Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 3127–3131, May 1987
Biochemistry

Properties of the duplex DNA-dependent ATPase activity of *Escherichia coli* RecA protein and its role in branch migration

(genetic recombination/protein–nucleic acid binding/DNA denaturation)

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Communicated by Irving M. Klotz, January 12, 1987 (received for review August 18, 1986)

ABSTRACT **We have investigated the double-stranded DNA (dsDNA)-dependent ATPase activity of recA protein. This activity is distinguished from the single-stranded DNA (ssDNA)-dependent ATPase activity by the presence of a pronounced lag time before the onset of steady-state ATP hydrolysis. During the lag phase there is little ATP hydrolysis. The duration of the lag phase, referred to as the lag time, is found to increase with the thermal stability of the dsDNA substrate. Increasing either the $MgCl_2$ or NaCl concentration increases the lag time, whereas increasing the temperature decreases the lag time. The lag time shows little dependence on recA protein concentration but is strongly dependent on ATP concentration. After the lag phase, a steady-state ATP hydrolysis rate is achieved that approaches the rate observed with ssDNA. The steady-state phase of the reaction is proportional to the concentration of recA protein–DNA complex and shows saturation behavior at ≈5 ± 1 base pairs per recA protein monomer. These results suggest that the lag phase represents a rate-limiting step in the dsDNA-dependent ATP hydrolysis reaction that requires a structural transition in the dsDNA and that involves a ternary complex of ATP, recA protein, and DNA. We propose that this transition involves the transient denaturation of the dsDNA to form regions of ssDNA. Elsewhere we demonstrate that the dsDNA-dependent ATPase activity is proportional to the rate of recA protein-catalyzed branch migration. We suggest that this activity is responsible for a polar polymerization that drives the branch migration reaction.**

The recA protein of *Escherichia coli* has been shown to play an essential role in genetic recombination (see ref. 1). *In vitro*, it can carry out a strand exchange reaction in which a single strand from linear double-stranded DNA (dsDNA) is reciprocally replaced with homologous circular single-stranded DNA (ssDNA), resulting in fully duplex circular DNA and linear ssDNA (2). This reaction consists of several distinct steps: *presynapsis*, the binding of recA protein to ssDNA; *synapsis*, the conjunction and homologous alignment of ssDNA and dsDNA; *D-loop formation*, the local denaturation of dsDNA and nascent heteroduplex formation; and *branch migration*, the polar exchange of ssDNA strands (see refs. 3–5).

Branch migration proceeds in a 5′ to 3′ polar direction with regard to the invading ssDNA and requires continual ATP hydrolysis (6–8). The apparent efficiency of the overall branch migration reaction is quite poor, with 100–1000 ATP molecules hydrolyzed per base pair of heteroduplex DNA formed; under optimal conditions, the apparent efficiency increases to 16 ATP molecules per base pair (9). This apparent inefficiency has led to a number of conceptual proposals for the mechanism of branch migration (5).

Implicit in the protein-catalyzed branch migration reaction is the requisite local denaturation of the dsDNA molecule either prior to or simultaneous with the strand-exchange event. Although it would seem plausible that recA protein should possess either a DNA "melting" activity or a DNA helicase activity, none has been found (10, 29) except with short oligonucleotides (12). RecA protein has been shown to "unwind" dsDNA as defined by topological studies, though no DNA denaturation was demonstrated directly (13).

In this paper, we describe in detail the properties of the dsDNA-dependent ATPase activity of recA protein that were described in abstract form (11). The existence of this activity was noted previously (14), but its significance was not readily apparent. We show that the time course of this ATPase activity displays a unique "lag," which is dependent on the melting temperature (t_m) of the DNA and during which there is essentially no ATP hydrolysis. Following the lag phase, a steady-state ATP hydrolysis rate typical for ssDNA is observed. This suggests that the lag phase is a rate-limiting step in the dsDNA-dependent ATPase activity involving a DNA structural transition. We propose that this transition is a local denaturation of dsDNA and that the ATPase activity of recA protein is activated by this transiently formed ssDNA. The implications of these results to the mechanism of the recA protein-catalyzed strand-exchange reaction are discussed.

