Direct Observation of Complexes Formed between recA Protein and a Fluorescent Single-Stranded Deoxyribonucleic Acid Derivative†

Marc S. Silver*,‡ and Alan R. Fersht*

ABSTRACT: The reaction of chloroacetaldehyde with single-stranded DNA (ssDNA) yields ϵDNA, a highly fluorescent substance. The binding of recA protein to ϵDNA nearly doubles its fluorescence yield. The enhanced fluorescence signals the formation of a recA–ϵDNA complex. This complex exhibits an ATPase activity as great as that of the corresponding recA–ssDNA complex. Addition of a saturating concentration of adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) to a solution of the recA–ϵDNA complex yields a further rise in fluorescence. Saturation with ATP produces the same rise. The nucleotide triphosphates have converted the recA–ϵDNA complex into the respective ATPγS–recA–ϵDNA and ATP–recA–ϵDNA complexes. The fluorescence changes that accompany the formation of the three complexes have enabled us to (1) establish by titration that recA protein binds to 6.0 ± 0.3 nucleotides of ϵDNA, (2) show that the binding of ATP to the recA–ϵDNA complex is highly cooperative under various conditions, with a Hill coefficient of 2.4–4.9 and K_{app} = 25 ± 2 μM, (3) show that the binding of ATPγS is also highly cooperative, with a Hill coefficient of 3.3–4.2 and $K_{app} \simeq 0.5$ μM, and (4) perform initial measurements on the rate at which recA protein transfers between polynucleotides. The experiments provide the first direct observation of an ATP–recA–ssDNA-like complex, and they illuminate some of the properties of such complexes.

The recA protein shows a remarkable range of activities for so small a molecule. Particularly interesting is its ability to catalyze ATP-dependent DNA strand assimilation. Studies with the recA protein are consequently providing important insights into genetic recombination mechanisms (Geider & Hoffmann-Berling, 1981; Radding, 1981). In particular, the structural features that homologous DNA molecules must possess if they are to undergo recA-promoted[1] strand assimilation are being explored (West et al., 1981; DasGupta & Radding, 1982). Complementary biochemical studies have broadly established the nature of the interactions between recA protein and polynucleotides and nucleotide triphosphates and the factors controlling the ability of recA protein to act as a protease (Weinstock et al., 1981a–c; Craig & Roberts, 1980). These experiments rely primarily on selective filter-binding assays to characterize the various recA–DNA complexes presumed to be involved. Data obtained with the ultracentrifuge and electron microscope provide supplementary information. A spectroscopic method that allows recA-catalyzed reactions to be continuously monitored would make it possible to examine more closely the nature of these complexes and the dynamics governing their formation and disappearance. We describe here such a method and the initial results it has yielded.

The essential reagent is ϵDNA, obtained by treating ssDNA with chloroacetaldehyde. This highly fluorescent modification of ssDNA has been known for nearly 10 years, but its great potential for investigating protein–ssDNA interactions does not appear to have been realized (Lee & Wetmur, 1973). The corresponding derivative of poly(rA), poly(ϵA), has been employed by several investigators (Ledneva et al., 1978; Toulmé & Hélène, 1980) but, as we shall see, is of no use in the recA protein system. The chloroacetaldehyde reaction converts adenosine to 1,N^6-ethenoadenosine and cytidine to 3,N^4-ethenocytidine. It is the high fluorescence of the former at neutral pH (λ_{max} ~405 nm) that renders ϵDNA and poly(ϵA) so useful (Leonard & Tolman, 1975).

Experimental Procedures

Materials

Commercial samples of ADP (Sigma), ATP (Sigma), and ATPγS (Boehringer) were used as received. Highly polymerized calf thymus dsDNA (Sigma) was converted to ssDNA by heating it for 20 min at 100 °C and then plunging it into an ice bath. The concentrations of all these nucleic acid derivatives were determined spectroscopically (Weinstock et al., 1981a). Thin-layer chromatography established that the ATYγS contained 25 ± 5% ADP, and all cited concentrations for ATPγS allow for this. Other purchased chemicals were of the highest purity available.

In all but two experiments, a single stock recA protein preparation was employed. It had been purified by Sephacryl S-300 filtration and is certainly >98% pure and binds 1.0 mol of nucleotide/mol of recA monomer (Cotterill et al., 1982). recA protein concentrations were determined spectrophotometrically (Cotterill et al., 1982).

Our procedure for synthesizing ϵDNA was based primarily on an earlier method for preparing poly(ϵA) from poly(rA) (Steiner et al., 1973). Chloroacetaldehyde was obtained by heating a mixture of 24 mL of $CH_2ClCH(OCH_3)_2$ (Aldrich), 10 mL of H_2SO_4, and 250 mL of water under reflux for 20 min and distilling the resultant homogeneous solution until ~130 mL of distillate had been collected. This distillate, when diluted to a volume of 150 mL, had a pH of 5 ± 0.5. It was redistilled until ~100 mL of distillate had been collected. The stock chloroacetaldehyde solution was obtained by diluting the second distillate to 130 mL. The principle synthesis of ϵDNA began with the addition of 29 mL of the chloroacetaldehyde

† From the Department of Chemistry, Imperial College of Science and Technology, London SW7 2AY, U.K. *Received April 16, 1982.* This work was supported by SERC Grant GR/A94249.

‡ Permanent address: Chemistry Department, Amherst College, Amherst, MA 01002.

[1] Abbreviations: ss, single stranded; ds, double stranded; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); NTP, nucleoside triphosphate; poly(ϵA), poly(1,N^6-ethenoadenylic acid); ϵDNA, product obtained by treating ssDNA with chloroacetaldehyde, which contains 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine residues; recA, recA protein; Tris, tris(hydroxymethyl)aminomethane.

