SgrAI is bound to primary site than when bound to secondary site sequences, explaining why SgrAI bound to primary site DNA readily assembles into the ROO filaments, but SgrAI bound to secondary-site DNA alone will not. The activated conformation has rapid DNA cleavage activity; thus DNA is rapidly cleaved by SgrAI in the ROO filament. ROO filaments assemble from SgrAI–DNA complexes, regardless of the state of cleavage of the DNA; hence the product of primary site DNA cleavage by SgrAI also stimulates SgrAI activity by inducing ROO filament formation. The formation of ROO filaments intuitively suggests cooperativity and rapid activation; however, until this study, the details of these effects were not known or quantified. In addition, the ROO filament structure suggests potential trapping of cleaved DNA, yet the rapid turnover in steady state reactions indicates that trapping does not occur (11).

In this two-part work, we measure individual steps in the run-on oligomer mechanism to address specific questions of cooperativity, trapping, filament growth mechanisms, and sequestration of activity using fluorophore-labeled DNA, kinetic FRET measurements, and reaction modeling with global data fitting. The first part of this work (18) developed reaction models for ROO filament formation and determined initial estimates of rate constants for assembly and disassembly of SgrAI–DNA complexes from the ROO filament. In this second of the two parts, we develop models further and globally fit all steps of the reaction pathway including DNA cleavage and product release. The reaction data were collected using four different experimental approaches. Each approach measures a different set of steps in the reaction pathway, and concentrations of activating and reporter DNA were varied in each timed reaction to provide additional information. The resulting data from a total of 22 different timed reactions were then fit globally to different reaction models of increasing complexity and ROO filament growth mechanisms, resulting in estimates of microscopic rate constants for each major step of the reaction pathway.

The resulting globally fit rate constants show several consistent trends across all models and mechanisms. First, the association of SgrAI–DNA complexes into the ROO filament is relatively slow and is 3−4 orders of magnitude slower than diffusion limited. We show that this characteristic is likely what provides the sequestering effect necessary to prevent cleavage of secondary sites in the host DNA upon activation of SgrAI via invading phage. Second, the corresponding dissociation of SgrAI–DNA complexes from the ROO filament is the next slowest step, and this rate constant is the same to the best of our estimation regardless of whether the bound DNA is cleaved or not. However, though it is the second slowest step of the overall reaction, this step is still fast enough to result in the rapid equilibration of individual SgrAI–DNA complexes into and out of the ROO filament. Therefore, trapping of SgrAI enzyme and/or product (cleaved) DNA within the ROO filament does not occur. Third, the DNA-cleavage rate is 4 orders of magnitude faster in the ROO filament than in isolated SgrAI–DNA complexes and is now comparable to those of other REs. Significantly, it is at least three times faster than the dissociation of SgrAI–DNA complexes from the ROO filament; hence cleavage of the DNA is likely upon every association of a SgrAI–DNA complex into the ROO filament, making the reaction mechanism efficient. The last step, product release (dissociation of the cleaved DNA from SgrAI) is fast and does not limit the overall DNA-cleavage reaction. Finally, we use the different reaction models and extracted rate constants to address issues of cooperativity and growth mechanisms of the ROO filament.

**Results**

**Overview of methodology**

Activated DNA cleavage by SgrAI involves several steps: 1) DNA binding by SgrAI, 2) assembly of SgrAI–DNA complexes into the filamentous assembly we call ROO filaments, 3) rapid DNA cleavage by SgrAI while in the ROO filament, 4) separation of the individual SgrAI–DNA complexes from the ROO filament, and then 5) dissociation of the cleaved DNA from SgrAI. In addition, SgrAI will cleave primary site DNA sequences when unoligomerized (i.e. not in the ROO filament), albeit at a slow rate (8). Also, the primary site DNA used to induce ROO filament formation and activate SgrAI (i.e. PC DNA in this work) is a mimic of the cleaved product DNA, which must self-anneal before binding to SgrAI, and this annealing can limit the reaction rates at lower concentrations of PC DNA.

Because the goal of this work is to estimate each individual, microscopic rate constant (forward and reverse) for each step, the reaction data were measured using three different approaches. In addition, the number of rate constants to be fit was reduced by estimating equilibrium dissociation constants ($K_D$), and fixing reverse rate constants to the fitted forward rate constant. This was done for the self-annealing of PC DNA ($K_D$ estimated in Ref. 18) and for binding of DNA to SgrAI. The $K_D$ of SgrAI for the DNA used in these studies was determined as described in the supporting information.

The association of SgrAI–DNA complexes into (and out of) the ROO filament was measured by placing FRET donor and acceptor fluorophores on different DNA molecules, resulting in a FRET signal when SgrAI bound to donor DNA assembles with SgrAI bound to acceptor DNA into the ROO filament (Fig. 2A). A titration was first performed to show that the FRET technique measures ROO filament assembly (described below). Timed reactions were then measured to provide the data to use in global fitting to reaction models and from which to determine rate constants for each step of the reaction (Data Sets 4 and 6 and Table S1). First, to isolate only ROO filament formation (and breakdown), DNA cleavage was blocked by excluding the required Mg$^{2+}$ cofactor from the reaction buffer (reactions of Data Set 4). This approach is similar to that used in the accompanying article (18), namely the approach to equilibrium method, although herein we use also a new DNA construct that is a shorter version of the acceptor labeled reporter DNA (i.e. 18M-1, an 18-bp DNA with one uncleaved primary recognition site). The 18-bp reporter DNA is used instead of the 40-bp reporter DNA of the accompanying article (18) because it will not significantly induce ROO filament formation by SgrAI. The 18-bp construct cannot induce ROO filament formation pre-

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*Run-on oligomerization and enzyme regulation*

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**Supporting information**

1. **Table S1**

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Table 1

Models used in data fitting

| Model                      | Type of data used in fitting | Major steps modeled | Disjoint reactions collected | Major model | Model Type | Strands Cleavage          | Major steps modeled | Major steps modeled | Major steps modeled |
|----------------------------|------------------------------|---------------------|-----------------------------|-------------|------------|---------------------------|---------------------|---------------------|---------------------|
| Full pathway 2-mers        | Independent or simultaneous cleavage of both strands | | | | | | | | |
| Full pathway 3-mers        | Independent or simultaneous cleavage of both strands | | | | | | | | |
| Full pathway 4-mers        | Independent or simultaneous cleavage of both strands | | | | | | | | |
| Full pathway 5-mers        | Independent or simultaneous cleavage of both strands | | | | | | | | |

A. Data Sets 4 and 6

B. Data Sets 5 and 7

Figure 2. Cartoon representation of reactions schemes. A. scheme for reactions of Data Sets 4 and 6 and FRET titrations. The change from green filled boxes to green filled boxes for the SgrAI dimer indicates activation, presumably via a conformational change. Both the FRET titration and the reactions of Data Set 4 stop before DNA cleavage, because of the absence of Mg^{2+}. B. scheme for reactions of Data Sets 5 and 7. Data Set 5 measures FRET as a function of time after mixing, whereas Data Set 7 measures the total amount of cleaved Flo-Rox-labeled DNA using PAGE and densitometry.

summarily because of the shortened flanking base pairs on either side of the 8-bp recognition site, because those flanking base pairs make contact to neighboring SgrAI–DNA complexes in the ROO filament (Fig. 1A) (8, 13, 16). Therefore, the activation of SgrAI can be controlled in reactions by using different concentrations of activator DNA (i.e. PC DNA, which self-associates into a 40-bp precleaved DNA containing a single primary recognition site cleaved in the SgrAI pattern and which was also used in the accompanying article (18)). SgrAI bound to self-annealed PC DNA will induce ROO filament formation that can incorporate (and thereby activate) SgrAI bound to the 18-bp reporter DNA. In total, six different timed reactions, each having different concentrations of DNA (all with excess SgrAI), were measured and comprise Data Set 4 (Table S1).

Reaction mechanism models created to globally fit the reaction data of Data Set 4 are summarized in Table 1 (the “filament assembly” mechanisms). It was not possible to build models allowing for infinitely long ROO filaments, although these models are theoretically possible. Instead, the modeling software limited ROO filaments to only four or five SgrAI–DNA complexes total. However, this simplification is adequate for modeling the data because previous analyses done in the accompanying article (18) showed that most ROO filaments are four or five SgrAI–DNA complexes in size or smaller when the total DNA concentration (corresponding to the total concentration of SgrAI recognition sites) is limited to 250 nm or lower, as is the case in these reactions (Table S1).

Next, the growth mechanism of the ROO filaments was modeled to allow for either “ends-only” or “breaks-in-the-middle” mechanisms. The ends-only mechanism limits ROO filament growth to only either end and via the addition of only single SgrAI–DNA complexes at a time. In contrast, the mechanism...
with breaks in the middle allows ROO filaments to grow via the association of two ROO filaments of any size (or single SgrAI–DNA complexes as well) and to dissociate via “breakage” at anywhere within the ROO filament, between any adjacent SgrAI–DNA complex. The implications and justifications for these mechanisms are discussed below.

