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Investigation of Binding between recA Protein and Single-Stranded Polynucleotides with the Aid of a Fluorescent Deoxyribonucleic Acid Derivative†

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ABSTRACT: The availability of ϵDNA, a fluorescent ssDNA derivative, has made it possible to examine quantitatively the interactions between recA protein and single-stranded polynucleotides. Fluorescence titrations of ϵDNA with recA protein and vice versa establish that each recA protein monomer covers 5.5 ϵDNA nucleotides and that the dissociation constant of the recA–ϵDNA complex is 10 nM. Fluorescence titrations of recA protein–ϵDNA mixtures with poly(dT) establish that each recA protein monomer covers 5.1 poly(dT) nucleotides and that the dissociation constant of the recA–poly(dT) complex is 0.03 nM. Observations on how the addition of ssDNA affects the fluorescence of recA protein–ϵDNA mixtures establish that the dissociation constant of the recA–ssDNA complex exceeds 20 μM. Stopped-flow kinetics in which excess recA protein binds to ϵDNA indicate that $k_2 = 6 \times 10^6$ M^{-1} s^{-1} for the process. A more approximate kinetic technique indicates that recA protein binds to ϵDNA at least one-tenth as fast as to poly(dT); the rate constant for dissociation of recA–ϵDNA exceeds that for recA–poly(dT) by at least 30-fold. ϵDNA is proven to be a versatile reagent for studying single-stranded polynucleotide–protein interactions. Not only can its own complexes with protein be investigated but also, under suitable circumstances, it can be used as a fluorescent probe to explore complexes incorporating nonfluorescent polynucleotides.

ϵDNA,[1] a highly fluorescent modified form of ssDNA, is readily prepared by treating ssDNA with chloroacetaldehyde (Lee & Wetmur, 1973; Silver & Fersht, 1982). The fluorescence of an ϵDNA solution is markedly enhanced by the addition of recA protein; subsequent introduction of ATP or ATPγS at suitable concentrations produces a further substantial rise in fluorescence. These fluorescence changes are useful for investigating the properties of recA protein. They have enabled us to establish tentative values for the stoichiometry governing the binding of recA protein to ϵDNA under various reaction conditions and to confirm that ATP and ATPγS bind to the recA–ϵDNA complex in a highly cooperative process (Silver & Fersht, 1982).

We describe here a more quantitative evaluation of the interactions between recA protein and ϵDNA and show how it is possible to use ϵDNA to probe the nature of the interactions between recA protein and other, nonfluorescent, single-stranded polynucleotides. All these experiments have been performed in the absence of any nucleoside triphosphates. We expect them to guide further efforts to study the far more complex systems incorporating NTP's. They also emphasize further the potential utility of ϵDNA for investigating protein–ssDNA interactions in other systems.

Experimental Procedures

Materials

We employed the same ϵDNA sample used previously (Silver & Fersht, 1982). ssDNA was prepared by heat denaturing highly polymerized calf thymus dsDNA (purchased from Sigma). Poly(dT) was purchased from P-L Biochemicals, Inc. One stock recA protein preparation was used for most experiments (Cotterill et al., 1982). Its purity exceeds 98%. Some experiments employed another batch isolated by Cotterill et al. of apparently greater purity. As far as we can tell, the two samples of recA protein used here and also the one employed in our earlier work behave identically in the fluorescence experiments described.

Methods

All fluorescence titrations were performed by adding small volumes of reactants to 1.0 mL of standard buffer contained in a cuvette thermostated at 25 °C, mixing by gentle shaking, and recording the fluorescence with a Perkin-Elmer MPF-44B instrument. Standard buffer consisted of pH 7.5 20 mM Tris-HCl holding 10 mM $MgCl_2$ and 1 mM dithiothreitol

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[1] Abbreviations: ss, single stranded; ds, double stranded; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); NTP, nucleotide triphosphate; poly(ϵA), poly(1,N^6-ethenoadenylic acid); ϵDNA, the product obtained by treating ssDNA with chloroacetaldehyde, which contains 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine residues; recA, recA protein in hyphenated expressions and equations; p(dT), poly(dT) in equations; SSB, *Escherichia coli* single-strand binding protein; Tris, tris(hydroxymethyl)aminomethane.

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Table I: Determination of Stoichiometry and Dissociation Constant of recA–ϵDNA Complex by Fluorescence Titration[a]

run no.	method[b]	[recA] (μM)[c]	ϵDNA nucleotides per recA protein monomer[d]	no. of points[e]	$K_{d\epsilon}$ (nM)[f]
1-5	A to ϵ	0.50-1.0	7.0 ± 0.2	16	19 ± 3
6-8	A to ϵ	0.21-0.24	6.4 ± 0.4	20	10 ± 1
1-8	A to ϵ	0.21-1.0	6.8 ± 0.2	36	14 ± 1
9-10[g]	A to ϵ	0.14-0.15	5.6 ± 0.1		5-10
11-13[g]	A to ϵ	0.08-0.10	5.2 ± 0.4		2-10
14-17	ϵ to A	1.2-1.5	6.0 ± 0.1	13	28 ± 3
18-22	ϵ to A	0.61-0.75	5.7 ± 0.2	14	8 ± 1
23-25	ϵ to A	0.24	5.2 ± 0.5	19	10 ± 1

[a] All experiments were performed under standard conditions. [b] A to ϵ signifies addition of recA protein to ϵDNA and ϵ to A the reverse. [c] [recA] at the equivalence point. [d] The average value (and standard error), obtained from the intersection of the extrapolated initial and final slopes of the titration curves, as illustrated in Figure 2. [e] Number of points used to calculate the tabulated $K_{d\epsilon}$. [f] The average value for $K_{d\epsilon}$, the dissociation constant for the recA–ϵDNA complex (eq 1). As explained in the text, it relies on expressing [ϵDNA] as recA protein equivalents, i.e., [ϵDNA] expressed as nucleotides divided by the number of ϵDNA nucleotides covered by a recA protein monomer. [g] Values for stoichiometries and $K_{d\epsilon}$'s for runs 9–13 were obtained by the curve-fitting procedure illustrated in Figure 1.