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reagent to a solution prepared from 37 mg of ssDNA, 6 mL of 1.7 M sodium acetate buffer (pH 5), and 24 mL of water. Reaction was allowed to continue at 40 °C for 280 min. The pH of the mixture was raised from 4.9 to 7.1 by the careful addition of NaOH and the εDNA isolated by ethanol precipitation. Several redissolutions and reprecipitations served to purify the product (Steiner et al., 1973). The solution obtained prior to the final precipitation was incubated at 40 °C overnight to hasten the decomposition of undesirable reaction intermediates that may have accumulated (Krzyzusia et al., 1981). About 20 mg of colorless εDNA resulted. Aqueous solutions of this material have shown no detectable change in spectroscopic properties during a 3-month period.

The concentration of εDNA was determined by phosphate analysis (Ames, 1966). In standard buffer, our material showed λ_{min} 244 nm (ϵ = 4700), a broad maximum at 250–267 nm (ϵ_{260} = 6300), and ϵ_{300} = 970. Although it is not important to know the extent of modification in the εDNA, the following procedure suggests that 75 ± 5% of the adenine and cytosine rings have been converted to the etheno derivatives [cf. Lee & Wetmur (1973)]. In a trial run, ssDNA was treated with chloroacetaldehyde overnight. The slightly yellow product isolated, assumed to be 100% modified, showed λ_{min} 247 nm and OD_{270}/OD_{260} = 1.03. The corresponding numbers for the starting material are 230 nm and 0.83. By interpolation, the εDNA used in our experiments is 82% or 70% modified, if the changes described depend linearly on the extent of modification.

Poly(εA) was similarly prepared from poly(rA). Its spectroscopic properties were in excellent agreement with those reported, and its concentration was determined as described elsewhere (Ledneva et al., 1978).

Methods

All fluorescence experiments were performed with a Perkin-Elmer MPF-44B instrument operated in the ratio mode, generally with λ_{ex} = 300 nm and λ_{em} = 400 nm. A cuvette of approximately 1.5-mL capacity was positioned in a thermostated cell holder; it was illuminated over a 0.4-cm path length. The absorbance of the solutions employed was almost always <0.02, but at the very end of some titrations, it rose to ~0.025.

All experiments were performed at 25 °C and, except when noted, in standard buffer, consisting of 20 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and 1 mM dithiothreitol. A typical run involved the addition of small volumes of the reactants to 1.0 mL of standard buffer. Solutions were mixed by gently inverting the stoppered cell several times. recA protein was handled either with plastic apparatus or with glassware that had been previously treated with dimethyldichlorosilane. However, Hamilton syringes used for the addition of recA protein in some titration experiments were merely rinsed with the recA protein solution prior to use.

Miscellaneous Points. (1) The fluorescence titrations for determining the stoichiometry for the binding of ATPγS to the recA–εDNA complex were performed on 200 μL of solution in a cuvette of ~300-μL capacity. The spectrophotometer was set at λ_{ex} = 310 nm and λ_{em} = 450 nm. (2) ATPase experiments, performed with, [γ-^{32}P]ATP (Amersham), determined the rate of release of radioactivity that was not adsorbed to activated charcoal. (3) In some experiments reported here and other unpublished ones, the order of mixing of reagents significantly affects what is observed. We have specified the order followed where that is a consideration. (4) All polynucleotide concentrations are reported as nucleotide residues.

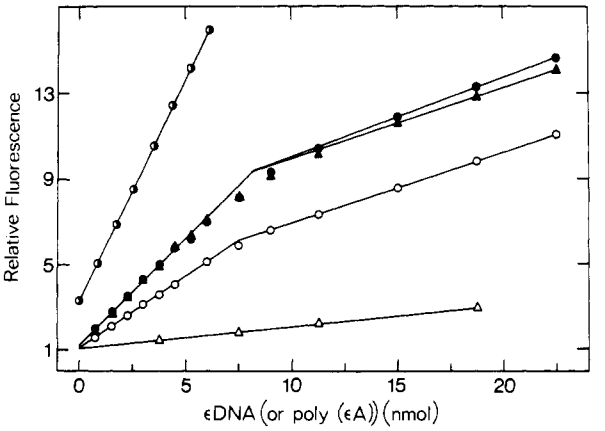

FIGURE 1: Titration of 1.24 nmol of recA protein at 25 °C in standard buffer. Aliquots of 750 μM εDNA solution were added to approximately 1 mL of the recA protein solution which also held 930 μM ATP (●), 140 μM ATPγS (▲), or no NTP (○). The two controls illustrate the addition of εDNA to 1.0 nmol of α-chymotrypsin in the presence of 140 μM ATPγS (△) and the attempted titration of 0.6 nmol of recA protein with poly(εA) (◑). The latter curve has been rescaled in order to fit it on the plot. It shows no change in slope in the region of 3.7 nmol of poly(εA).

Results

Two critical observations lie at the heart of all that follows. First, addition of recA protein to a solution of εDNA greatly enhances the fluorescence of εDNA at 400 nm. For example, the fluorescence yield from a mixture of 0.6 μM recA protein with 6.4 μM εDNA in standard buffer at 25 °C is ~80% higher than the sum of the separated individual components. The complex responsible for this enhanced fluorescence will be designated as the recA–εDNA complex. Second, addition of saturating concentrations of ATP (≥500 μM) or ATPγS (≥30 μM) to the solution of recA–εDNA complex formed in the preceding experiment causes a further substantial rise in fluorescence. Under any particular set of conditions, rises in fluorescence for the two NTP's are identical. The experiments described below examine the utility of these fluorescence changes for exploring the behavior of recA protein.