To gain more information on steps following ROO filament formation (including DNA cleavage and product release), we also used an approach where the reporter DNA is doubly labeled with FRET donor and acceptor, respectively); however, 6 contains reaction data from the same approach as used in the timed reactions of Data Set 7. Data Set 5–7, with a total of 22 timed reactions) was performed separately to include DNA cleavage, as well as subsequent steps. These models are also summarized in Table 1 (i.e. “full pathway” models) and were tested for their ability to fit the experimental timed reaction data. Despite the differing degrees of complexity (maximum ROO filament length and filament growth mechanisms, independent or simultaneous DNA strand cleavage, and cleavage with the ROO filament), fitting with these models resulted in very similar extracted microscopic rate constants and similar measures of the quality of fit (see Tables 2–6, Fig. S5, and below).

**FRET titration showing ROO filament formation**

FRET between donor (Flo, 6-carboxyfluorescein) and acceptor (Rox, Rhodamine-X or 5(6)-carboxy-X-rhodamine) labeled DNA (see “Experimental procedures” and Fig. S1) was used to investigate the equilibrium association of SgrAI–DNA complexes into run-on oligomer (ROO) filaments. DNA cleavage by SgrAI was prevented from occurring by excluding the necessary Mg$^{2+}$ cofactor from the reaction buffer and instead using Ca$^{2+}$ as a mimic, which inhibits DNA cleavage (8). A limiting concentration (50 nm) of acceptor labeled reporter DNA (Rox–18M-1) was mixed with 2–4 μM SgrAI and then titrated with increasing concentrations of DNA containing donor (fluorescein)-labeled DNA (either Flo–18M-1 or Flo–PC). The fluorescence emission (using 498-nm excitation, the excitation maximum of Flo, the donor) was measured at 508–700 nm before and after each addition in this titration. The emission contained emitted light from both donor Flo (λemission = 520 nm) and acceptor Rox (λemission = 608 nm) fluorophores. To isolate the acceptor emission caused by FRET, the contributions from donor emission, as well as that which occurs from the acceptor in the absence of donor, were subtracted (see “Experimental procedures”).

The resulting emission spectra increased with increasing concentrations of total Flo–PC DNA (Fig. 3A). In Fig. 3 we show emissions as a function of total [EP40] (for enzyme product of 40 bp, which is SgrAI bound to self- annealed Flo–PC DNA; Fig. 2A) rather than Flo–PC DNA concentration to allow comparison with the other titrations (Table 7). This is reasonable because SgrAI binds to self- annealed PC DNA with a K_d in the low nanomolar range (18), and SgrAI is in the micromolar range in the titrations, so nearly all PC DNA should be bound by SgrAI. The increase in FRET observed in Fig. 3A is plotted versus total [EP40] in Fig. 3B (red) and fit to the Hill equation giving K_1/2 (i.e. [EP40] giving the half-maximum FRET signal) of 0.50 ± 0.02 μM. The Hill coefficient for the fit, a measure of cooperativity on total [EP40], was found to be 2.5. These data are summarized in Table 7. For comparison, no such increase in in FRET is seen when SgrAI is absent (Fig. 3B, black).

A second titration was performed to test the assumption that SgrAI bound to the shorter DNA (18M-1) would not significantly induce ROO filament formation (8). In this case, instead of Flo–PC DNA, Flo–18M-1 was titrated into the reaction with Rox–18M-1 and excess SgrAI, and FRET was measured. Fig. S2A shows the result. Little FRET is found until very high concentrations of Flo–18M-1 (and consequently Flo–ES18 for Enzyme Substrate of 18 bp, SgrAI bound to Flo–18M-1). The Hill plot in Fig. S2B shows a curve that has not reached saturation even at 3.5 μM ES18. This result is also summarized in Table 7, along with the similar titration performed in the accompanying article (18) with Rox–40–1 (a 40-bp DNA with a single primary site) and Flo–PC.

Table 2. Rate constants for ROO filament assembly from global data fitting of Data Set 4

| Reaction model | association rate constant (k_a) | dissociation rate constant (k_d) |
|----------------|-------------------------------|----------------------------------|
| Filament assembly 5EO | 4 x 10^6 (4 x 10^7 to 7 x 10^7) | 0.06 (0.012–0.15) |
| Filament assembly 5BM | 3 x 10^6 (3 x 10^7 to 1.5 x 10^8) | 0.01 (9 x 10^-4 to 0.09) |
| Filament assembly 4EO | 2 x 10^6 (2 x 10^6 to 1 x 10^7) | 0.017 (0.002–0.07) |
| Filament assembly 4BM | 2 x 10^6 (1 x 10^6 to 3 x 10^7) | 0.05 (0.002–0.12) |

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Table 3
Quality of fit parameters of global fitting of Data Set 4

| Reaction model | Number of reaction data sets used in global fitting<sup>a</sup> | Number of reaction data points total | Number of fitted parameters (rate constants + baseline/scaling constants) | $\chi^2$/DoF<sup>b</sup> | $\sigma$<sup>b</sup> |
|----------------|-------------------------------------------------|-------------------------------------|---------------------------------------------------------------------|--------------------------|--------------------------|
| Filament assembly 5EO | 6 | 998 | 7 + 12 = 19 | 2.5 | 0.021 |
| Filament assembly 5BM | 6 | 998 | 7 + 12 = 19 | 2.7 | 0.022 |
| Filament assembly 4EO | 6 | 998 | 7 + 12 = 19 | 2.5 | 0.020 |
| Filament assembly 4BM | 6 | 998 | 7 + 12 = 19 | 2.1 | 0.019 |

<sup>a</sup> See Table S1 for reaction data set details.

<sup>b</sup> DoF is the degrees of freedom, calculated from the number of data points $N$ and number of fitted parameters. $\chi^2$/DoF and $\sigma$ are as defined by the authors of the modeling software (37, 38).

Table 4
Rate constants for ROO filament assembly from global fitting of Data Sets 5–7

EP$_{\alpha}\gamma$, SgrAI bound to self-annealed PC DNA, ES$_{\alpha}\gamma$, SgrAI bound to uncleaved 18M-1 DNA, EP$_{\alpha}\gamma$, SgrAI bound to cleaved 18M-1 DNA. FitSpace boundaries calculated at $0.95(\chi^2/(\chi^2)_{\text{null}})$ threshold boundary. See Tables S6–S14 for reactions and equations used in modeling and global data fitting.

| Reaction model | $k_{\text{EP}_{\alpha}\gamma}$ | $k_{\text{EP}_{\alpha}\gamma}$ dissociation (k$_{-1}$) | $k_{\text{EP}_{\alpha}\gamma}$ or $k_{\text{EP}_{\alpha}\gamma}$ dissociation (k$_{-1}$) | $k_{\text{ES}_{\alpha}\gamma}$ or $k_{\text{ES}_{\alpha}\gamma}$ dissociation (k$_{-1}$) |
|----------------|-----------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| Full pathway 2-mers independent strand cleavage | $2 \times 10^5$ (200 to 1000) | $1 \times 10^{-5}$ (100 to 1000) | $5 \times 10^{-8}$ (500 to 1000) | $0.03 (0.02–0.04)$ |
| Full pathway 2-mers independent strand cleavage | $2 \times 10^5$ (200 to 1000) | $1 \times 10^{-5}$ (100 to 1000) | $5 \times 10^{-8}$ (500 to 1000) | $0.03 (0.02–0.04)$ |
| Full pathway 3-mers | $1.4 \times 10^5$ (8 to 5 × 10^5) | $0.03 (1.8 \times 10^{-5}–0.02)$ | $2 \times 10^5$ (200 to 1000) | $0.07 (0.07–0.09)$ |
| Full pathway 4EO | $1.7 \times 10^5$ (6 to 7 × 10^5) | $0.03 (0.017–0.04)$ | $2.5 \times 10^5$ (200 to 1000) | $0.08 (0.07–0.1)$ |
| Full pathway 4BM | $1.2 \times 10^5$ (6 to 3 × 10^5) | $0.03 (3 \times 10^{-6}–1)$ | $2 \times 10^5$ (200 to 1000) | $0.08 (0.07–0.10)$ |

<sup>a</sup> The overall $\chi^2$ was affected little by values within the tested range.

Table 5
Additional rate constants from global fitting of Data Sets 5–7

See Tables S6–S14 for reactions and equations used in modeling and global data fitting.

| Reaction model | Self-association of PC DNA forward rate constant ($k_2$) | DNA cleavage within run-on oligomer (k$_{\text{ES}_{\alpha}\gamma}$) | Dissociation of cleaved 18M-1 from SgrAI (k$_{-1}$) | DNA cleavage by isolated ES$_{\alpha}$ (SgrAI bound to 18M-1) (k$_{\text{ES}_{\alpha}\gamma}$) |
|----------------|-------------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| Full pathway 2-mers independent strand cleavage | $2 \times 10^4$ (4 × 10^3 to 8 × 10^4) | $0.5 (0.2–0.8)$ | $3 (0.8)$ | $7 \times 10^{-4}$ (4 × 10^{-4} to 10 × 10^{-4}) |
| Full pathway 2-mers independent strand cleavage | $2 \times 10^4$ (4 × 10^3 to 8 × 10^4) | $0.5 (0.2–0.8)$ | $3 (0.8)$ | $7 \times 10^{-4}$ (4 × 10^{-4} to 10 × 10^{-4}) |
| Full pathway 3-mers | $2 \times 10^4$ (5 × 10^3 to 6 × 10^4) | $0.5 (0.2–1.0)$ | $3 (0.8)$ | $7 \times 10^{-4}$ (4 × 10^{-4} to 10 × 10^{-4}) |
| Full pathway 4EO | $2 \times 10^4$ (3 × 10^3 to 5 × 10^4) | $0.5 (0.3–25)$ | $3 (0.3–1.0)$ | $7 \times 10^{-4}$ (4 × 10^{-4} to 10 × 10^{-4}) |
| Full pathway 4BM | $2 \times 10^4$ (1 × 10^3 to 6 × 10^4) | $0.5 (0.3–6)$ | $3 (0.4–1.0)$ | $7 \times 10^{-4}$ (4 × 10^{-4} to 10 × 10^{-4}) |

<sup>a</sup> FitSpace 0.95(\chi^2/(\chi^2)_{\text{null}}) threshold boundary.