MATERIALS AND METHODS

Chemicals and Buffers. All chemicals were reagent grade. ATP was from P-L Biochemicals and NADH and phospho*enol*pyruvate were from Sigma. Unless otherwise noted, the buffer used in all experiments consisted of 20 mM Tris·HCl, pH 7.5/4 mM $MgCl_2$/0.1 mM dithiothreitol/2 mM phospho*enol*pyruvate/0.5 mM ATP.

DNA and Protein. ssDNA and dsDNA were isolated from bacteriophage M13mp7 using the procedures of Messing (15). Concentrations were determined using molar extinction coefficients of 8780 and 6500 $M^{-1}\cdot cm^{-1}$ at 260 nm for ssDNA and dsDNA, respectively. Polynucleotides were from P-L Biochemicals. Concentrations were determined using molar extinction coefficients of 6000 and 8100 at 260 nm for poly(dA)-poly(dT) and poly(dT), respectively, and 6600 at 262 nm for the alternating copolymer poly(dA-dT).

RecA protein was purified from strain KM 1842 (16); its concentration was determined using an extinction coefficient of 27,000 $M^{-1}\cdot cm^{-1}$ at 280 nm. Lactate dehydrogenase and pyruvate kinase were from Sigma.

ATPase Assays. ATP hydrolysis was measured using a spectrophotometric assay (17) exactly as described (18), except that lactate dehydrogenase and pyruvate kinase were each used at 50 units/0.5 ml.

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Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; t_m, melting temperature.

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RESULTS

Duplex DNA-Dependent ATPase Activity Displays a Lag Phase Prior to Attainment of the Steady-State ATP Hydrolysis Rate. Upon addition of recA protein to a solution containing ssDNA and ATP, the ssDNA-dependent ATP hydrolysis rate is observed to be independent of time after a delay of <10 sec (Fig. 1). However, if dsDNA is used as the DNA substrate in an identical assay, then a pronounced lag in ATP hydrolysis is observed (Fig. 1). After this lag phase, a nearly linear steady-state ATP hydrolysis rate is obtained that approaches the steady-state rate observed with ssDNA (data for dsDNA at longer times are not shown). The lag is not due to any rate-limiting step in the coupled ATP regenerating/NADH oxidizing enzyme system nor is it observed with other ssDNA, such as poly(dT), under these conditions.

To quantitatively discuss the behavior of the lag phase, we define the "lag time" as the time required to attain the steady-state ATP hydrolysis rate. Experimentally, the lag time is determined by extending the linear steady-state portion of the kinetic time course back to zero absorbance change. A similar definition was employed in the abortive initiation assay for RNA polymerase (19). For the conditions employed in Fig. 1, the lag time as defined in this way is 176 sec for poly(dA-dT), 250 sec for poly(dA)·poly(dT), and 500 sec for linear M13 DNA. Coincidently, the stability of these DNA molecules to thermal denaturation follows the order: poly(dA-dT) < poly(dA)·poly(dT) < M13 dsDNA, with t_m values of 65°C, 76°C, and 84°C, respectively (not shown). These results suggest that dsDNA stability may be an important variable in determining the duration of the lag phase.

Effects of $MgCl_2$ Concentration, NaCl Concentration, and Temperature on the Lag Time. If the lag phase in the dsDNA-dependent ATPase activity of recA protein involves a structural transition in the dsDNA that is related to the t_m of the DNA (e.g., a transient denaturation), then factors that have an effect on the t_m of the DNA should also affect the lag time. Fig. 2 demonstrates the effect of $MgCl_2$ concentration on the observed ATP hydrolysis rate stimulated by poly(dA-dT) versus M13 ssDNA. We use the homopolymer duplex DNA in these studies because the distinction between the lag phase and the terminal steady-state phase is very prominent; similar trends are always observed with natural M13 dsDNA. Fig. 2 shows that there is no lag discernible in the M13 ssDNA