Fluorescence Titrations. The expectation that recA protein binds strongly to εDNA suggests performing fluorescence titrations to define accurately the number of nucleotides covered by a recA monomer (Toulmé & Hélène, 1980). Two procedures are possible, for εDNA can be added to recA protein, or vice versa. Since a titration may be performed in the presence of saturating concentrations of ATP or ATPγS or in the absence of any NTP, a total of six kinds of titrations may be attempted. All have been tried. Figures 1 and 2 give one example of each kind and illustrate a few control experiments. The difference in slope between the initial and final segments of each titration is greater in Figure 2 than it is for the corresponding titration in Figure 1 because the contribution of the fluorescence at 400 nm from excess recA protein, which the final portion of Figure 2 reflects, is so modest.

The equivalence point for each titration was obtained by determining the point of intersection of the least-squares straight lines passed through its initial and terminal phases. Table I summarizes the results obtained.

Binding of ATP and ATPγS to the recA–εDNA Complex. As previously stated, addition of a high concentration of ATP or ATPγS to a solution of the recA–εDNA complex affords a substantial fluorescence enhancement. Figure 3 graphically demonstrates this effect for ATP. Saturating concentrations of the two nucleotides produce the same enhancement, within experimental error.

Table I: Results of Fluorescence Titrations To Determine the Stoichiometry for the Binding of recA Protein to ϵDNA[a]

run	method[b]	$[recA]_0$ (μM)	$[\epsilon DNA]_0$ (μM)	$[ATP]_0$ (mM)	$[ATP\gamma S]_0$ (μM)	nucleotides per recA monomer[c]
1–3	ϵ to A	0.6–1.2				5.7 ± 0.1
4–6	ϵ to A	0.6–1.2		0.93–1.4		6.0 ± 0.1
7–9	ϵ to A	0.6–1.2			60–140	6.3 ± 0.1
1–9	ϵ to A					6.0 ± 0.2
10[d]	ϵ to A	1.2			60	5.9 ± 0.1
11[d]	ϵ to A	0.6			35	6.4 ± 0.1
12–14	A to ϵ		3.7–7.4			7.3 ± 0.1
15–16	A to ϵ		3.7–7.4	0.78–1.85		7.7 ± 0.1
17–21[e]			1.4–7.4		30–140	8.9 ± 0.2
12–21	A to ϵ					8.3 ± 0.3

[a] All experiments were performed at 25 °C in standard buffer. Small aliquots of either 750 μM ϵDNA or 124 μM recA protein were added to ~1 mL of solution containing the other reagents at the stated concentrations. [b] ϵ to A signifies addition of ϵDNA; A to ϵ, addition of recA protein. [c] The average value for this ratio at the equivalence point for the indicated runs. Figures 1 and 2 illustrate the experiments from which these data derive. Standard errors are also given. [d] Runs 10 and 11 employed a second (190 μM) and third (122 μM) batch of recA protein. The errors in the last column represent the estimated uncertainties in the individual titrations. [e] The two runs with 1.4 μM ϵDNA were titrated with 24.8 μM recA protein.

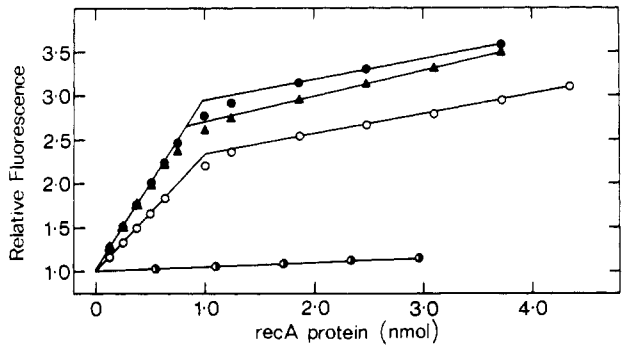

FIGURE 2: Titration of 7.5 nmol of ϵDNA at 25 °C in standard buffer. Aliquots of 124 μM recA protein solution were added to about 1 mL of the ϵDNA solution in the presence or absence of NTP. The symbols (●), (▲), and (○) have the same meaning as in Figure 1. The control (◑) describes the addition of recA protein to a solution holding 4.4 nmol of poly(ϵA) and 57 μM ATPγS. It shows no detectable change in slope. The slope is so slight because the added recA protein barely perturbs the intensity of the highly fluorescent poly(ϵA) solution.

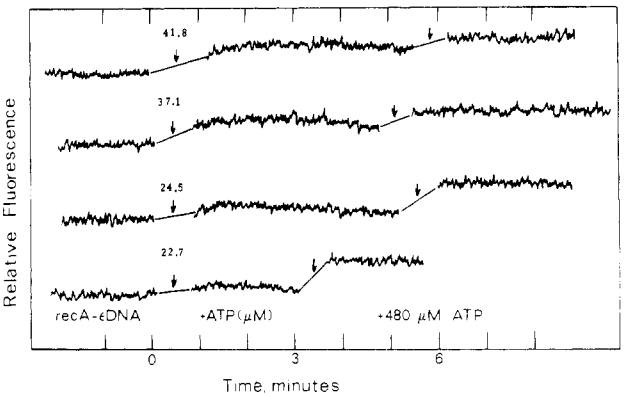

FIGURE 3: Fluorescence changes that characterize the binding of ATP to the recA–ϵDNA complex. The extreme left of each run represents the fluorescence of 7.4 μM ϵDNA plus 0.61 μM recA protein in standard buffer (this was the same for each run, but the traces have been displaced vertically). At the first arrow, [ATP] was increased to the level shown; at the second, it was increased to >480 μM. Traces of actual recordings obtained are shown, but the straight segments represent periods when the pen returned to zero as a reagent was added (the same holds for Figure 6). The final fluorescence intensity average was 1.16 ± 0.01 relative to the initial value for 13 runs in this experiment.