<sup>b</sup> 0.99(\chi^2/(\chi^2)_{\text{null}}) threshold boundary.

Table 6
Quality of fit parameters of global fitting of Data Sets 5–7

| Reaction model | Number of reaction data sets used in global fitting | Number of reaction data points total | Number of fitted parameters (rate constants + baseline/scaling constants) | $\chi^2$/DoF<sup>a</sup> | $\sigma$<sup>a</sup> |
|----------------|-------------------------------------------------|-------------------------------------|---------------------------------------------------------------------|--------------------------|--------------------------|
| Full pathway 2-mers independent strand cleavage | 22 | 3454 | 8 + 50 = 58 | 2.3 | 0.020 |
| Full pathway 2-mers | 22 | 3454 | 8 + 50 = 58 | 2.7 | 0.021 |
| Full pathway 3-mers | 22 | 3454 | 10 + 50 = 60 | 3.3 | 0.020 |
| Full pathway 4EO | 22 | 3448 | 10 + 50 = 60 | 1.7 | 0.019 |
| Full pathway 4BM | 22 | 3448 | 10 + 50 = 60 | 1.8 | 0.021 |

<sup>a</sup> DoF is degrees of freedom, calculated from the number of data points $N$ and number of fitted parameters. Values of $\chi^2$/DoF closer to 1 indicate better fits.

<sup>b</sup> $\sigma$ is the “$\sigma$ with respect to the fit,” another measure of how well the experimental data agree with the simulated.

The affinities of SgrAI–DNA complexes to each other within the ROO filament. The data of Table 7 indicate that the ROO filament forms more readily (requiring lower concentrations) when the DNA contains longer flanking regions, as in case of the longer DNA constructs PC DNA and 40-1 (each having 16-bp flanking the primary recognition sequence). In contrast, SgrAI bound to 18M-1 (with just 5 flanking bp on either side of the primary recognition sequence) only weakly self-associates into the ROO filament (Table 7). However, the assembly formed between SgrAI–DNA complexes where both DNAs have longer flanking regions (i.e., PC DNA and 40-1) has the lowest $K_{12}$ (0.16 $\mu M$; Table 7). When one DNA is shorter (18M-1) and one longer (PC DNA), the $K_{12}$ is intermediate (0.5 $\mu M$; Table 7). These results are consistent with the cryo-EM
structure of the run-on oligomer formed by SgrAI bound to PC DNA, showing contacts between the flanking DNA of one SgrAI–DNA complex to adjacent SgrAI–DNA complexes (Fig. 1). The shorter flanking DNA of 18M-1 cannot make these contacts; hence self-association of ES18 into the ROO filament is much weaker. This also explains why SgrAI activation depends on DNA length, and why 18M-1 is unable to provide activation (8, 16). Similarly, results from mutagenesis of residues at this protein–DNA interface show the importance of these contacts between the flanking DNA and neighboring SgrAI–DNA complexes in the ROO filament. For example, mutations removing a positive charge or introducing a negative to this interface disrupt activated cleavage by SgrAI, also presumably by weakening the ROO filament (13).

Dissociation of ROO filament with DNA cleavage and the absence of DNA trapping

To investigate whether trapping of SgrAI bound to product DNA occurs, the FRET titration discussed above with DNAs Rox–18M-1 and Flo–PC (but without Ca\(^{2+}\) present) was repeated, and then Mg\(^{2+}\) was added to allow DNA cleavage by SgrAI. Upon cleavage and dissociation of Rox–18M-1 from SgrAI, the shortened DNA duplex is unstable and rapidly dissociates irreversibly to single strands, making the reaction irreversible. Spectra were taken before and after the addition of 10 mM MgCl\(_2\). Fig. S3 shows that after 30 min, very little FRET signal remains, indicating that very few complexes containing both SgrAI–Rox–18M-1 and SgrAI/Flo–PC remain. Thus, no significant trapping of Rox–18M-1 in ROO filaments occurs.

**Timed approach to equilibrium reactions to measure ROO filament assembly (Data Set 4)**

FRET was used to detect the association of SgrAI–Rox–18M-1 complexes (i.e. Rox–ES\(_{18}\)) with SgrAI bound to two copies of Flo–PC (Flo–EP\(_{40}\)) (Fig. 2A). Table S1 provides information on the independent timed reactions of Data Set 4, and Fig. 4 shows time traces of the data with their global fits (using model filament assembly 4BM; see Table 1 and above for descriptions of models). Models were created to simulate the reaction and predict concentrations of species at a given time after reaction initiation (given appropriate starting concentrations and rate constants).

Note that in all models (see Tables S3–S5 for reaction equilibrium modeled and equations used to simulate timed FRET data), it was assumed that the kinetics of assembly and disassembly of the ROO filament are independent of ROO filament size; for example, the addition of a single SgrAI–DNA complex to a ROO filament of size 3 (having three SgrAI–DNA complexes) has the same association and dissociation rate constants as it would when associating to a ROO filament of size 4. This essentially assumes that interactions occur *only* between adjacent SgrAI–DNA complexes within the ROO filament and that assembly is noncooperative. This assumption is tested and discussed further below.

In addition to modeling the self-association of PC DNA and DNA binding by SgrAI, the associations of interest in this work are those that form the ROO filament: 1) association of Rox–ES\(_{18}\) (SgrAI bound to Rox-labeled 18-mer) and Flo–EP\(_{40}\) (SgrAI

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**Figure 3.** Titration of Rox–18M-1, SgrAI, with Flo–PC DNA in the presence of 10 mM Ca\(^{2+}\). A, 50 nM Rox–18M-1, 2 μM SgrAI, and 10 mM CaCl\(_2\) with PC DNA (1:9 Flo–PC:PC) at 25 °C. Emission spectra were taken with 498-nm excitation and corrected for Flo emission, dilution, and Rox emission caused by absorption at 498 nm. B, average corrected intensities from A (wavelengths 602–612 nm) versus total EP\(_{40}\) concentration (SgrAI bound to self-annealed Flo–PC DNA, consisting of 1:9 Flo–PC:PC) filled red circles. Fit to Hill plot blue line gives K\(_{1/2}\) = 0.50 ± 0.02 μM, Hill coefficient = 2.5 ± 0.3, and r = 0.998. Control (black circles and line) performed exactly the same, but without SgrAI.

**Table 7**

| Reporter\(^a\) | Donor | K\(_{1/2}\) (μM) | Hill coefficient |
|---|---|---|---|
| 50 nM Rox–18M-1 | Flo–PC | 0.5 ± 0.02 μM | 2.5 ± 0.3 |
| 50 nM Rox–18M-1 | Too weak to measure | 1.2 ± 0.4 |
| 50 nM Rox–40–40 \(^b\) | Flo–PC | 0.16 ± 0.03 μM | 1.1 ± 0.1 |

\(^a\) See Figs. 2 and 3 and Fig. S2. All experiments were performed in the presence of 10 mM CaCl\(_2\).

\(^b\) When plotted versus [SgrAI–Flo–DNA] such as [Flo–ES\(_{18}\)] or [Flo–EP\(_{40}\)].
bound to self-annealed Flo–PC, mimicking a 40-bp cleaved DNA, which gives the FRET signal; and 2) self-association of Flo–EP₄₀, which does not give a FRET signal. ES₁₈ does not self-associate significantly, as assessed by the FRET titration (Fig. S2), and hence is not modeled, nor are ROO filaments with two adjacent ES₁₈ (Tables S3–S5). Table 2 summarizes the resulting rate constants derived from fitting each of the four filament assembly models to the six different reactions measured in Data Set 4. A total of seven independent rate constants, and twelve baseline or scaling constants (for relating simulated concentrations of species to FRET signals measured in the timed reactions) were fit in each model (Table 3). Also shown in Table 3 is the χ²/degrees of freedom, a measure of the quality of the fit with 1 being optimal, which was found to be between 2.1–2.7 for fitting of the four different models to the same data (Data Set 4). The fitted rate constants for the self-association and dissociation of EP₄₀ with itself were found to be very similar to those reported previously for EP₄₀ and ES₁₈ (Tables S3–S5). The 0.95 χ² threshold error boundaries (see “Experimental procedures” and supporting information for how this is estimated) are given in parentheses for each rate constant (Table 2). The values and boundaries overlap for each independent rate constant determined from the four different models, as is also true for the two types of associations (EP₄₀ + EP₄₀ and EP₄₀ + ES₁₈) (see also Fig. S5, A–D). Because all four models give similar quality of fit parameters (Table 3), it can be concluded that the data and these models cannot distinguish between the different types of ROO filament growth mechanisms (i.e. breaks in the middle versus ends only) and that models that limit filament size to four versus five SgrAl–DNA complexes per ROO filament give similar rate constants.