(Silver & Fersht, 1982). Polynucleotide concentrations are reported as nucleotide residues. Standard errors are given for all average values.

Stopped-Flow Fluorescence Kinetics. The fluorescence changes that result when equal volumes of two reactant solutions are mixed in the stopped-flow fluorometer were monitored with a storage oscilloscope and an Aminco DASAR computer with output to a Bryans XY/t recorder (Fersht et al., 1975; Mulvey & Fersht, 1978). Excitation was at 300 nm with a 20-nm band-pass; emission was monitored through a CW 12 cut-off filter. In the measurements of the rate of binding of recA protein to ϵDNA, one syringe held the protein and the other ϵDNA. Efforts to measure the rate of transfer of recA protein between polynucleotides involved mixing poly(dT) with a solution holding recA protein plus ϵDNA or ϵDNA with a solution holding recA protein plus ssDNA.

Results

Measurement of Dissociation Constant for recA–ϵDNA Complex. Fluorescence titrations that involve the addition either of recA protein to 3.7–7.4 μM ϵDNA or of ϵDNA to 0.6–1.2 μM recA protein show a significant curvature near the equivalence point because of dissociation of the recA–ϵDNA complex (Silver & Fersht, 1982). We have now extended these measurements to progressively lower reagent concentrations in order to evaluate $K_{d\epsilon}$, the dissociation constant for the recA–ϵDNA complex (eq 1). Figure 1 illustrates

$$K_{d\epsilon} = [\text{recA}][\epsilon\text{DNA}]/[\text{recA–}\epsilon\text{DNA}] \quad (1)$$

three of the runs performed. Each titration coincidentally provides another determination of the stoichiometry of the recA–ϵDNA complex (Table I), a quantity of importance for the calculations below.[2] As previously, the point of intersection of the extrapolated initial and final linear segments of the experimental titration curve (Figure 2, P) was taken as defining the stoichiometry of the recA–ϵDNA complex.

For runs involving the addition of recA protein to ϵDNA, the following procedure has afforded the tabulated values for $K_{d\epsilon}$ [cf. Toulmé & Hélène (1980)]. It has been applied to every point that lies near the equivalence point for a run and that deviates significantly from the ideal titration curve, e.g.,

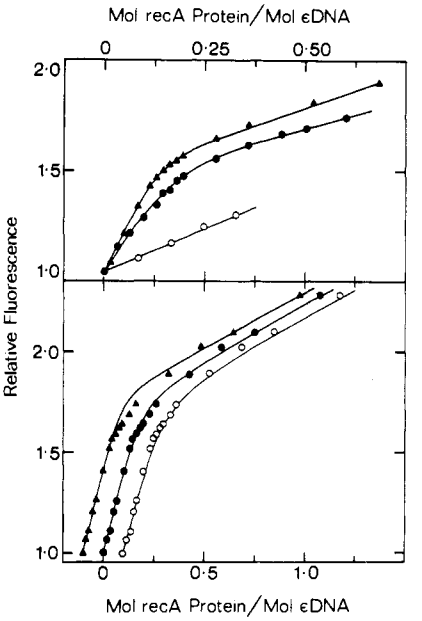

FIGURE 1: Fluorescence titrations of ϵDNA with recA protein at low [ϵDNA] under standard conditions. Experimental points and theoretical titration curves are shown. The upper panel describes the following: (a) Addition of 24.4 μM recA protein to 1.5 nmol of ϵDNA (▲). This is run 7 of Table I. Calculation of the curve assumes each recA protein monomer binds 7.0 ϵDNA nucleotides and $K_{d\epsilon}$ = 10 nM. (b) Addition of 6.1 μM recA protein to 0.375 nmol of ϵDNA (●). This is run 11 of Table I. Calculation of the curve assumes each recA protein monomer binds 5.5 ϵDNA nucleotides and $K_{d\epsilon}$ = 5 nM. (c) Addition of 24.4 μM recA protein to 1.5 nmol of ϵDNA in the presence of 30 μM poly(dT) (○). The lower panel illustrates run 10 of Table I (addition of 24.4 μM recA protein to 0.75 nmol of ϵDNA). It shows curves calculated if each recA protein monomer binds 5.5 nucleotides of ϵDNA and if $K_{d\epsilon}$ = 5 (▲), 10 (●), and 15 (○) nM. For clarity of illustration, the first and last curves are displaced horizontally.

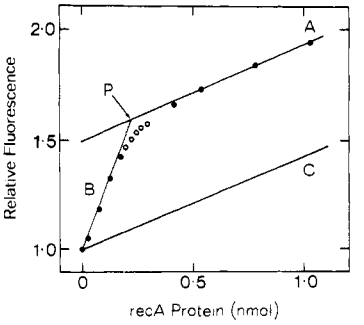

FIGURE 2: Plot of run 7, Table I, to illustrate methods for calculating tabulated data. See text for explanations.

[2] Polynucleotide concentrations are expressed as recA protein equivalents for all calculations of K_{eq}'s. These are obtained by dividing the polynucleotide concentration in nucleotides by the number of nucleotides per recA protein monomer in the appropriate complex. The recA protein concentration is calculated in terms of the monomeric unit since the stoichiometry of the oligomeric proteins is unknown.