These factors give the titration curves in Figures 1 and 2 their characteristic forms. In each figure, the two titrations that include an NTP give a sharper change near the equivalence point than does the one without. The initial rise in fluorescence for the latter reflects solely the fluorescence enhancement associated with recA–ϵDNA complex formation. With the other two, we see this enhancement reinforced by the contribution from the conversion of that complex into what is most conveniently designated an ATP–recA–ϵDNA or ATPγS–recA–ϵDNA complex. Furthermore, the titration curves in the presence of ATP and ATPγS are nearly superimposable in Figure 1 and match closely in Figure 2. The following two qualitative observations on the nature of the NTP–recA–ϵDNA complexes are worth noting: (1) addition of ADP to a solution of recA–ϵDNA complex results in a slight decrease in the measured fluorescence; (2) addition of either NTP to a solution of recA–ϵDNA complex does not affect the measured intensity if Ca^{2+} is substituted for Mg^{2+} in the standard buffer or if no divalent cation is present.

When less than a saturating concentration of ATP (25–60 μM) is mixed with preformed recA–ϵDNA complex, the measured fluorescence changes in a characteristic way. As Figure 3 shows, it rapidly rises to a maximum value, remains constant for 0.5–3 min, and then commences a slow descent. If allowed to proceed long enough, the runs show a final fluorescence that generally lies slightly below the original level. The difference between the highest value reached and the original one, ΔF, is greater when a larger ATP concentration is introduced. The observations suggest a method for determining the apparent dissociation constant, K, and the degree of cooperativity characterizing the ability of ATP to convert the recA–ϵDNA complex into the ATP–recA–ϵDNA one.

The experiments are best done by preparing a stock solution containing recA protein and ϵDNA at the desired concentrations. The following three-step procedure is used for each data point (see Figure 3): (1) record the fluorescence of 1.0 mL of the stock; (2) add 0.5–5 μL of a relatively dilute ATP solution and record the time-dependent fluorescence change, thus determining ΔF; and (3) after the measured fluorescence has peaked, add 5–10 μL of a second ATP solution that is sufficiently concentrated to convert all the recA–ϵDNA complex into ATP–recA–ϵDNA. The difference between the final, constant fluorescence and that in step 1 defines ΔF_m.

Since the effect of [ATP] upon the ATPase activity of the recA–ssDNA complex has been treated successfully in terms of the Hill equation (Weinstock et al., 1981b), the same approach has been attempted for the fluorescence experiments. The data for each set of runs have been plotted according to the appropriate form of the Hill equation:

$$\log [\Delta F/(\Delta F_m - \Delta F)] = h \log [ATP] - \log K \quad (1)$$

As Figure 4 illustrates, each plot displays satisfactory linearity.

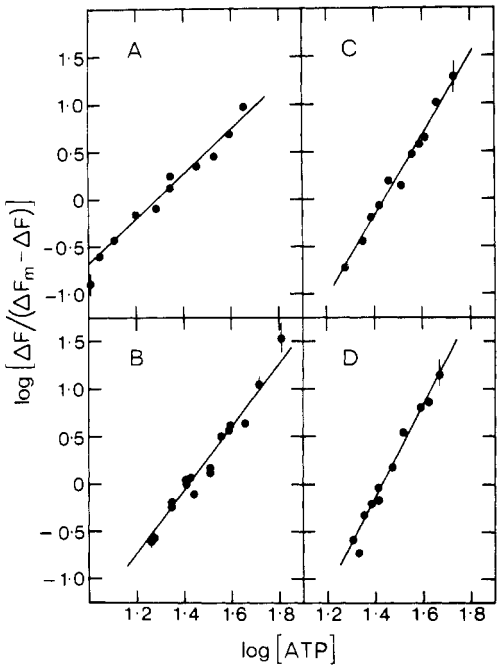

FIGURE 4: Binding of ATP to the recA–ϵDNA complex under standard conditions. The data are plotted according to the Hill equation (eq 1) with [ATP] values expressed in micromolar: (A) 1.2 μM recA protein, [ϵDNA]/[recA] = 3.1; (B–D) 0.61 μM recA protein and [ϵDNA]/[recA] = 6.1, 12, and 18, respectively.

Table II: Cooperative Binding of ATP and ATPγS to recA–ϵDNA Complexes[a]

[recA] (μM)	[ϵDNA]/[recA]	$[NTP]_0$ (μM)	h[b]	K_{app}[b] (μM)
1.2[c]	3	10–46	2.4	20
0.6[c]	6	19–66	3.3	26
0.6[c]	12	19–54	4.3	28
0.6[c]	18	20–48	4.9	27
0.6[d]	12	20–49	2.5	33
0.8[e]	27	14–50	3.3	18
1.2[f]	3	0.3–1.2	3.3–4.2	0.4–0.6
0.5[f]	18	0.2–1.1	3.3–3.4	0.4–0.5

[a] Performed under standard conditions as described in the text. [b] h is the slope of the Hill equation plot (or its kinetic equivalent), and K_{app} is the [NTP] required for half-saturation. [c] In these ATP binding experiments, no correction to $[ATP]_0$ was made for the minor hydrolysis that occurred during the time required for the measured fluorescence to reach its maximum. The average value for K_{app} is 25 ± 2 μM. [d] This ATPase run with ϵDNA gave V_m = 3.7 ± 0.1 M min^{-1} for 480–750 μM ATP, so k_{cat} = 6.3 min^{-1}. The Hill plot consisted of six points, encompassed a range for V/V_m of 0.2–0.75, and showed a correlation coefficient of 0.991. [e] For this ϕX174 ssDNA promoted ATPase at pH 8.1, 30 °C, k_{cat} = 7 min^{-1} (Weinstock et al., 1981b). [f] Analysis of these ATPγS binding experiments is described in the text. We have indicated the range of possible values for h and K_{app}.