**DNA cleavage pathway measured via FRET with singly labeled reporter (Rox–18M-1) and activator (Flo–PC) DNA (Data Set 6)**

Data Set 6 (see Fig. 2A for a reaction schematic) is similar to Data Set 4; however, 10 mM MgCl₂ is included in the reaction allowing DNA cleavage by SgrAl. Following initiation by the addition of Rox–18M-1, emission intensities were recorded at both 520 nm (Flo emission) and through the 590 cut-on filter (Flo and Rox emissions). The 520-nm recording was used to remove Flo emission contributions from the 590 cut-on filter data (following appropriate scaling; see “Experimental procedures”). This corrected filter data was used in global data fitting along with reactions of Data Sets 5 and 7 described below. Table S1 summarizes the different reactions of Data Set 5.

**DNA cleavage pathway measured via FRET with doubly labeled reporter (Flo–18M–1–Rox) and unlabeled activator DNA (PC DNA) (Data Set 5)**

Data Set 5 utilizes doubly labeled reporter DNA (Flo–18M–1–Rox), unlabeled PC DNA, and allows for DNA cleavage by SgrAl caused by the presence of Mg²⁺ in the reaction buffer (see Fig. S2B for a reaction schematic). Once the reporter DNA is cleaved and dissociated from SgrAl, and the two strands of the DNA duplex dissociate into single strands, the FRET signal between the Flo and Rox fluorophores is lost (Fig. S2B). We expect the dissociation into single strands to be irreversible because the predicted Tₘ (melting temperature) of the two halves of the cleaved 18M-1 DNA is 9–11 °C, and reactions were performed at 25 °C. We also tested this irreversibility in a number of ways. Fig. 5A shows a before and after emission (using excitation of Flo at 498 nm) from a reaction (with Flo–18M-1–Rox), showing loss of FRET following the reaction (increase in Flo emission at 520 and loss of Rox emission at 605 nm). If annealing of the cleaved Flo–18M-1–Rox occurred, no such loss would occur. Further, additional reactions were performed in the presence of excess unlabeled single-stranded complimentary DNA to prevent any reannealing of cleaved product DNA. No differences in the recorded data were found (data not shown). Finally, additional evidence of the irreversibility of dissociated cleaved 18M-1 is shown in Fig. S3, where complete loss of FRET between Rox–18M-1 and Flo–PC in assemblies with SgrAl was seen following the addition of 10 mM MgCl₂.

Timed FRET reactions were performed as described above for Data Set 6; however, the DNA used was unlabeled PC DNA and doubly labeled 18M-1 (Flo–18M–1–Rox). The data were recorded following the addition of SgrAl to the premixed solution using excitation at 498 nm and emission at 520 nm, which followed the unquenching of Flo caused by the loss of FRET between Flo and Rox as the substrate (Flo–18M–1–Rox) DNA was cleaved and strand-separated (Fig. 2B). All timed reaction data sets are summarized in Table S1, and an example of a measured reaction with an analytical fit is shown in Fig. 5B. Fig. 5 (C and D) shows Hill plots of the analytically derived rate constants versus the concentration of total [EP₄₀]. These give...
of 0.2 \pm 0.1 \mu M when N (the Hill coefficient, a measure of cooperativity) is forced to 1 (Fig. 5C) and $K_{1/2}$ of 2 \mu M when N is allowed to be fit, giving 0.4 \pm 1 (Fig. 5D). These results do not support the presence of positive cooperativity of DNA cleavage and release by SgrAI on total EP_{40} concentration. Global fitting of Data Set 5 is done together with Data Sets 6–7 and is described below.

**DNA cleavage pathway measured via doubly labeled reporter (Flo–18M-1–Rox), unlabeled activator (PC) DNA, and denaturing PAGE (Data Set 7)**

This data set is similar to Data Set 5 (see Fig. 2B for reaction schematic) in using Flo–18M–1–Rox, varied concentrations of PC DNA, and excess SgrAI in the presence of 10 mM MgCl$_2$; however, instead of FRET measurements, the total amount of cleaved DNA (whether bound to SgrAI or not) was measured by quenching 5-\mu l aliquots of a 100-\mu l reaction and analyzing via denaturing PAGE and densitometry of Flo– and Rox-containing bands. The reactions were repeated with different concentrations of PC DNA, resulting in varied concentrations of SgrAI bound to PC DNA (i.e., total [EP$_{40}$]). Table S1 summarizes the nine reactions and the results of analytical fits to each, an example of which is shown in Fig. 6A. Fig. 6B shows a Hill plot of the analytically fitted rate constants, giving a $K_{1/2}$ of 0.5 \mu M and a Hill coefficient $N$ of 1.0 \pm 0.2. This indicates that half-maximal DNA cleavage occurs at \sim 0.5 \mu M total [EP$_{40}$] and that the rate constant for DNA cleavage by SgrAI under these conditions, although dependent on total [EP$_{40}$], does not appear to be cooperative on total [EP$_{40}$] (because the Hill coefficient is \sim 1). Data Set 7 data are fit together with that of Data Sets 5 and 6 in global fitting to five different reaction models (described below).

**Global fitting of DNA-cleavage reaction data (Data Sets 5–7)**

Reaction data from Data Sets 5–7 described above were fit together (globally) using five different reaction mechanism models (full pathway models of Table 1, equations given in Tables S6–S14). Fig. 7 summarizes one of the simpler models (full pathway 2-mers), which includes the reversible association of single-stranded activator (PC) DNA (with rate constants $k_1$ and $k_{-1}$), the reversible binding of self-annealed PC DNA and substrate DNA (18M–1) by SgrAI (rate constants $k_2$, $k_{-2}$, $k_3$, and $k_{-3}$), and then the reversible association of SgrAI–DNA com-

Figure 5. Data Set 5 analysis. A, before (blue) and after (red) fluorescence emission scans (excitation, 498 nm) of 10 nM Flo–18M–1–Rox with SgrAI in the presence of 10 mM MgCl$_2$. The peak at 520 nm corresponds to the Flo emission maximum, and the emission at 605 nm corresponds to that of Rox. Before reaction (and DNA cleavage) by SgrAI, a strong Rox signal is seen largely because of FRET from Flo (a small amount of Rox emission derives from Rox absorbance at 498 nm). After reaction with SgrAI (and DNA cleavage and strand separation), the Flo emission increases because of reduced FRET to Rox, resulting in reduced Rox emission. AU, arbitrary units. B, fluorescence emission data at 520 nm with excitation at 498 nm (red filled circles) of a Data Set 5 reaction of 10 nM Flo–18M–1–Rox, excess SgrAI, and in the presence of 10 mM MgCl$_2$. Data after 100 s are fit to a single exponential function (blue line), giving a rate constant of $5 \times 10^{-7} \pm 4 \times 10^{-8}$ s$^{-1}$, and $r = 0.996$. C, analytically derived rate constants for Data Set 5 versus total EP$_{40}$ concentration (SgrAI bound to self-annealed PC DNA) shown as red filled circles. Hill analysis (blue line) with Hill coefficient forced to 1 gives a $K_{1/2}$ of 0.2 \pm 0.1 \mu M, and $r = 0.990$ and an upper asymptote of 5.8 \pm 0.9 \times 10^{-5}$ s$^{-1}$. D, Hill plot with fitted Hill coefficient. $K_{1/2} = 2 \pm 50 \mu M$, $n = 0.4 \pm 1$, $r = 0.991$. 

**Figure 6. Data Set 5 analysis.**
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**Figure 6. Data Set 7 analysis.** A. Example of analytical data fitting from densitometric scan data. The data (red circles) were fit to a single exponential function (blue line) to give a rate constant (see “Experimental procedures”). The fit gives 0.037 min$^{-1}$ for the rate constant, with $r = 0.984$. B. Hill plot of Data Set 7 single exponential rate constants, plotted versus the total concentration of EP$_{40}$ (SgrAI bound to self-annealed PC DNA). $K_{1/2} = 0.5 \pm 0.5 \mu M$, and Hill coefficient $n = 1.0 \pm 0.3$, upper asymptote, 0.22 $\pm$ 0.12 s$^{-1}$, and $r = 0.995$.