Table II: Determination of Stoichiometry of the recA-p(dT) Complex and Equilibrium Constant for Transfer of recA Protein from ϵDNA to Poly(dT)[a]

no. of runs	$[recA]_0$ (μM)[b]	$\frac{[\epsilon DNA]_0}{[recA]_0}$[b]	poly(dT) nucleotides per recA protein monomer[c]	no. of points[d]	$K_{\epsilon T}$
8	0.12-0.25	3.1-7.4	4.7 ± 0.1	8	210 ± 30
7	0.49-2.44	1.5-3.6	5.2 ± 0.2	4	420 ± 100
5	0.61-1.83	5.0-7.5	5.3 ± 0.2	7	300 ± 40
3	0.61-0.95	9.7-15.1	5.4 ± 0.2	4	300 ± 40
23[e]	0.12-2.44	1.5-15.1	5.1 ± 0.1	23	290 ± 30
8[f]	0.11-0.71	0.7-2.9	5.2 ± 0.2		290 ± 30

[a] All experiments were performed under standard conditions. See text for details of calculations. [b] Poly(dT) was added to a solution, the composition of which is defined by columns 2 and 3. [c] The average value (and standard error), obtained by extrapolating the portion of the titration curve of steepest slope to the fluorescence intensity corresponding to complete transfer of recA protein from ϵDNA to poly(dT). [d] Number of points used to calculate the tabulated $K_{\epsilon T}$ that governs the transfer of recA protein from ϵDNA to poly(dT), as defined by eq 5. Some runs gave more than one reliable point; some gave none. [e] Summary of all runs above. [f] In these runs recA protein was added to a mixture of ϵDNA + poly(dT) (see Figure 4). Column 2 specifies [recA] at the equivalence point for conversion of all the poly(dT) to its recA protein complex, and column 3 defines $[\epsilon DNA]_0/[p(dT)]_0$.

the open circles for run 7 in Figure 2. This amounts to 1–2 points per run at high reactant concentrations, comparable to those previously employed (runs 1–5), but to 5 or more at lower concentrations. The latter titrations give points with greater deviations and provide more reliable values for $K_{d\epsilon}$.

The analysis assumes that only three relevant species are present in solution: recA protein, ϵDNA, and recA–ϵDNA complex. Two stoichiometric equations express this condition:

$$[recA]_{TOT} = [recA] + [recA\text{–}\epsilon DNA]$$

$$[\epsilon DNA]_{TOT} = [\epsilon DNA] + [recA\text{–}\epsilon DNA]$$

Let θ designate the fraction of $[\epsilon DNA]_{TOT}$ that is complexed at any point:

$$\theta = [recA\text{–}\epsilon DNA]/[\epsilon DNA]_{TOT}$$

Substitution of the three expressions into eq 1 yields eq 2.

$$K_{d\epsilon} = [(1/\theta) - 1]([recA]_{TOT} - \theta[\epsilon DNA]_{TOT}) \quad (2)$$

Equation 2 determines $K_{d\epsilon}$ once θ is evaluated and $[\epsilon DNA]_{TOT}$ is converted to recA equivalents.[2] The latter is readily done by dividing $[\epsilon DNA]_{TOT}$, expressed as nucleotides, by the stoichiometry deduced for the recA–ϵDNA complex in the titration under examination. Figure 2 explains the evaluation of θ. Line A and line C, parallel to line A but passing through the initial point of the titration experiment, define respectively F_{100} and F_0 for each experimental point. F_{100} and F_0 represent the relative fluorescence corresponding to 100% and 0% conversion of the ϵDNA present into recA–ϵDNA complex. Since experiment provides F_{obsd}, $\theta = (F_{obsd} - F_0)/(F_{100} - F_0)$.

The method just outlined has supplied the values of $K_{d\epsilon}$ for runs 1–8 (Table I). A similar treatment has been applied to runs 14–25, where ϵDNA was added to recA protein. Here, $\theta = [recA\text{–}\epsilon DNA]/[recA]_{TOT}$, and eq 3 defines $K_{d\epsilon}$. (Again,

$$K_{d\epsilon} = [(1/\theta) - 1]([\epsilon DNA]_{TOT} - \theta[recA]_{TOT}) \quad (3)$$

experiments at lower concentrations of recA protein provide the more accurate estimates of $K_{d\epsilon}$.)

Reliable analysis of runs 9–13 requires searching for theoretical titration curves that reproduce the experimental data. Figure 1 shows that the following procedure successfully meets this goal. It begins with eq 4, obtained by rearranging eq 2.

$$\text{let } \chi = [recA]_{TOT} + [\epsilon DNA]_{TOT} + K_{d\epsilon}$$

$$\theta = [\chi - (\chi^2 - 4[\epsilon DNA]_{TOT}[recA]_{TOT})^{1/2}]/(2[\epsilon DNA]_{TOT}) \quad (4)$$

Equation 4 defines θ for all points in the calculated curve in terms of the amount of recA protein added, an assumed value for $K_{d\epsilon}$ and an assumed stoichiometry for the recA–ϵDNA complex (needed[2] to obtain $[\epsilon DNA]_{TOT}$). Values for the latter two quantities are tried that are roughly comparable to those already obtained for runs 1–8 ($K_{d\epsilon}$ = 1–50 nM; $[\epsilon DNA]/[recA]$ = 5–7 in the recA–ϵDNA complex), until a satisfactory fit between theory and experiment is achieved (note curves in lower panel of Figure 1). The comparison is made by empirically converting θ to relative fluorescence. As Figure 2 illustrates, the calculated rise in relative fluorescence may be represented as the sum of a contribution from the intrinsic recA protein fluorescence (defined by experimental line C) and that from recA–ϵDNA complex formation (obtained by multiplying each calculated θ by the measured difference between the values for the y intercept of lines A and C).

The conclusion that $K_{d\epsilon}$ = 10 nM and that each recA protein monomer covers 5.5 ϵDNA nucleotides adequately summarizes the data in Table I. These numbers provide a consistent basis for treating the experiments described below.

Fluorescence Titrations of recA Protein with Poly(dT) in the Presence of ϵDNA. Sequential additions of 0.5–1-μL aliquots of 920 μM poly(dT) to a solution containing 0.6 μM recA protein plus 9.2 μM ϵDNA cause repeated decreases in the fluorescence of the solution. Indeed, as the left-hand curve in Figure 3 shows, the initial fall in relative fluorescence is proportional to the total amount of poly(dT) introduced. The affinity of recA protein for poly(dT) is very great [McEntee et al., 1981; note the control (lowest) titration in the top panel of Figure 1]. It must be that we are detecting the progressive transformation of the original recA–ϵDNA complex into the more stable recA–poly(dT) one. The experiment represents a titration of recA protein with poly(dT).