Table II demonstrates that the four derived values for K_{app}, the value of [ATP] required for $\Delta F = \Delta F_m/2$, are in good agreement. The magnitude of the Hill coefficient establishes that ATP converts recA–ϵDNA into ATP–recA–ϵDNA in a highly cooperative process under all conditions examined. The degree of cooperativity rises as the ratio [ϵDNA]/[recA] is increased, but the difference between the h values for the two runs at excess ϵDNA is barely significant. It is not experimentally feasible to increase the [ϵDNA] further.

The recA–ϵDNA complex is an effective ATPase under conditions nearly identical with those employed for one set of binding runs. The two experiments afford reasonably similar values for h and K_{app} (Table II; comparable ATPase data from the literature are also shown).

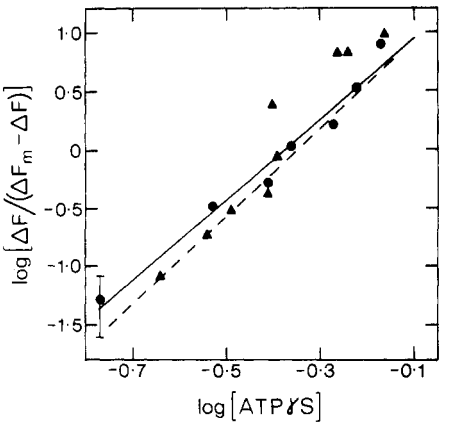

FIGURE 5: Binding of ATPγS to the recA–ϵDNA complex under standard conditions. The data are plotted according to the Hill equation (eq 1) with [ATPγS] values expressed in micromolar. The broken line is the least-squares straight line through the lowest five (▲) points. The experiment employed 1.2 μM recA protein and 3.7 μM ϵDNA. The solid line is the least-squares straight line through all (●) points. That experiment involved 0.5 μM recA protein and 8.9 μM ϵDNA. The analysis, explained in more detail in the text, assumed that ATPγS binds to the recA–ϵDNA complex with a stoichiometry of 1:1.

The method described for evaluating the binding of ATP to the recA–ϵDNA complex has also been applied to ATPγS. Complexes between ATPγS and recA–ssDNA have figured prominently in previous work with recA protein, primarily because they are readily captured in filter-binding assays (Weinstock et al., 1981c). The high stability of the ATPγS–recA–ϵDNA complex has proven a liability in the fluorescence investigations of ATPγS binding. Analysis of the experimental data requires making allowance for the substantial fraction of the added ATPγS that is bound to recA protein. Furthermore, experiments with excess recA protein witness a reproducible discontinuity in fluorescence at [ATPγS] ≃ 0.8 μM [Figure 5 at log [ATPγS] = −0.4; the point shown at log $[\Delta F/(\Delta F_m - \Delta F)]$ = 0.39 represents three identical determinations] when the solution slowly grows perceptibly hazy. This is one of two instances where enzyme aggregation has caused a problem. It apparently does not affect the value for ΔF_m. The measured ΔF_m for the hazy solution is identical with that for a clear solution obtained by saturating the pure recA–ϵDNA complex with a single addition of ATPγS. As a result of these difficulties, the ATPγS binding data are not as reliable as those for ATP. Table II lists the range of values for h and K_{app} that is compatible with our experiments and Figure 5 displays the Hill equation plots. The entries in Table II summarize several alternative analyses of the data acquired. We can assert confidently that ATPγS converts recA–ϵDNA to ATPγS–recA–ϵDNA in a highly cooperative process that shows a $K_{app} \simeq 0.5$ μM. This value of K_{app} agrees with the value of K_i = 0.6 μM obtained from ATPγS inhibition of the ATPase reaction (Weinstock et al., 1981c).

The tight binding of ATPγS to the recA–ϵDNA complex presents the opportunity of determining the stoichiometry of that binding by fluorescence titration. Meeting the requirement that [recA–ϵDNA] greatly exceed the dissociation constant for the ATPγS complex proved difficult. Enzyme aggregation foiled our efforts to use 17 μM recA protein ([ϵDNA]/[recA] = 7.6). Solutions of 4.92 μM recA protein ([ϵDNA]/[recA] = 13.7) appeared to remain homogeneous. Triplicate determinations give [ATPγS]/[recA–ϵDNA] = 0.78 ± 0.04. Various other procedures have given values of 0.5–1.7 for this stoichiometry (Weinstock et al., 1981c).

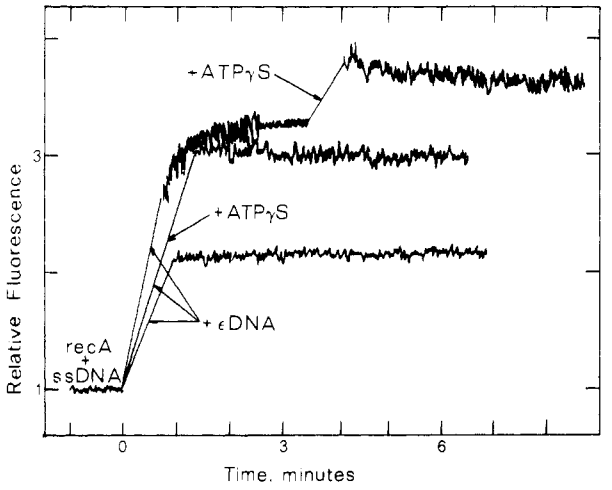

FIGURE 6: Demonstration of the transfer of recA protein from ssDNA to ϵDNA. The extreme left records the fluorescence of a solution containing 0.6 μM recA protein plus 80 μM ssDNA. The lowest run also held 30 μM ATPγS. At the designated point, [ϵDNA] was increased to 3.6 μM, and, after ~10 s (middle trace) or 3 min (upper trace), [ATPγS] was raised to 30 μM. A fast pen response was initially used in the latter in order to demonstrate the occurrence of a detectable time-dependent rise in fluorescence.