**Figure 7. Schematic cartoon of full pathway 2-mers reaction model.** SgrAI is shown in gray (low activity conformation) or green (high activity conformation), and DNA is shown in brown for PC DNA and yellow or purple for uncleaved or cleaved 18M-1 DNA, respectively. Numbered rate constants are shown and correspond to the forward and reverse rate constants (see also Table S8) for association of two PC DNA molecules to a semicontinuous 40-mer ($k_4$, $k_{-4}$); binding of SgrAI to self-annealed PC DNA to create EP$_{40}$ ($k_1$, $k_{-1}$); binding of SgrAI to 18M-1 DNA to create ES$_{18}$ ($k_2$, $k_{-2}$); association of ES$_{18}$ with EP$_{40}$ (two orientations for association are possible) ($k_3$, $k_{-3}$); cleavage of 18M-1 DNA (although both top and bottom strands are cleaved independently, only a single cleavage event is shown for simplicity) in ES$_{18}$ to create EP$_{18}$ ($k_5$); dissociation of EP$_{40}$ from EP$_{18}$ (for simplicity, these rate constants are set to be identical for the association and dissociation of EP$_{40}$ and ES$_{18}$ ($k_6$, $k_{-6}$); and dissociation of EP$_{18}$ into SgrAI and cleaved 18M-1 ($k_7$, $k_{-7}$) considered equivalent to dissociation of uncleaved 18M-1 DNA from SgrAI). Cleaved 18M-1 dissociates into single strands and is considered irreversible (not shown).

complexes (EP$_{40}$ and ES$_{18}$) into larger assemblies (rate constants $k_5$ and $k_{-5}$). Although self-assembly of EP$_{40}$ does occur, it is not modeled in this particular reaction model, and also ROO filaments of ES$_{18}$ and EP$_{40}$ are limited to size 2 with no larger filaments modeled. Cleavage of substrate DNA (18M-1) is rapid and irreversible in the ROO filament (rate constant $k_5$). Complexes that now contain EP$_{18}$ ($P$ for product, i.e. the cleaved 18-mer) dissociate into EP$_{40}$ and EP$_{18}$ ($k_6$ and $k_{-6}$, same rate constants as with uncleaved 18M-1 in ES$_{18}$), and the cleaved 18M-1 DNA dissociates irreversibly from SgrAI (rate constant $k_7$). Other models include more complexity; the five global models of the full DNA cleavage pathway (summarized in Table 1) differ in several attributes including size of ROO filament (2–4 SgrAI–DNA complexes total) and growth mechanism (breaking in the middle, i.e. BM; or ends only, i.e. EO) as described above, as well as whether or not self-association of EP$_{40}$ is included in the model. The cleavage of each individual strand of DNA in the 18M-1 DNA duplex is modeled in full pathway 2-mers independent strand cleavage, and the model of full pathway 2-mers is exactly the same with the exception that only one step is modeled for duplex DNA cleavage. The models of full pathway 3-mers, full pathway 4EO, and full pathway 4BM all have this simplification as well and, in addition, differ in the independence of DNA cleavage by different ES$_{18}$ in the same ROO filament (Table 1). Of course, DNA cleavage by each ES$_{18}$ is expected to be independent, as cleavage of each strand is also, but simplifications are necessitated by limitations of the modeling software, and simulations show that only very few of the ROO filaments contain more than one copy of ES$_{18}$ (Fig. S7C). Another assumption and simplification that is present in all
models is that ES18 and EP18 behave the same with respect to association to EP40, meaning that the state of cleavage of the bound 18M-1 DNA does not affect the affinity of the SgrAl–DNA complex to a SgrAl–DNA complex containing PC DNA. The similar association and dissociation rate constants found in the accompanying article with ES40 and ES40 support this assumption (18). The similar resulting fits (described below) and extracted rate constants from all five models indicate that these simplifications have a minimal effect on data fitting.

Fig. 8 shows individual plots (simulated and experimental data) for representative reactions from using Data Sets 5–7 and using the model of full pathway 4BM. Tables 4 and 5 show the derived rate constants for each reaction step along with error boundaries (plotted in Fig. S5), and the quality of fit parameters are given in Table 6. The average of fitted rate constants for the self-association of EP40 (i.e. $k_4$) for the three models having such an association step is $1.4 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$, with a range of $1.2 \times 10^5$ to $1.7 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ (Table 4 and Fig. S5A). These are ~10-fold slower than those found in the fitting of Data Set 4 (Table 2 and Fig. S5A), containing approach to equilibrium reaction data measured without Mg$^{2+}$. Previous work also showed that this association rate constant was 10-fold slower in the presence of 10 mM Ca$^{2+}$ relative to that measured without divalent cations (18), suggesting a divalent cation effect on the association rate constant of SgrAl–DNA complexes into the ROO filament. Error boundaries of the rate constant indicate a range from $8 \times 10^4$ to $7 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ among the five models, and the model of full pathway 4BM, the most sophisticated model, gives an error interval (at a $0.95 \chi^2$ threshold) of $6 \times 10^4$ to $3 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ (Table 4).

Dissociation rate constants ($k_{-4}$) of EP40 from other EP40 fit best to 0.03 s$^{-1}$, although the analysis also suggests these to have a range of $3 \times 10^{-6}$ to 1.0 s$^{-1}$ (Table 4 and Fig. S5B). An average of 0.03 s$^{-1}$ for this rate constant was also found in the fitting of Data Set 4 (Table 2) but with tighter boundary limits of $9 \times 10^{-6}$ to 0.15 s$^{-1}$. Thus, the presence of the divalent cation (Mg$^{2+}$ in the current case, no divalent cation for Data Set 4) does not appear to affect this rate constant in the current analysis, consistent with that observed using ES40 and EP40 (18).

The association rate constant for ES18 + EP40 (or EP18 + EP40 when 18M-1 is cleaved; $k_4$; Table 4 and Fig. S5C) was found to be $2 \times 10^5$ to $2.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ in the five models, similar to that found for EP40 + EP40 (Table 4). Error analysis indicates a fairly tight boundary of $2.3 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ (Fig. S5C), likely because this rate constant limits the overall reaction rates measured in all three Data Sets 5–7 (in most reactions).

The dissociation rate constant found for ES18 from EP40 (or EP18 from EP40) (i.e. $k_{-4}$) of 0.06 s$^{-1}$ was on average faster than that of EP40 from EP40 ($k_{-4}; 0.03$ s$^{-1}$), with a range of 0.04–0.08 s$^{-1}$ in the various models, and fairly constrained error boundary of 0.04–0.10 s$^{-1}$ (Table 4 and Fig. SSD). This step appears to limit the overall reaction rates of the DNA-cleavage reactions at the higher concentrations of PC DNA (and total [EP40]), when the association of ES18 and EP40 is no longer rate-limiting. For comparison, the average rate constant for the same reaction from fitting the approach to equilibrium data (i.e. Data Set 4) to the four different models is 0.05 s$^{-1}$, very similar to that found here in the full DNA cleavage pathway ($k_{-4};$ Table 2 and Fig. SSD); however, the error boundaries are far less constrained by the models with the mechanism of breaks in the middle (i.e. types 4BM and 5BM) compared with the ends-only mechanism (i.e. types 5EO and 4EO; Table 2 and Fig. SSD).

The DNA-cleavage rate constant ($k_5$) was fit to an average of 0.7 s$^{-1}$ and ranged from 0.5–0.8 s$^{-1}$ (Table 5 and Fig. S5E). The error analysis for the simpler three models were more constrained than the two more sophisticated models, with error boundaries of 0.2–1.0 s$^{-1}$ (Table 4 and Fig. S5E). For the more complex models, the boundaries are much larger, but a closer look at the error calculation for this parameter reveals a peak at the best-fit parameter (see Fig. S6E for that of full pathway 4BM), with a sharp drop-off for lower values but a shallower drop-off for higher. The shallow drop-off at the higher end is because only two or three data points constrain the upper limit of this rate constant. These derive from two reactions of Data Set 7 (reactions 408 and 409; Table S1) having the highest con-
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centration of PC DNA (and total [EP40]), in which association into the ROO filament is no longer rate-limiting. Data Set 7 data with lower concentrations of PC DNA (and therefore lower total [EP40]) are limited by the association of ES18 into the ROO filament, hence contributing little to defining the DNA-cleavage rate constant. Data from the other experimental data sets (i.e. Data Sets 5 and 6) measure steps including those after DNA cleavage, are limited by the slower rate constant for dissociation of EP18 from ROO filament, and therefore also do not help to define the upper limit of the DNA-cleavage rate constant.

Finally, release of cleaved reporter DNA (18M-1) from SgrAI, also referred to here as product release (k−), was fit to 3–10 s−1 but really was constrained by the data only to a lower limit of 0.4–1.0 s−1, depending on the model (Table 4 and Fig. S5F). This is likely because earlier steps limit the reaction kinetics measured in these experiments, such that they provide little data on the product release step itself. The estimated lower limit of 0.4–1.0 s−1 is similar to the estimate for the dissociation of uncleaved reporter DNA (18M-1) from SgrAI of 0.6 s−1, which is based on the measured equilibrium constant and estimates of the forward, diffusion limited, association rate constant (see supporting information).

Pathway of reporter DNA-bound SgrAI in DNA-cleavage reactions

A simulation was done to follow ES18 and EP18 (SgrAI bound to the reporter DNA 18M-1) during the DNA-cleavage reaction. Fig. S7A shows the simulation using the model of full pathway 4BM and its fitted rate constants (Tables 4 and 5) and plots the concentrations of ES18 (or EP18) alone (green), or in ROO filaments of size 2 (red), 3 (cyan), or 4 (blue), scaled for the number of ES18 (or EP18) present. These are plotted for several different initial conditions, differing in the concentration of total PC DNA (which changes the concentration of total EP40) and all with 10 nM reporter DNA (18M-1) and 1 μM SgrAI starting concentrations.

Most of the ES18 (and/or EP18) was found to be isolated from EP40 (i.e. not in an ROO filament) during most of the reaction time, and Fig. S7B shows that the majority of these are ES18 (blue lines) rather than EP18. Because product release (dissociation of cleaved 18M-1 DNA from SgrAI, k−) is fast (Table 5 and Fig. S5F), very little SgrAI is bound to cleaved 18M-1 DNA (i.e. EP18 plotted in red in Fig. S7B) is found. Most of the SgrAI bound to 18M-1 DNA is bound to uncleaved DNA (i.e. ES18), requiring association with EP40 to accelerate DNA cleavage, or alternatively, in time, to cleave DNA itself using the unaccelerated DNA cleavage with rate constant of 9 × 10−4 s−1 (k7; Table 5).