Such a titration is best done by performing several additions of poly(dT), as described, and then jumping [poly(dT)] to >15 μM. This defines a final minimum relative fluorescence, F_∞, indicated by a terminal horizontal line in Figure 3. The measured fluorescence corresponding to F_∞ is found to equal the sum of the separate fluorescences of recA protein and ϵDNA under the experimental conditions. A line is drawn through the initial experimental points and extrapolated until it intersects the horizontal line corresponding to F_∞. The point of intersection defines the amount of poly(dT) required to combine with all the recA protein present.

Table II summarizes the data from many such titrations. They have been divided into four groups. The first represents all those at the lowest recA protein concentrations employed. The next three indicate titrations that used excess recA protein, equivalent amounts of recA protein and ϵDNA, and excess ϵDNA, respectively. Figure 3 illustrates one run from each

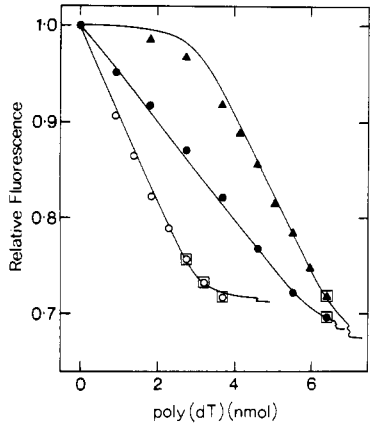

FIGURE 3: Fluorescence titrations of recA protein with 920 μM poly(dT) in the presence of ϵDNA under standard conditions. From left to right the runs contained respectively 0.61 nmol of recA protein + 9.2 nmol of ϵDNA (○; ϵDNA/recA = 15), 1.22 nmol of recA protein + 7.5 nmol of ϵDNA (●; ϵDNA/recA = 6), and 1.22 nmol of recA protein + 3.7 nmol of ϵDNA (▲; ϵDNA/recA = 3). To obtain the theoretical curves shown, it was assumed that a recA protein monomer can bind 5.5 ϵDNA nucleotides or 5.01 (○, ●) or 5.45 (▲) poly(dT) nucleotides, that $K_{d\epsilon}$ = 10 nM, and that K_d for the recA–poly(dT) complex is 300 times smaller. See text for explanation of calculations. The horizontal line at the end of each curve designates the final measured relative fluorescence, obtained by adding a large excess of poly(dT). The significance of the boxed points is explained in the text.

of the last three categories. In each titration the recA protein–poly(dT) equivalence point has been estimated by extrapolating the *steepest* descending portion of the experimental titration curve to F_∞, as explained above. Note that these lines of maximum slope are not shown in Figure 3.

There are several "boxed" data points at the bottom of Figure 3. These points lie above those extrapolated lines of steepest descent just mentioned. They also preferably lie appreciably above F_∞. Only two such points arise in most titrations of the kind under examination. Their relative fluorescence exceeds that expected for total conversion of the poly(dT) added into recA–poly(dT) complex. If this enhanced fluorescence signifies the presence of residual excess recA–ϵDNA complex, these points enable us to evaluate $K_{\epsilon T}$. $K_{\epsilon T}$, defined by eq 5, represents the equilibrium constant governing

$$\text{recA–}\epsilon\text{DNA} + \text{p(dT)} \overset{K_{\epsilon T}}{\rightleftharpoons} \text{recA–p(dT)} + \epsilon\text{DNA} \quad (5)$$

transfer of recA protein from ϵDNA to poly(dT).

Calculation of $K_{\epsilon T}$ from the experimental data assumes that only the four species that appear in eq 5 need to be considered (the concentration of free recA protein is negligible). The usual stoichiometric conditions then give eq 6, where [Aϵ]

$$K_{\epsilon T} = \frac{([\text{recA}]_{TOT} - [A\epsilon])/([\epsilon\text{DNA}]_{TOT} - [A\epsilon])}{[A\epsilon]([\text{p(dT)}]_{TOT} + [A\epsilon] - [\text{recA}]_{TOT})} \quad (6)$$

abbreviates [recA–ϵDNA]. [Aϵ] in turn is specified by eq 7

$$[A\epsilon] = [\text{recA–}\epsilon\text{DNA}]_0(F_{obsd} - F_\infty)/(1 - F_\infty) \quad (7)$$

where F_0, the initial relative fluorescence, is 1.0, and [recA–ϵDNA]$_0$, the initial concentration of recA–ϵDNA complex, is determined by [recA]$_0$, [ϵDNA]$_0$, and $K_{d\epsilon}$. Our best evaluations of $K_{\epsilon T}$, summarized in the last column of Table II, rely on the assumption that $K_{d\epsilon}$ = 10 nM and that a recA protein monomer can cover 5.5 ϵDNA nucleotides or the number of poly(dT) nucleotides estimated from the equivalence point for the particular run under examination.[2]

Addition of recA Protein to Mixtures of ϵDNA and Poly(dT). Figure 4 illustrates the typical fluorescence changes

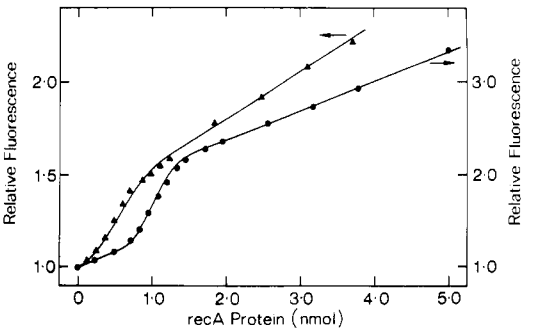

FIGURE 4: Fluorescence titrations of ϵDNA–poly(dT) mixtures with recA protein under standard conditions. A solution of 123 μM recA protein was added to 3.75 nmol of ϵDNA + 1.3 nmol of poly(dT) (▲) or to 3.1 nmol of ϵDNA + 4.0 nmol of poly(dT) (●). Calculation of the theoretical curves shown assumes that each recA protein monomer can bind 5.5 ϵDNA nucleotides or 5.26 (▲) or 5.37 (●) poly(dT) nucleotides, that $K_{d\epsilon}$ = 10 nM, and that K_d for the recA–poly(dT) complex is 300 times smaller.