Detection of the Transfer of recA Protein between Polynucleotides. If ϵDNA is added to recA protein or vice versa, the measured fluorescence reaches its final value by the time observation begins, ~20 s after mixing. That is not the case if ϵDNA is added to a solution containing recA protein plus ssDNA. When 3.7 μM ϵDNA is added to a mixture of 0.6 μM recA with 80 μM ssDNA, the last 20–25% of a rapid rise in fluorescence is seen (note the upper curve of Figure 6). The ultimate value, attained within 2 min of mixing, is identical with that found when the same concentrations of recA protein and ϵDNA are combined. Addition of 80 μM ssDNA to the latter solution fails to affect its fluorescence significantly.

A large number of such experiments have been performed. We shall focus on typical observations that best illuminate the properties of the various recA–ϵDNA complexes with which we have been concerned or that mostly clearly indicate the potential utility of fluorescent ϵDNA for investigating protein–nucleic acid interactions. We are currently attempting to acquire more thermodynamic and kinetic data for some of the processes described with the aid of conventional and stopped-flow fluorometers.

Only one interpretation of the fluorescence change seen when ϵDNA is added to the solution of the recA–ssDNA complex warrants consideration. We are detecting the relatively slow transfer of recA protein from ssDNA to ϵDNA, possibly by a dissociation mechanism. The rate of transfer is too fast to measure accurately with the Perkin-Elmer fluorometer. Since under the specified conditions transfer is more than half complete by the time recording commences, the half-life for the process is considerably less than 30 s. The corresponding value for k, >1.4 min^{-1} (Table III), represents a conservative lower limit. The ability of 3.7 μM ϵDNA to strip recA protein from 80 μM ssDNA so effectively proves, moreover, that recA protein preferentially binds to the former polynucleotide.

A simple experiment, illustrated in Figure 6, confirms that recA protein transfers from ssDNA to ϵDNA in the experiment described. It relies upon the fact that a high concentration of ATPγS "freezes" recA protein to the polynucleotide upon which it resides for the few minutes required to record a stable fluorescence intensity (Weinstock et al., 1981c, and below). The crucial experiment is performed by adding ϵDNA to a solution of recA–ssDNA in the cuvette, inverting the cuvette twice, adding ATPγS ~10 s after the addition of ϵDNA, mixing, and recording the final fluorescence intensity. The measured value of 518 exceeds that obtained (365) when ATPγS is introduced prior to the addition of ϵDNA, which fixes recA protein to ssDNA. It lies below that measured (650) if ATPγS addition is delayed until 3 min after the addition of ϵDNA, when transfer of recA protein to ϵDNA is complete. When the addition of ATPγS occurred ~25 s after that of ϵDNA, the final fluorescence intensity was 605 (not shown in Figure 6).

Table III: Apparent First-Order Rate Constants Governing the Transfer of recA Protein from One Polynucleotide to Another

reaction	NTP	k^c (min^{-1})
recA–ϵDNA + poly(dT)[a]	none	>6[d]
	ATP	0.13
	ATPγS	≤0.002
recA–ssDNA + ϵDNA[b]	none	>1.4[d]
	ATP	0.3–1
	ATPγS	≤0.007

[a] These three experiments employed 0.6 μM recA protein, 3.7 μM ϵDNA, 25 μM poly(dT), and, where appropriate, 770 μM ATP or 60 μM ATPγS. [b] These three experiments utilized 0.6 μM recA protein, 80 μM ssDNA, 3.7 μM ϵDNA, and, where appropriate, 960 μM ATP or 25 μM ATPγS. [c] The text explains the origins of the tabulated values of k. [d] Preliminary results with the stopped-flow fluorometer indicate these rate constants exceed 20 min^{-1} and that the kinetic processes governing these transfers are reasonably complicated.

The fluorescence experiments confirm the extreme inertness of the ATPγS–recA–ssDNA complex (Weinstock et al., 1981c). Note, for example, the relatively constant fluorescence intensity characterizing the lowest curve in Figure 6. The rate of transfer of recA protein to ϵDNA from this complex is too slow to be evaluated accurately by the fluorescence technique. We have estimated the rate as follows. When recA protein, ssDNA, and ATPγS (0.6, 80, and 25 μM, respectively) are mixed and 3.7 μM ϵDNA is added, the initial instantaneous rise in fluorescence associated with the introduction of ϵDNA is followed by a very slow subsequent rise. Let us assume that the latter corresponds to the formation of ATPγS–recA–ϵDNA and that eventually all the recA protein would be converted to that complex under these conditions. The measured increase in fluorescence after 46 min corresponds to ~25% of the total expected rise. The half-life for the transfer is certainly ≥100 min, corresponding to $k \leq 0.007$ min^{-1} (Table III). Weinstock et al. (1981c) report a comparable half-life for the exchange of ATPγS in the ATPγS–recA–ssDNA complex at 37 °C and pH 7.5.

recA protein complexes incorporating ATP are of the most biochemical interest. Consider what happens in the experiment just described when 960 μM ATP is substituted for ATPγS. The instantaneous rise in fluorescence attendant upon the addition of ϵDNA is followed by a further modestly rapid increase. The final fluorescence intensity, reached after 15–20 min, is identical (within experimental error) with that obtained for a solution holding just ATP, recA protein, and ϵDNA at the same concentrations. A first-order plot of the kinetic data is biphasic, corresponding to an initial slope of 1 min^{-1} and a final one of 0.3 min^{-1} (Table III). There are two important points: (1) the ATP–recA–ssDNA complex transfers recA protein more rapidly than does the corresponding ATPγS complex but less rapidly than does recA–ssDNA; and (2) recA protein binds more strongly to ϵDNA than to ssDNA, in the presence of ATP.