Fig. S7C shows the percentage (relative to total 18M-1 DNA) that is in a ROO filament with more than one copy of ES18 and/or EP18. As can be seen, this species occurs in only a very low amount, 0.08% or less. This simulation was done to determine whether or not FRET between multiple copies of Flo–18M-1–Rox bound to SgrAI in the same ROO filament should be considered in the modeling; however, because of its low quantity, this adjustment was not necessary.

Tests for evidence of cooperativity in models

To see whether the models such as filament assembly 5EO could reproduce the cooperativity of ROO filament formation seen in Fig. 3 (see supporting information and Fig. S8), the fraction of SgrAI–DNA complexes in ROO filament was predicted, as well as the predicted FRET signal (even in a modified model allowing ROO filaments up to 9-mer; see supporting information and Tables S15 and S16) at different total [EP40]. When plotted versus total [EP40] and fit to the Hill equation, no cooperativity is evident (Fig. S8, A and B). Hence the models derived here do not intrinsically result in ROO filament formation that is cooperative on total [EP40].

To introduce cooperativity into a model, the full pathway 4BM model was altered to include serially slower dissociation rate constants with increasing ROO filament size (see supporting information). This could be imagined to result from a greater number of favorable interactions made between the SgrAI–DNA complexes in the ROO filament when more complexes are present. The resulting effect on the quality of the fits is shown in Table S2. As can be seen, the fits for cooperativity factors of 5 or below are similar but very much poorer than with a cooperativity factor of 10. In particular, the simulations of data from Data Set 5 (which is most influenced by ROO filament dissociation rates) fit most poorly with larger cooperativity factors and hence slower ROO filament dissociation rates.

Discussion

In this two-part work, measurements of various equilibria and reactions were made to better understand the ROO filament mechanism used by SgrAI in DNA cleavage. Most of these measurements use FRET to measure rates of association of SgrAI–DNA complexes and rates of DNA cleavage and release. FRET was also used to measure the concentration dependence of assembly of SgrAI–DNA complexes into ROO filaments, to obtain measures of affinity and cooperativity (Table 7). This latter measurement involves titrating a low concentration of one type of SgrAI–DNA complex with increasing concentrations of another and measuring the signal that arises when they are in close proximity to each other. For the kinetic measurements, various reaction schemes were used to isolate particular steps in the reaction pathways and/or to allow for simplified models with fewer rate constants to be fit. In this way, models of increasing complexity could be fit to data sets with greater confidence. Model fitting also allowed for the investigation of cooperativity, ROO filament growth mechanisms, and trapping of cleaved DNA.

This second part of the two-part work focuses on global fitting of DNA cleavage data to test various models and extract microscopic rate constants for each step of the reaction pathway. Table 1 summarizes the different models used in global data fitting in the current work, and Tables 2, 4, and 5 summarize their fitted rate constants, as well as their estimated error boundaries, which are also summarized graphically in Fig. S5. Table 8 gives the final best estimates of these rate constants considering all measurements including those in the accompanying article (18).
Run-on oligomer filament growth formation

The different ROO filament growth and dissolution mechanisms (i.e. ends only versus breaks in the middle; Table 1) were tested and resulted in very similar fitted rate constants (compare rate constants and error boundaries in Fig. S5). However, at least with the self-association of EP40, we argue in the accompanying article (18) that the three-dimensional structure and lack of cooperativity seen in association measurements suggest that the breaks-in-the-middle model and not the ends-only model is most consistent with these data. In the breaks-in-the-middle model, ROO filaments may form from other ROO filaments, and importantly, ROO filaments may break into smaller ROO filaments by the breaking of any contact between any adjacent SgrAl–DNA complexes. Hence in the current work, we favor filament assembly 5BM for fitting of Data Set 4 data and full pathway 4BM for fitting data from Data Sets 5–7, because these both contain the breaks-in-the-middle mechanism and for the largest ROO filaments possible to model.

Table 8 summarizes rate constants from the favored model (i.e. full pathway 4BM). Also included in Table 8 are estimates of error on each rate constant, determined from the quality of fit of the experimental data. However, because the association and dissociation of EP40 was less constrained by the data used in this model, error boundaries for rate constants $k$ and $k_{-4}$ are instead taken from the accompanying article (18), which measure only these steps and give better estimates of these rate constants. The rate constants of Table 8 and their boundaries justify some assumptions and simplifications in our modeling: first, our modeling precluded ROO filaments larger than four or five SgrAl–DNA complexes, but the extracted rate constants and simulations indicate that larger ROO filaments are not predicted for the concentrations of DNA used in the assays (18). Second, again because of limitations in modeling, it was necessary in certain models (full pathway 4BM only) to make the assumption that when DNA cleavage occurs within an ROO filament, this occurs for all SgrAl–DNA complexes (in this case, ES40) prior to ROO filament dissociation (i.e. we model simultaneous DNA cleavage in any ROO filament with more than one ES40). This assumption is justified by the limits on ROO filament dissociation and DNA-cleavage rate constants, showing that DNA cleavage is significantly faster than ROO filament dissociation. In addition, the fraction of ROO filament with greater than one ES18 and/or EP18 is very small (Fig. S7C).

Cooperativity

Most measures of cooperativity, as described under “Results,” are either inconclusive or indicate no cooperativity on PC DNA concentration (or total [EP40]) in ROO filament assembly or DNA cleavage. These tests include: 1) Hill plots of Data Set 5 and Data Set 7 data (Figs. 5, C and D, and 6B), 2) simulations of ROO filament concentrations and of FRET signal (Fig. S8), 3) introducing cooperativity into a full pathway 4BM to fit data from Data Sets 5–7 (Table S2), 4) FRET titrations of ES40 with EP40 (18) (Table 7), and finally 5) simulation of ROO filament size distributions when cooperativity is included and comparing to that found by EM (18). However, one measure did show cooperativity in binding SgrAl–DNA complexes to ROO filament, specifically, of ES18 to EP40 to form the ROO filament (Fig. 3B). This uses FRET between Rox–18M–1 bound to SgrAl and Flo–PC bound to SgrAl (Fig. 2A). A fit of the data to the Hill equation provides a Hill coefficient, $N$, of $2.5 \pm 0.3$ (Table 7). The Hill coefficient, $N$, is a measure of cooperativity, and $N > 1$ indicates positive cooperativity. A Hill coefficient of $2.5 \pm 0.3$ can be interpreted as ES18 binding cooperatively with two or three copies of EP40. Although we feel from analysis of the cryo-EM structure of the ROO filament that association of SgrAl–DNA complexes into the ROO filament should not be cooperative, and the similar FRET titration ES40 with EP40 shows no evidence of cooperativity (18), the reduced number of contacts from the shortened 18M–1 flanking DNA (relative to PC DNA) may require additional EP40 binding to stabilize the ROO filament. Our attempts to see whether our models, which do not have cooperativity specifically built in, would lead to simulated data that would show a Hill coefficient greater than 1 did not (Fig. S8). Introduction of such cooperativity to models in the form of decreasing dissociation rate constants with longer ROO filament fits equally well to the observed experimental data (Table S2) when the cooperativity was low but deviates significantly when higher (Table S2). Therefore, our experimental kinetic data are consistent with some level of cooperativity in binding ES18 to EP40, although it cannot conclusively distinguish between a low

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Table 8

| Reaction step $^a$ | Forward rate constant | Reverse rate constant |
|-------------------|-----------------------|----------------------|
| PC DNA + PC DNA $\rightarrow$ PCDS $^a$ | $k_1 = 2 \times 10^5$ M$^{-1}$ s$^{-1}$ ($10^5$ to $6 \times 10^5$ M$^{-1}$ s$^{-1}$) | $k_{-1} = 8$ s$^{-1}$ (0.4–225 s$^{-1}$) |
| SgrAl + PCDS $\rightarrow$ ES40 | $k_1 = 10^4$ M$^{-1}$ s$^{-1}$ | $k_{-1} = 0.06$ s$^{-1}$ |
| SgrAl + 18M–1 DNA $\rightarrow$ ES18 $^a$ | $k_1 = 10^4$ M$^{-1}$ s$^{-1}$ | $k_{-1} = 0.6$ s$^{-1}$ |
| Associations and dissociations of EP40 with EP40 | $k_1 = 3 \times 10^5$ M$^{-1}$ s$^{-1}$ (1.1 $\times 10^5$ to $7 \times 10^5$ M$^{-1}$ s$^{-1}$) | $k_{-1} = 0.017$ s$^{-1}$ (0.007–0.02 s$^{-1}$) |
| Associations and dissociations of ES18 with EP40 (and EP18 with EP40) | $k_1 = 2 \times 10^5$ M$^{-1}$ s$^{-1}$ (2 $\times 10^5$ to $3 \times 10^5$ M$^{-1}$ s$^{-1}$) | $k_{-1} = 0.08$ s$^{-1}$ (0.07–0.1 s$^{-1}$) |

$^a$ PCDS, self-anneled PC DNA; EP40, SgrAl bound to self-anneled PC DNA; ES40, SgrAl bound to 18M–1 DNA. Association and dissociation rate constants of EP40, or ES18 (or EP18) with EP40 are considered to be independent of the size of the run-on oligomer filament. The ranges given in parentheses correspond to boundaries determined by FitSpace at the 0.95 $\chi^2$ threshold (see “Experimental procedures”).