observed when ϵDNA–poly(dT) mixtures are titrated with recA protein (at appropriate reagent concentrations). Three phases are seen. The fluorescence rises gradually initially. The slope of that rise is characteristic of the intrinsic fluorescence of isolated recA protein. During this stage of the experiment the fluorescence is measured ~5 min after each addition of recA protein, when the high initial fluorescence has relaxed to a lower final, time-independent value. A more rapid rise in fluorescence then ensues, to be followed eventually by a terminal gradual increase that parallels the first phase. The last two segments resemble the fluorescence titration of ϵDNA with recA protein (cf. Figure 1).

The onset of the rapid rise in fluorescence should signal complete conversion of the poly(dT) present to the recA–poly(dT) complex. The composition of the complex is defined by the point of intersection of the extrapolated line of steepest ascent with the extrapolated line through the initial points (these lines are absent from Figure 4). Eight titrations give 5.2 ± 0.2 poly(dT) nucleotides per recA monomer (Table II, last line). The Discussion explains how the theoretical curves shown in Figures 3 and 4 were calculated.

Relative Affinity of recA Protein for ϵDNA and ssDNA. The introduction of high concentrations of ssDNA into solutions of the recA–ϵDNA complex slightly reduces the fluorescence of those solutions. For example, 200 μM ssDNA lowered the fluorescence of a solution holding 3 μM ϵDNA plus 0.6 μM recA protein from 0.466 to ≥0.447. The sum of the separate fluorescences of ϵDNA and recA protein at these concentrations was 0.260. Introduction of 200 μM dsDNA, instead, produced approximately half as great a fall in fluorescence.

Data of this kind set an upper limit to $K_{\epsilon S}$, the equilibrium constant controlling the transfer of recA protein from ϵDNA to ssDNA (eq 8). The calculation resembles others already

$$\text{recA–}\epsilon\text{DNA} + \text{ssDNA} \overset{K_{\epsilon S}}{\rightleftharpoons} \text{recA–ssDNA} + \epsilon\text{DNA} \quad (8)$$

explained. It assumes that only the four species that appear in eq 8 and free recA protein need be considered, that $K_{d\epsilon}$ = 10 nM, and that each recA protein monomer can bind 5.5 nucleotides of ssDNA (we have not measured this number) or ϵDNA.[2] Application of the usual stoichiometric conservation equations to the particular experiment cited above gives the following: [recA–ϵDNA] = 0.5 μM prior to the addition of ssDNA and ≥0.45 μM after; in the final solution, [ϵDNA] ≤ 0.1 μM, [recA] = 0.05 μM, [recA–ssDNA] ≤ 0.1 μM, and [ssDNA] = 38 μM. The calculated value for $K_{\epsilon S}$ is $\leq 6 \times 10^{-4}$.

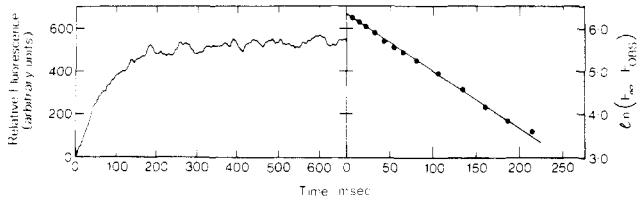

FIGURE 5: Detection of fluorescence rise characterizing mixing of recA protein with ϵDNA in the stopped-flow fluorometer. The left side shows the recorder trace obtained (excitation at 300 nm, 5-ms time constant, 20-nm band-pass). The right side presents a plot of the experimental kinetic data obtained according to the first-order rate law (k_{obsd} = 13.3 s^{-1}). Concentrations after mixing were 2.35 μM recA protein and 1.2 μM ϵDNA.

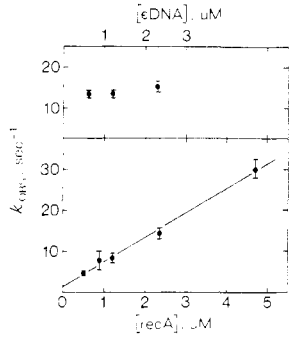

FIGURE 6: Determination of rate constant for binding of recA protein to ϵDNA. The bottom panel plots k_{obsd} values (obtained from data like that shown in Figure 5) as a function of $[recA]_0$. The straight line has a slope of 6.0×10^6 M^{-1} s^{-1} and an intercept of 1.5 s^{-1}. The points represent the average and standard deviation for 6, 4, 6, 16, and 6 independent runs at each $[recA]_0$ (reading from left to right), measured for $[\epsilon DNA]_0$ = 0.6, 0.6, 0.6, 0.6–2.3, and 1.2 μM, respectively. The top panel shows that k_{obsd} for 2.35 μM $[recA]_0$ is independent of $[\epsilon DNA]_0$, within experimental error.

A dozen such runs yield $K_{\epsilon S} \leq 5 \times 10^{-4}$ as the preferred value.