Complementary experiments have been performed in which recA protein distributes itself between ϵDNA and poly(dT). Since the affinity of recA protein for poly(dT) is so great (McEntee et al., 1981a), these experiments have been mostly performed in the following way. The desired recA–ϵDNA complex is prepared, a high concentration of poly(dT) is added, and the fall in fluorescence characterizing the transfer of recA protein from ϵDNA to poly(dT) is recorded. Unlike the experiments involving the transfer of recA protein from ssDNA to ϵDNA, it is here possible to assume that the concentration of the polynucleotide acceptor, poly(dT), remains constant during a run. If 1.5–3.6 μM poly(dT) is added to a mixture of 0.3 μM recA protein with 0.9 μM ϵDNA, we see the very end of a time-dependent fall in fluorescence. However, under the standard conditions specified in Table III with $[poly(dT)]_0$ = 29 μM, transfer is complete within 20 s (the half-life is certainly <7 s, corresponding to $k > 8$ min^{-1}). If 25 μM ATPγS is added before the poly(dT), under the same conditions <8% of the expected fall in fluorescence occurs in 37 min ($k < 0.002$ min^{-1}). Most interesting is the transfer of recA protein in the presence of high [ATP]. Under standard conditions, the reaction obeyed the first-order rate law to better than three half-lives, $k = 0.13$ min^{-1}. Indeed, five runs, incorporating a range of concentrations, gave excellent first-order plots and identical rate constants [average $k = 0.13 \pm 0.01$ min^{-1} for 0.6–1.8 μM recA protein, 3.7–40 μM ϵDNA, and 12–29 μM poly(dT)]. When the recA–ϵDNA complex is mixed with a saturating concentration of ATP in the absence of poly(dT), the fall in fluorescence is negligible during the period required for these kinetic measurements (note the final traces in Figure 3).

Discussion

The assumption that the fluorescence data described under Results reveal the existence of recA–ϵDNA, ATPγS–recA–ϵDNA, and ATP–recA–ϵDNA complexes renders those data readily understandable. It provides a theoretical interpretation that is consistent with previous studies on the interactions of recA protein with ssDNA. Filter-binding assays have captured recA–ssDNA and ATPγS–recA–ssDNA complexes, while an ATP–recA–ssDNA complex must be implicated in the ssDNA-promoted ATPase activity of recA protein (McEntee et al., 1981a; Weinstock et al., 1981a). Additional telling fluorescence observations are the following: (1) complexes between recA protein and poly(ϵA) are not detectable (controls in Figures 1 and 2); (2) if Ca^{2+} is substituted for Mg^{2+} or if no divalent cation is present in the standard buffer, the fluorescence enhancement attributed to the conversion of the recA–ϵDNA complex into the ATP–recA–ϵDNA ones is not seen; (3) addition of ADP to a solution of the recA–ϵDNA complex causes the fluorescence intensity to fall slightly; and (4) the fluorescence data imply that the recA–ssDNA and recA–ϵDNA complexes rapidly transfer recA protein to suitable acceptor polynucleotides while for the corresponding ATPγS complexes those transfers are extremely slow. All four points find close analogy in earlier work, employing other techniques (Cotterill et al., 1982; McEntee et al., 1981a; Weinstock et al., 1981a): (1) recA protein binds poorly to poly(rA); (2) filter-binding assays fail to detect an ATPγS–recA–ssDNA complex in the absence of a divalent cation (Ca^{2+} affords that complex in reduced yield but does not support ATPase activity); (3) ADP appears to promote the dissociation of recA protein from ssDNA; and (4) filter-binding assays establish that the recA–ssDNA complex is highly mobile while the corresponding ATPγS one is extremely inert.

These several arguments justify the conclusion that the interactions of recA protein with ϵDNA and ssDNA are similar. Two other observations corroborate the point. ϵDNA and ssDNA support the ATPase activity of recA protein with comparable efficiency (Table II), and the relative ease of transfer of recA protein from the three kinds of complexes identified is comparable for the two polynucleotides (Table III). The enhanced affinity of ϵDNA for recA protein is experimentally advantageous. We believe it derives, at least in part, from the disrupted secondary structure of ϵDNA. The etheno derivatives that characterize ϵDNA cannot participate in base pairing.

In brief, fluorescence studies with ϵDNA should provide biochemically relevant insights into the behavior of recA protein and other proteins that interact strongly with ssDNA. The experiments performed thus far with recA protein (1) define the stoichiometry governing its binding to ϵDNA under various conditions, (2) quantify the binding of ATP to the recA–ϵDNA complex, (3) quantify the binding and stoichiometry of the binding of ATPγS to the recA–ϵDNA complex, and (4) provide initial estimates on the rate at which recA protein transfers between polynucleotides. Our discussion will focus on the titration experiments and on the data bearing on the properties of the ATP–recA–ϵDNA complex.

Stoichiometry for the Binding of recA Protein to ϵDNA. The fluorescence titration procedures described offer a convenient empirical method for standardizing recA protein solutions. The experiments may be performed rapidly (15–20 min per run) and yield highly reproducible equivalence points, which are readily estimated by eye to ±5% under the specified conditions. Table I summarizes our results and displays titration data for two other batches of recA protein (runs 10 and 11).