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degree and a complete absence of cooperativity. It should be noted that the DNA-cleavage rate depends on the concentration of SgrAI–DNA complexes, because association of these complexes must occur prior to activation, and therefore are cooperative in that sense, but the reactions are not cooperative in terms of the classic definition defined by the Hill equation.

Interpretations and significance of fitted rate constants

From the above analyses, several important conclusions can be drawn for solution reactions done with SgrAI in vitro. First, because ES18 (SgrAI bound to 18M-1 DNA) does not self-associate at the reaction temperature, activation of DNA cleavage in ES18 requires the addition of EP40 (SgrAI bound to self-annealed PC DNA). This association is rate-limiting at concentrations of total EP40 below 250 nM, and therefore the overall rate of DNA cleavage increases with increasing total EP40 concentration in this range (13, 16). The association is rate-limiting because of the relatively slow association rate constant, $k_5 = -1.2 \times 10^5$ M$^{-1}$ s$^{-1}$ (Table 8), which is approximately 3–4 orders of magnitude slower than diffusion limited (19, 20).

At higher concentrations of EP40 downstream steps limit the overall reaction rate to a greater degree. DNA cleavage is fast within the ROO filament, $k_9 = 0.8$ s$^{-1}$ (Table 1), and is 3 orders of magnitude faster than in isolated (i.e. not in an ROO filament) ES18 ($k_9 = 9 \times 10^{-4}$ s$^{-1}$; Table 1). Further, the accelerated DNA-cleavage rate constant is now comparable with that measured for other restriction endonucleases including EcoRV (21) (0.6 ± 0.06 s$^{-1}$) and EcoRI (12) (0.34 ± 0.07 s$^{-1}$). Dissociation of ES18 from EP40 in the ROO filament is estimated to occur with a rate constant $k_{-5}$ of 0.08 s$^{-1}$ (Table 8). This dissociation rate constant is slower than the DNA-cleavage rate constant by a factor of 10, indicating that upon association with EP40 the ES18 is more likely to cleave DNA than to dissociate, resulting in a form of commitment and efficiency in the reaction.

One aspect of the reaction mechanism determined from this work is the relatively fast association and dissociation of SgrAI–DNA complexes from the ROO filament, preventing long-term trapping. Simulations to estimate the time ES18 and EP18 spend in the ROO filament show 200–300 s or less (Fig. S7A). Following dissociation of EP18 from the ROO filament, product release (dissociation of cleaved 18M-1 from SgrAI) appears to be very fast, and only a lower limit of its rate constant ($k_7 > 0.4$ s$^{-1}$; Table 1) was possible to determine. Hence, release of cleaved DNA in this scenario is not rate-limiting. The shorter DNA 18M-1 was used to prevent the rebinding of cleaved 18M-1 DNA, thereby making its dissociation from SgrAI irreversible (because this DNA when cleaved dissociates irreversibly into separated single strands). The in vivo situation is distinct in this respect, because SgrAI cleavage recognition sites will be present in longer DNA that do not dissociate into single strands. Still, trapping should not occur given the rapid association and dissociation of SgrAI–DNA complexes from the ROO filament, and the breaks-in-the-middle mechanism ensures even those complexes buried in the filament access to dissociation.

An important result that the association of SgrAI–DNA complexes (i.e. ES18 and EP40 in our reactions) is rate-limiting is significant. Given likely concentrations of DNA in the cell (estimated as 3 nM for 1 copy per cell for 1 DNA copy), association of SgrAI–DNA complexes on separate DNA molecules is predicted to be very slow (2 × 10$^5$ M$^{-1}$ s$^{-1}$ × 3 × 10$^{-9}$ M × 3 × 10$^{-8}$ M = 1.8 × 10$^{-12}$ M/s or 1.8 × 10$^{-3}$ nm/s). However, when present on the same DNA molecule, association is greatly accelerated by local concentration effects. Each 10-fold increase in concentration caused by local concentration effects increases the association rate 100-fold. Hence, the combination of the slow, second-order rate-limiting association rate constant and local concentration effects results in sequestration of activated DNA cleavage by SgrAI to sites within the same DNA molecule. This would result in rapid cleavage of both primary and secondary sites in invading phage DNA, with minimal damage to the host genome and in particular to likely unmethylated and unprotected secondary sites.

Conclusions

Only recently has filament formation by non-ATP or GTPases been appreciated. New imaging technologies have allowed for large-scale screening of protein localization in cells and have revealed filament formation by many metabolic and other enzymes, sometimes coinciding with particular phases of the cell cycle, with certain stress conditions, or as part of signaling pathways (1–7, 22–29). Run-on oligomers, or ROO filaments, are by definition filaments, although not all ROO filaments form filaments as large and as stable as others. The SgrAI ROO filament has been observed to form filaments composed of up to 20 or more SgrAI–DNA complexes in vitro (16, 30). These may be more limited in size in vivo (to number of recognition sites in DNA, usually 10–20 per phage genome including both primary and secondary sites) and may be short-lived. Much larger filaments (several microns in length) formed by other enzymes can be visualized in cells and have been found to be stable for minutes to hours (3–5, 7, 22–25). Only for a handful of such enzymes is the effect (i.e. activating or inhibiting) on enzymatic activity known (27, 28, 31–36). However, the particular advantages of forming a filament to control enzyme behavior is largely unexplored. Functions for filament formation have been proposed in the various systems in which they have been found, including sequestration of enzyme activity, rapid enzyme activation or inhibition, storage, fine-tune buffering of metabolic activity, in forming cytoskeleton-like structures, protein stabilization, developmental switching, rapid cell proliferation, stress coping, metabolic channeling, and finally intracellular transportation (2, 6, 16). However, few studies have investigated the kinetics of filament or ROO filament formation and none with the level of detail here.

Only one other theoretical model to understand the enzyme kinetics of enzyme filament formation and its function in enzyme turnover has been published (27); however, this model (of CTP synthase assembly) is derived from theory and based on several assumptions including positive cooperativity, the presence of a nucleation step, and growth limited to only the ends of filaments. This model is not a simulation but a derived mathematical equation, with some coarse data fitting (six data points from in vivo data imaging). The authors conclude that the filaments act as a reservoir and buffer to maintain a constant concentration of the enzyme in the cell, which can respond to the environment to increase or decrease that buffered concentra-
tion rapidly as needed (for example, in starvation conditions). Our modeling differs in that it is an explicit simulation rather than a singularly derived equation, and we test the assumptions of cooperativity, nucleation, and growth kinetics. We also use a considerably larger set of experimental data and derive the individual microscopic rate constants, not only for ROO filament assembly but for enzyme catalysis and product release as well. These models and derived microscopic rate constants will be used in simulations to investigate different starting conditions and conditions in vivo in future works.

In conclusion, our study quantifies individual steps and affinities in the assembly of ROO filaments, DNA cleavage, filament disassembly, and release of product (cleaved DNA) from the enzyme. We test various models for ROO filament assembly and find that cooperativity is not required to fit the experimental data, although some cooperativity with certain shorter DNA substrates may be present. In addition, our data are consistent with different growth mechanisms (ends only or breaking in the middle), but we find that the lack of observed cooperativity combined with structural analysis of the ROO filament is most consistent with a model allowing for disassembly at any junction within the filament. We also find that DNA cleavage is accelerated ~1,000-fold to a rate constant of ~0.8 s⁻¹. This is much faster than dissociation of the ROO filament, although ROO filament dissociation is fast enough to prevent significant trapping of product complexes. Importantly, we propose that the slow, second-order rate-limiting association step to form the ROO filament serves the purpose of sequestering activated DNA cleavage, particularly cleavage of secondary sites, on invading DNA and away from damaging the host genome.

**Experimental procedures**

**Protein and DNA preparation**

SgrAI enzyme and DNA were prepared as described (18). The sequences of DNA oligonucleotides used are shown (Sequences 1–4).

**Data Set 5: Fluorescence measurements with doubly labeled 18M-1 (Flo–18M-1–Rox)**

Doubly labeled 18M-1 (Flo–18M-1–Rox) was prepared by annealing single-stranded Flo–18M-1–bot (possessing a covalently linked 5′ fluorescein (6-FAM = 6-carboxyfluorescein) connected to the 5′ phosphate via a trans-4-amino cyclohexanol linker, Fig. S1A; excitation = 495 nm; emission = 520 nm), and single-stranded Rox–18M-1–top (possessing a covalently linked 5′ rhodamine-X or 5(6)-carboxy-X-rhodamine connected to the 5′ phosphate via a 6-amino hexan-1-ol linker, Fig. S1B; excitation = 575 nm; emission = 603 nm), both prepared synthetically from a commercial source (Sigma-Genosys, Inc.). Reactions were carried out in 1.5 ml volume in a 2 ml cuvette with constant stirring and consisted of 10 nM Flo–18M-1–Rox, 50 nM to 1 μM SgrAI, and 0–1 μM unlabeled PC DNA in buffer A (50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 1 mM DTT, pH 8.0) supplemented with 10 mM MgCl₂ and at 25 °C. Fluorescence measurements were taken using a ISS PC-1 fluorimeter with 495-nm excitation and emission monitored at 520-nm emission via monochromator and 1-mm (8-nm spectral width) slits. Intensity measurements were taken in 0.1-ms readings and averaged over 10 iterations for readings approximately every 1.1 s. After DNA cleavage, dissociation from SgrAI, and strand separation, FRET between the Flo and Rox labels is lost, resulting in an increase in Flo emission (Fig. S2A).