Kinetic Experiments. When 0.6–2.3 μM ϵDNA reacts with 0.5–4.7 μM recA protein, the fluorescence of the polynucleotide rises markedly.[3] The rate of this rise can be measured with the stopped-flow fluorometer. No rise is seen when 0.97 μM ϵDNA is mixed with buffer or when 2.35 μM recA protein is combined with 0.54 μM poly(ϵA), a polynucleotide to which recA protein binds poorly (Silver & Fersht, 1982). The kinetic experiments are best performed with $[recA]_0 \gg [\epsilon DNA]_0$; the captions to Figures 5 and 6 summarize the conditions employed (note that the specification $[recA]_0 \gg [\epsilon DNA]_0$ refers to recA protein equivalents[2] of ϵDNA). Under these circumstances the rise in fluorescence follows a simple exponential curve, and the corresponding first-order rate plot defines the pseudo-first-order rate constant, k_{obsd} (Figure 5). Figure 6 demonstrates that the k_{obsd}'s measured for 0.5–4.7 μM recA protein depend linearly on $[recA]_0$ and those obtained at 2.35 μM recA protein are independent of $[\epsilon DNA]_0$. The bottom plot in Figure 6 has a slope of 6×10^6 M^{-1} s^{-1} and a y intercept of 1.5 s^{-1}.

Efforts to quantify the rates of transfer of recA protein between polynucleotides have thus far proved less successful. Consider the mixing of ϵDNA with a solution containing ssDNA and recA protein in the stopped-flow machine. The rise in fluorescence that ensues is readily monitored but is quantitatively uninterpretable. It stems from the combination of ϵDNA with both recA protein that is originally bound to ssDNA and with free recA protein. The latter is unfortunately present at relatively high concentrations since recA protein binds to ssDNA so poorly.

The fall in fluorescence that attends the mixing of poly(dT) with a solution containing ϵDNA and recA protein should, in principle, be more amenable to study. The concentration of poly(dT) can be set at a high, constant value; the disappearance of the recA–ϵDNA complex is observed. Nevertheless, we have yet to find experimental conditions that yield a simple first-order decay in fluorescence.

The failures just described led us to attempt to estimate the relative rate constants for the binding of recA protein to ϵDNA and poly(dT) by a different approach. The experiments, performed with the Perkin-Elmer instrument, involve adding recA protein to a mixture of the two polynucleotides (cf. the titrations of Figure 4). The fluorescence is recorded for the first 1–3 min, as it falls to its final value. The final value in all these runs corresponds to total positioning of recA protein on poly(dT). The addition of 0.6 μM recA protein to 14 μM ϵDNA plus 4.3 μM poly(dT) is typical. The earliest measured fluorescence, obtained $\sim$15 s after mixing, shows an enhancement over the final fluorescence that is $\sim$30% of the maximum possible. That maximum is established by measuring the fluorescence of a solution containing 0.6 μM recA protein plus 14 μM ϵDNA. The experiment is interpreted as establishing that at least 0.18 μM recA–ϵDNA has been formed initially upon the addition of recA protein. When this and several other experiments are analyzed by the technique explained in the section below, it is concluded that the rate constant for the binding of recA protein to ϵDNA is at least one-tenth that for its binding to poly(dT). Similar experiments involving the addition of recA protein to ϵDNA/ssDNA mixtures proved uninformative.

Treatment of Competition between ϵDNA and Poly(dT) for Added recA Protein. If $-d[\epsilon DNA]/dt = k_\epsilon[\epsilon DNA][recA]$ and $-d[p(dT)]/dt = k_t[p(dT)][recA]$, division of the first expression by the second, followed by integration between t = 0 and time t yields

$$k_\epsilon/k_t = \ln ([\epsilon DNA]_0/[\epsilon DNA])/\ln ([p(dT)]_0/[p(dT)])$$

When applied to the earliest recorded fluorescence for eight runs, the equation indicates $k_\epsilon/k_t \geq 0.13 \pm 0.02$. The calculations assume that each recA protein monomer can bind 5.5 ϵDNA or 5.1 poly(dT) nucleotides.

Discussion

The titration experiments described quantitatively establish the stoichiometry and strength of binding of recA protein to ϵDNA and to poly(dT) in the absence of a nucleoside triphosphate. Consider first the recA protein–ϵDNA system (Table I and Figure 1). Titrations 14–25 rely on the addition of ϵDNA to recA protein. They are consistent with our previous suggestion, on the basis of three titrations, that a recA protein monomer covers 5.7 $\pm$ 0.1 ϵDNA nucleotides (Silver & Fersht, 1982). This procedure of adding ϵDNA to recA protein was deemed the preferred one for estimating the stoichiometry of the recA–ϵDNA complex. It is not so useful for evaluating $K_{d\epsilon}$, however, because it cannot be extended to the low reactant concentrations required for the best determinations of that quantity. The high intrinsic fluorescence of the ϵDNA being added reduces the sharpness of the break in the titration curve near the equivalence point. This problem grows worse as the reactant concentrations are lowered. The values of 5.5 ϵDNA nucleotides/recA protein monomer and $K_{d\epsilon}$ = 10 nM adequately summarize the data for runs 18–25. Within experimental error, these values are independent of reactant concentrations in those runs. The same values fit runs

[3] The concentrations of reactants after mixing are specified for stopped-flow experiments.

Table III: Dissociation Constants for Three recA–Polynucleotide Complexes under Standard Conditions

complex	K_d (nM)[a]
recA–p(dT)	0.03
recA–ϵDNA	10
recA–ssDNA	⩾20000

[a] The polynucleotide concentrations are expressed as recA protein equivalents—that is, the concentration in nucleotides divided by the number of nucleotides covered by a recA protein monomer. For recA–ssDNA, the latter is assumed to be 5.5.

9–13 of Table I satisfactorily (see Figure 1). Successful analysis of runs 9–13 is important because they involve the addition of recA protein to ϵDNA at the lowest reagent concentrations we could manage.

Runs 1–8 confirm that, at higher reactant concentrations, addition of recA protein to ϵDNA gives an apparent greater coverage of ϵDNA by recA protein (Silver & Fersht, 1982). This may derive from the heterogeneous distribution of fluorescent sites on ϵDNA, as previously suggested, but it is not obvious why the effect vanishes in runs 9–13. It is gratifying that the two titration procedures appear to approach common values for both stoichiometry of coverage and $K_{d\epsilon}$ as the reagent concentrations are reduced.