Earlier experiments of various kinds have indicated that each recA protein monomer binds to 4–10 nucleotides of ssDNA [e.g., see Craig & Roberts (1980) and McEntee et al. (1981b)]. Our determinations all lie within that range. Nevertheless, the order of addition of the reagents appears to affect the titration results significantly, and it is instructive to examine why this may be so. The stoichiometry values obtained by adding ϵDNA to recA protein are more readily interpreted. The procedure maximizes the likelihood that recA protein fully coats ϵDNA throughout the titration, since it is in excess until the equivalence point. The good agreement among the results from runs 1–9 confirms this expectation. The conclusion that each recA monomer covers 6.0 ± 0.3 nucleotides of ϵDNA probably represents the best available estimate for the stoichiometry of binding between the protein and single-stranded polynucleotides.

Titrations 12–21, where recA protein is added to ϵDNA, give larger values for the ratio [ϵDNA]/[recA] at the equivalence point. We think these experiments overestimate the ability of recA protein to cover ϵDNA. The fluorescent regions of ϵDNA cannot be homogeneously distributed, since the adenosine residues are not so distributed and the modification reaction with chloroacetaldehyde may not act randomly [cf. Ledneva et al. (1978)]. The finding that recA protein binds far more strongly to ϵDNA than it does to ssDNA suggests that recA protein may bind preferentially to the modified regions of the ϵDNA strands. Should this be so, when ϵDNA is in excess, recA protein will afford a fluorescence enhancement that overestimates the total degree of coverage of ϵDNA. This describes exactly the situation that pertains at the early stages of titration 12–21, when recA protein is added to ϵDNA.

The fact that the extent of overestimation is greatest for runs 17–21, which includes ATPγS, accords with the preceding

explanation. Recall that recA protein binds essentially irreversibly to ϵDNA in the presence of ATPγS. Runs 17–21 should therefore most clearly reveal the initial preferred attachment of recA protein to the modified regions of ϵDNA, and they do. The high mobility of recA protein in the simple recA–ϵDNA complex affords the protein ample opportunity to reposition itself on the ϵDNA strands during the course of titrations 12–14. This repositioning reduces the extent to which those runs overestimate the ability of recA protein to cover ϵDNA. Perhaps fortuitously, the ATP–recA–ϵDNA complex exhibits both intermediate mobility and an intermediate degree of overestimation in runs 15–16.

ATP–recA–ϵDNA Complex. ATP converts recA–ϵDNA into ATP–recA–ϵDNA in a highly cooperative process under all conditions examined (Table II). The values for h and K_{app} obtained from the Hill equation are very close to those obtained for the ϵDNA- and ssDNA-stimulated ATPase reactions. It is gratifying that such different techniques are in good agreement, for the critical fluorescence observations are completed when the ATPase reaction has barely commenced. This agreement reinforces our conviction that we are justified in ascribing the fluorescence change seen, when ATP is added to the recA–ϵDNA complex, to the formation of an ATP–recA–ϵDNA complex.

We do not yet know what that fluorescence change represents, at a molecular level. Preliminary studies establish the feasibility of examining the binding of ATP to the recA–ϵDNA complex with a stopped-flow fluorometer. They indicate that it will not be easy to establish a quantitative link between the fluorescence and ATPase data. The measured fluorescence intensity in the ATP binding runs clearly does not depend solely upon the instantaneous ATP concentration. The fluorescence intensity noticeably rises initially (Figure 3), but ATP is converted to ADP without a detectable lag (Weinstock et al., 1981a). Qualitatively, the fall in fluorescence seen after the peak value is reached (Figure 3) coincides with falling [ATP]. Quantitatively, the ATPase rate estimated fluorometrically (via the appropriate Hill plot) consistently exceeds that measured conventionally by at least 2-fold.

What do the fluorescence experiments contribute to the question of the role of ATP in the recA protein system? It is helpful to start by contrasting the behavior of ATP and ATPγS. Both readily form ternary ssDNA complexes, but ATP is cleaved some 5000 times more rapidly (Craig & Roberts, 1981) and apparently binds to recA–ϵDNA ~50 times less tightly. The following highly schematic equation (where NTP–recA–ϵDNA symbolizes the complex detected in the fluorometer and responsible for the cleavage reactions) suggests one possible way to reconcile these facts:

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

$$\text{NTP–recA–}\epsilon\text{DNA} \xrightarrow{k_3} \text{NDP} + \text{P}_i + \text{recA–}\epsilon\text{DNA}$$

For ATPγS, $k_2 \gg k_3$ and binding experiments afford a true equilibrium dissociation constant, $K(k_2/k_1)$. For ATP, $k_3 \gg k_2$. The k_3 route offers a relatively rapid pathway for breakdown of the critical ATP complex. ATP binding experiments thus measure an apparent dissociation constant for ATP, $K(k_3/k_1)$, that significantly exceeds its true dissociation constant from ATP–recA–ϵDNA.

The data in Table III provide the first firm evidence that the ATP–recA–ϵDNA and ATP–recA–ssDNA complexes release recA protein far more rapidly than do the corresponding complexes with ATPγS (perhaps the ATP complexes have escaped detection in filter-binding experiments because of this lability). It is thus tempting to link that release to the cleavage of ATP in the k_3 step of the above equation. However, if the two processes are coupled, the coupling is quite inefficient under our experimental conditions. The rate constant of 0.13 min^{-1} for the transfer of recA protein from ϵDNA to poly(dT) in the presence of ATP is far smaller than the ATPase turnover number of 6.3 min^{-1}. The discrepancy with unmodified ssDNA is less but still substantial. The primary function of the ATPase activity may well lie elsewhere (Cox & Lehman, 1981; Weinstock et al., 1981a).

Acknowledgments

A sabbatical leave from Amherst College to M.S.S. enabled him to participate in this project.

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