**Data Sets 4 and 6: Fluorescence measurements with Rox-labeled 18M-1 (Rox–18M-1) and Flo-labeled PC DNA (Flo–PC)**

These data were measured as described (18) for Data Sets 1–3 in that work, but with Rox-labeled 18M-1 (Rox–18M-1) (Rox–18M-1–top annealed to unlabeled 18M-1–bot) and Flo–PC (Flo labeled PC top and unlabeled PC–bot). In the case of Data Set 4, SgrAI was added last to the mixture of DNA in buffer A (with no divalent cation) at 25 °C (1.5 ml of total volume), and emission was followed through the 590-nm cut-on filter (measuring Rox and Flo emission signals) and at 585 nm (to monitor Flo emission). Data Set 6 differed in the inclusion of 10 mM MgCl₂, adding Rox–18M-1 last to the reaction and following the Flo emission at 520 nm. The corrected filter data used in data fitting was calculated as shown below. For Data Set 6,

\[
\text{Correction factor ratio } C = \frac{I(\text{Flo–PC})_{590\text{cof}}}{I(\text{Flo–PC})_{520}} \quad \text{(Eq. 1)}
\]

Correlated filter data (reaction, t)

\[
= I(\text{reaction, } t)_{590\text{cof}} - C \times I(\text{reaction, } t)_{585} \quad \text{(Eq. 2)}
\]

For Data Set 4,

\[
\text{Correction factor ratio } C = \frac{I(\text{Flo–PC})_{590\text{cof}}}{I(\text{Flo–PC})_{585}} \quad \text{(Eq. 3)}
\]

Correlated filter data (reaction, t)

\[
= I(\text{reaction, } t)_{590\text{cof}} - C \times I(\text{reaction, } t)_{585} \quad \text{(Eq. 4)}
\]

where \(I(\text{Flo–PC})_{590\text{cof}}\), \(I(\text{Flo–PC})_{520}\), and \(I(\text{Flo–PC})_{585}\) are the intensities from a solution containing only Flo–PC and measured through the 590-nm cut-on filter and using a monochromator at 520 and 585 nm, respectively.

**Data Set 7: DNA-cleavage reactions using Flo–18M-1–Rox and denaturing PAGE**

Doubly labeled 18M-1 (Flo–18M-1–Rox) was prepared as described above for Data Set 5. The reactions were performed in 100 μl of total volume and consisted of 50 nM Flo–18M-1–Rox, 1 μM SgrAI, and 0–1 μM unlabeled PC DNA in buffer A at 25 °C in 100 μl of total volume. The reactions were initiated by mixing solution A (containing Flo–18M-1–Rox) with solution B (containing SgrAI and PC DNA) at time = 0. At various times after mixing, 5-μl aliquots were removed and quenched in a separate tube with an equal volume of quench solution (containing 80% formamide, 50 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol). An initial 2.5 μl of aliquot was taken from

\[
18M-1-top \quad 5'-\text{GAGTCCAACCGTGCTGAG-3'}
18M-1-bot \quad 3'-\text{CTCAGGTTGGCCACGACTC-5'}
\]

PC-top \quad 5'-\text{GATGGCTGCTGGGTTCTTAC-3'}
PC-bot \quad 3'-\text{CTACGCACCCAGAAGTGTTGGCC-5'}

Sequences 1–4

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each solution (A and B) before mixing to represent time = 0 s. Aliquots were analyzed by electrophoresing on 20% acrylamide (19:1 acrylamide:bis-acrylamide) gels containing 4 M urea and 1× TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) and scanned with a Phoros phosphoimager (Bio-Rad) for Flo and Rox fluorescent bands. The bands were integrated for their intensity using the software ImageLab (Bio-Rad). The percentage of DNA cleaved was calculated by dividing the intensity of the cleaved DNA band over the sum of the cleaved and uncleaved DNA bands. These data, as a function of time after initiation, were used in subsequent analyses and data fitting.

FRET titrations to measure association of DNA-bound SgrAI into ROO filament

Titrations were performed with 50 nM Rox–18M-1, 2–4 μM SgrAI, and varied concentrations of Flo–PC DNA or Flo–18M-1 in buffer A supplied with 10 mM CaCl2, in 1.5 ml of total and maintained at 25 °C. Care was taken to ensure an excess of SgrAI over that of the DNA. Excitation was done using 498 nm, and emission spectra were collected (in a ratio with excitation) following 2 min of incubation with constant stirring after each addition of Flo-DNA. Flo labeled DNA was a mixture of labeled and unlabeled (1:9 Flo–PC:PC and 1:4 Flo–18M-1:18M-1). The resulting spectra were corrected for dilution of the added DNA, for Flo emission (using a reference Flo-DNA only spectrum scaled by relative emission at 570 nm), and for Rox emission caused by absorbance at 498 nm (using the spectrum before added Flo-DNA). The resulting average emissions at 602–612 nm (or 603–613 nm) were plotted versus concentration of Flo-DNA and fit to the Hill equation (see below). In a separate experiment, the titration was repeated without the presence of divalent cations, using 200 nM Rox–18M-1, 2 μM SgrAI, and 2 μM Flo–PC. After recording the spectrum, 10 mM MgCl2 was added. The spectra were recorded 15 and 30 min later, and the corrected spectrum calculated for evidence of residual FRET (to test for trapping of the Rox–18M-1 in the oligomer).

Analytical fitting of data

The Flo emission (Data Set 5) and DNA cleavage (Data Set 7) data were fit using the software Kaleidagraph (Synergy Software) to a single exponential function, and a rate constant was determined in Equation 5,

$$y = a + b \times (1 - e^{-kt-c}) \quad \text{(Eq. 5)}$$

where $y$ is the parameter to be fit (increase in FRET signal, fluorescence, or percentage of cleaved DNA); $a$ and $b$ are constants to be fit in each reaction; $k$ is the rate constant; $t$ is time; and $c$ is a time correction for data where initiation is later than time = 0.

The titration data were fit using Kaleidagraph (Synergy Software) and the following Hill equation,

$$y = a + b \times \frac{[EP_{40}]^n}{(K_{1/2})^n + [EP_{40}]^n} \quad \text{(Eq. 6)}$$

where $y$ is the average of the intensities at 602–612 nm (or 603–613 nm) of the fluorescence emission at each total concentration of EP$_{40}$ (equal to half the concentration of added PC DNA); $K_{1/2}$ is the concentration of EP$_{40}$ (total) where the average emission intensity is half-maximal; and $N$ is the Hill coefficient (a measure of the cooperativity of the reaction).

Global data fitting

Global data fitting was performed with Kintek Global Kinetic Explorer version 6.2.170301 (Kintek Global Kinetic Explorer Corp.) (37–39). Data fitting was as described in the accompanying article (18), and equations for each model are provided in the supporting information.

Error analysis

The FitSpace module of Kintek GKE (39) was used to determine error boundaries for fitted rate constants at the 0.95 $\chi^2$ threshold, meaning that values within these boundaries result in $\chi^2$, the sum of the squares of residuals between experimental and fitted values, less than or equal to the (minimum or best $\chi^2$)/0.95 (39). In some cases, limits on parameters tested were imposed as described. In the case of models using Data Sets 5–7 (Table 1), weighting was used to allow each independent data set (each “experiment” in Kintek GKE) equal weighting in the FitSpace calculation (to avoid overweighting of those data sets with higher $\chi^2$). Weights were calculated as 1000/($\chi^2$ for that particular data set) for each data set.

Data simulations

Where indicated, rate constants from Kintek GKE data fitting were used with new or existing models to predict concentrations of different reaction species as a function of time in the reaction, including at equilibrium, to address specific questions or to compare with other experimental data. In such cases, the equations used for the model and the rate constants being used are given (or reference to previously presented models and rate constants given). For the introduction of cooperativity into models, the dissociation rate constants for SgrAI–DNA complexes from ROO filament were made serially slower by a factor ($X$) with larger ROO filament. For example, if the rate constant for dissociation is 0.08 s$^{-1}$, and $X$ is 1.5, then the dissociation of SgrAI–DNA complexes from a ROO filament is 0.08/1.5 s$^{-1}$ when the ROO filament is composed of two SgrAI–DNA complexes, 0.08/1.5$^2$ s$^{-1}$ when composed of three SgrAI–DNA complexes, and (0.08/ (1.5)$^3$) s$^{-1}$ when composed of four SgrAI–DNA complexes.

Author contributions—C. K. P. and N. C. H. formal analysis; C. K. P., J. L. S., C. B., L. E. B., J. S., C. H., and N. C. H. investigation; C. K. P., J. L. S., C. B., L. E. B., J. S., C. H., and N. C. H. writing-original draft; N. C. H. project administration. C. K. P., N. C. H. validation; N. C. H. visualization; N. C. H. methodology; N. C. H. curation; N. C. H. supervision; N. C. H. funding acquisition; N. C. H. writing-review and editing; N. C. H. conceptualization; N. C. H. resources; N. C. H. data curation; N. C. H. supervision; N. C. H. funding acquisition; N. C. H. validation; N. C. H. visualization; N. C. H. methodology; N. C. H. writing-original draft; N. C. H. project administration.

Acknowledgments—We thank Robert M. Blumenthal for helpful discussions.

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