Titration of recA protein–ϵDNA mixtures with poly(dT) enables us to characterize the interactions between recA protein and poly(dT) in some detail (Table II and Figure 3). The run on the right in Figure 3 is especially interesting. Since it contains a considerable excess of recA protein over ϵDNA, initially added poly(dT) merely consumes that excess protein. Almost no fall in fluorescence is seen. As the "end point" for the titration is approached, the fall in fluorescence accelerates. In a sense, ϵDNA behaves here as an indicator. Because of this, data derived under these conditions are very reliable. We have not established how far [ϵDNA]/[recA] can be reduced and still provide a reliable titration.

The data for poly(dT) in Table II show no significant dependence on reagent concentrations or $[\epsilon DNA]_0/[recA]_0$. The average value of 5.1 nucleotides of poly(dT) per recA protein monomer is quite close of the corresponding number for ϵDNA. Both values agree well with estimates for the extent of coverage of ssDNA by a recA protein monomer, on the basis of recent sedimentation experiments (West et al., 1980). RecA protein binds far more strongly to poly(dT) than to ϵDNA; $K_{\epsilon T}$, the equilibrium constant for the transfer of recA protein from ϵDNA to poly(dT) (eq 5), is ~300. A simple thermodynamic analysis establishes that the dissociation constant for the recA–p(dT) complex is ~0.03 nM (Table III).

Efforts to use ϵDNA to explore recA protein–ssDNA interactions have been less successful because those interactions, at least in the absence of an NTP, are so weak. $K_{\epsilon S}$, the equilibrium constant governing the transfer of recA protein from ϵDNA to ssDNA (eq 8), is certainly $<5 \times 10^{-4}$. This means that the dissociation constant for the recA–ssDNA complex exceeds 20 μM. Our data provide some quantitative measure of the enormous range in affinity that recA protein exhibits toward single-stranded polynucleotides [Table III; cf. McEntee et al. (1981)]. Secondary structure present in ssDNA but absent from ϵDNA or poly(dT) may contribute significantly to this ordering (Krauss et al., 1981; Flory & Radding, 1982).

The titration experiments provide no persuasive evidence for more than one kind of binding site on recA protein under the reaction conditions employed. They are adequately explained without introducing cooperative binding by the protein. Although the electron microscopy studies of Dunn et al. (1982) imply cooperative binding of recA to ssDNA, the parallel observation that recA itself forms filaments in solution in the absence of DNA (Flory & Radding, 1982; Cotterill & Fersht, 1983) means that the classical description of the consequences of cooperative binding need not apply. For example, cooperative binding is usually analyzed in terms of a monomer (or small oligomer) binding initially weakly to DNA, followed by tighter binding of successive monomers. But, if the protein is already oligomerized in solution, it will tend to bind in long tracts. The good match between experimental data and theoretical titration curves seen in Figures 3 and 4 demonstrates these points. The Appendix explains the calculation of these curves. All the calculations employ the unique values $K_{d\epsilon}$ = 10 nM, $K_{\epsilon T}$ = 300, and 5.5 ϵDNA nucleotides/recA protein monomer and the recA–p(dT) stoichiometry determined for the particular run being analyzed. The two runs in Figure 4 are most informative because they, like the control in the top panel of Figure 1, graphically show the preferential binding of recA protein to poly(dT) rather than to ϵDNA. The initial rise in fluorescence is merely that attributable to the intrinsic fluorescence of the protein. The enhanced fluorescence associated with formation of the recA–ϵDNA complex is detected only after all the poly(dT) is converted to its recA protein complex.

The binding of excess recA protein to ϵDNA has been investigated with the stopped-flow fluorometer. If the scheme

$$\text{recA} + \epsilon\text{DNA} \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \text{recA–}\epsilon\text{DNA}$$

describes the binding of recA protein to ϵDNA, the experiments give $k_{obsd} = k_1 + k_2[recA]$. The lower plot in Figure 6 establishes that $k_2 = 6 \times 10^6$ M^{-1} s^{-1}. The y intercept requires $k_1 = 1.5$ s^{-1}. This value, if meaningful, implies $K_{d\epsilon}$ ~ 250 nM for the dissociation of the recA–ϵDNA complex, ~25 times greater than the estimate on the basis of titration data. Because of the well-established propensity of recA protein to undergo self-association (McEntee et al., 1981; Flory & Radding, 1982; Cotterill & Fersht, 1983), the interpretation of the kinetics is, at least, tentative. However, the data are consistent with a two-step binding process where the observed value for k_2 is that for the first step and not the overall process [e.g., pp 115–116 of Fersht (1977)].

Otherwise, the yield of useful kinetic data has been disappointing. Substantial fluorescence changes are seen when ϵDNA is added to a mixture of recA protein with ssDNA or when poly(dT) is added to a mixture of recA protein with ϵDNA. These "stripping" reactions are monitored with the stopped-flow fluorometer. As already explained, quantitative analysis of the kinetic data has yet to be realized. Qualitatively, the rate at which excess poly(dT) strips the recA–ϵDNA complex is appreciably slower than expected if $k_1 = 1.5$ s^{-1} under the reaction conditions.

Comparable transfers of SSB between polydeoxynucleotides have been studied (Schneider & Wetmur, 1982). They are governed by a diffusion-controlled direct-transfer mechanism. The binding of recA protein to ϵDNA is appreciably weaker than that of SSB to polydeoxynucleotides. The recA protein stripping reactions could involve initial dissociation of the original complex. If so, they would afford values for k_1 processes under conditions closely resembling those holding in the titrations. Furthermore, stripping reactions proceeding by a dissociative mechanism result in subsequent competition between the donor and acceptor polynucleotides for free recA protein. Such experiments thus should also define the relative rate constants for the binding of recA protein to ssDNA vs.

ϵDNA and to ϵDNA vs. poly(dT). The latter can also be estimated from the initial time-dependent fluorescence decay seen when recA protein is added to an ϵDNA–p(dT) mixture. The experiments show $k_2(\epsilon DNA)/k_2(poly(dT)) \geq 0.1$. Given that the equilibrium dissociation constant for recA–ϵDNA exceeds that for recA–poly(dT) by ~300 times, k_1 for the former must exceed k_1 for the latter by >30-fold. For these two recA protein complexes at least, the dissociation rate constant is an important, or perhaps the major, determinant of their relative stabilities.

The experiments described here quantitatively establish some features of the interactions between recA protein and single-stranded polynucleotides. They can guide the design of comparable experiments, of greater biochemical relevance, that incorporate NTP's into the reaction medium. One wonders, for example, what a table analogous to Table III will look like. We think the binding of recA protein to ssDNA is relatively weak even in the presence of ATP. Introduction of SSB into the critical ssDNA–ATP–recA complex may lend a necessary thermodynamic stability to that complex but at the cost of preventing the recycling of recA protein (Cox & Lehman, 1982). The present studies also reveal that the potential utility of ϵDNA for studying the nature of protein–ssDNA interactions is greater than originally anticipated. It is clear that under suitable circumstances ϵDNA can be used to elucidate the properties of complexes formed between proteins and nonfluorescent polynucleotides.

Appendix

Calculation of Theoretical Titration Curves in Figures 3 and 4. Essentially the same treatment applies to both experiments. The need is to calculate [recA–ϵDNA] in a mixture of recA protein, ϵDNA, and poly(dT). It is assumed that five species are present: the recA–ϵDNA and recA–poly(dT) complexes plus free recA protein, ϵDNA, and poly(dT). These are here abbreviated Aϵ, AT, A, ϵ, and T, respectively; the subscript T designates the total stoichiometric concentration of a substance.

The calculation utilizes three conservation conditions ($[A]_T = [A] + [A\epsilon] + [AT]$; $[\epsilon]_T = [\epsilon] + [A\epsilon]$; $[T]_T = [T] + [AT]$) and two defined equilibrium constants, $K_{d\epsilon}$ (eq 1) and $K_{\epsilon T}$ (eq 5). Equation A1 results from appropriate substitutions into

$$K_{\epsilon T} = \frac{\left([A]_T - [A\epsilon] - \frac{[A\epsilon]K_{d\epsilon}}{[\epsilon]_T - [A\epsilon]}\right)([\epsilon]_T - [A\epsilon])}{[A\epsilon]\left([T]_T + [A\epsilon] + \frac{[A\epsilon]K_{d\epsilon}}{[\epsilon]_T - [A\epsilon]} - [A]_T\right)} \quad (A1)$$

the definition of $K_{\epsilon T}$. It can be rearranged to eq A2, a cubic

$$(K_{\epsilon T} - 1)[A\epsilon]^3 + [A\epsilon]^2[(2 - K_{\epsilon T})[\epsilon]_T + (1 - K_{\epsilon T}) \times (K_{d\epsilon} + [A]_T) + K_{\epsilon T}[T]_T] - [A\epsilon][(2 - K_{\epsilon T})[A]_T[\epsilon]_T + [\epsilon]_T^2 + K_{d\epsilon}[\epsilon]_T + K_{\epsilon T}[T]_T[\epsilon]_T] + [A]T[\epsilon]_T^2 = 0 \quad (A2)$$

equation, one of whose roots defines [recA–ϵDNA], as required. Since $K_{\epsilon T} = 300 \gg 1$ or 2, eq A3 is actually satisfactory for our needs. In applying this equation, it has been consistently assumed that $K_{d\epsilon} = 10$ nM, $K_{\epsilon T} = 300$, and each recA protein monomer covers 5.5 ϵDNA nucleotides. The coverage of poly(dT) by recA protein is estimated for the run under analysis, by the methods explained in the text.

$$[A\epsilon]^3 + [A\epsilon]^2([T]_T - [\epsilon]_T - K_{d\epsilon} - [A]_T) + [A\epsilon][\epsilon]_T\left([A]_T - [T]_T - \frac{[\epsilon]_T}{K_{\epsilon T}} - \frac{K_{d\epsilon}}{K_{\epsilon T}}\right) + \frac{[A]_T[\epsilon]_T^2}{K_{\epsilon T}} = 0 \quad (A3)$$

The calculated values of [recA–ϵDNA] have been translated into relative fluorescence empirically. For titrations of ϵDNA–recA protein mixtures with poly(dT), this relies upon two fluorescence measurements—that made prior to the addition of any poly(dT), f_0, with an assigned relative fluorescence of 1.0, and that made after the addition of excess poly(dT), f_∞, with a relative value $F_\infty = f_\infty/f_0$. The concentration of recA–ϵDNA corresponding to f_0 is calculated from eq A3; that corresponding to f_∞ is taken as zero. The theoretical curve is obtained by calculating [recA–ϵDNA] from eq A3 and converting these concentrations to relative fluorescences with the relationship

$$\text{rel } F = F_\infty + (1 - F_\infty)[\text{recA–}\epsilon\text{DNA}]/[\text{recA–}\epsilon\text{DNA}]_0$$

To generate the curves in Figure 4, the relative fluorescence is set at 1.0 at the titration origin, prior to any addition of recA protein ([recA–ϵDNA] = 0). The slope of the final linear rise in fluorescence for the experimental titration curve is taken to reflect the intrinsic fluorescence of recA protein. A parallel line through the origin (absent from Figure 4 but designated C in Figure 2) defines that increasing contribution to the total relative fluorescence at any point. To this is added the contribution from recA–ϵDNA complex formation. Equation A3 establishes [recA–ϵDNA] and thus the fraction of ϵDNA that is complexed at any point. Multiplication of that fraction by the difference between the y intercepts of the two parallel lines (cf. lines A and C in Figure 2) establishes the predicted contribution from recA–ϵDNA complex formation to the total calculated relative fluorescence.

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