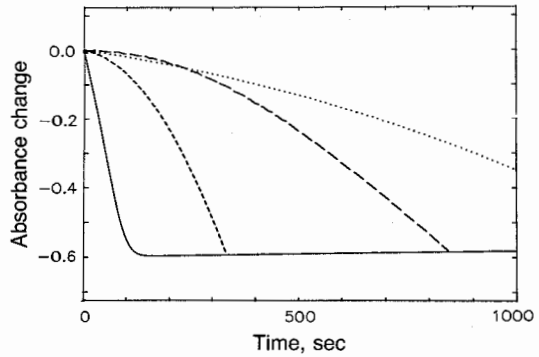

FIG. 1. Time course of ATP hydrolysis by recA protein using various DNA substrates. Reactions were carried out in standard buffer at 37°C using 2 μM recA protein and 30 μM DNA: single-stranded M13 (solid line); poly(dA-dT) (short dashed line); poly(dA)·poly(dT) (long dashed line); and linear duplex M13 (dotted line). The *y* axis represents the extent of ATP hydrolysis; a decrease of 1 absorbance unit corresponds to the hydrolysis of 0.16 mM ATP. In the M13 ssDNA experiment, the NADH concentration was 0.1 mg/ml and the NADH was exhausted after an absorbance decrease of ≈0.6; for the dsDNA experiments, the NADH concentration was 0.2 mg/ml.

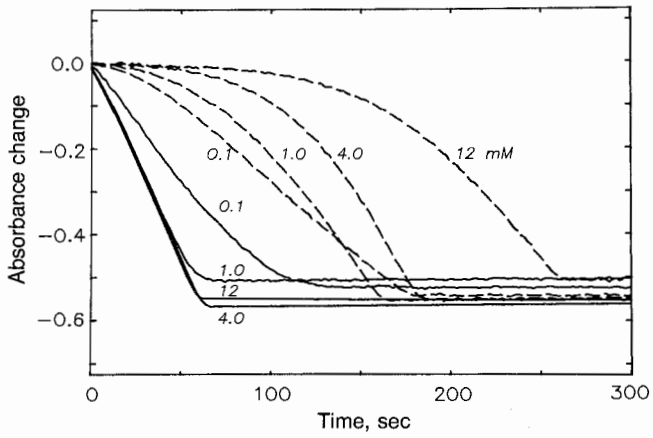

FIG. 2. Effect of $MgCl_2$ concentration on the time course of the dsDNA-dependent ATP hydrolysis reaction. Reactions were carried out in standard buffer containing 0.1 mM ATP and the $MgCl_2$ concentrations indicated at 45°C using 4 μM recA protein and 70 μM DNA: single-stranded M13 DNA (solid lines); poly(dA-dT) (dashed lines). The t_m of the poly(dA-dT) is 53°C, 62°C, 65°C, and 66°C at 0.1, 1.0, 4.0, and 12 mM $MgCl_2$, respectively.

data and there is no effect of $MgCl_2$ concentration on the steady-state ATP hydrolysis rates, except at 0.1 mM $MgCl_2$. In contrast, with dsDNA, increasing the $MgCl_2$ concentration brings about a progressive increase in the lag time. The lag is then followed by a steady-state hydrolysis rate that is comparable to the rate observed with ssDNA. In Table 1, the lag time values obtained from Fig. 2 are tabulated. The length of these lag times closely parallels the dependence of the t_m on $MgCl_2$ concentration (see figure legend).

Since NaCl has an effect on the stability of dsDNA, its effect was investigated also. The time course of ATP hydrolysis resembled that displayed in Fig. 2, and the lag times obtained are reported in Table 1. Again, as expected, increasing the NaCl concentration results in an increase in the lag time; a similar increase is also obtained for native M13 dsDNA (20). Although these results are consistent with the interpretation that the lag phase is a rate-limiting kinetic step that involves a DNA melting event, the NaCl data are equivocal because increasing the NaCl concentration results in a decreased affinity of recA protein for ssDNA (21).

The equilibrium between native and denatured duplex DNA is also perturbed by temperature, and its effect on the lag phase is summarized in Table 1. At 25°C, the lag time is increased to at least 1350 sec, since a terminal steady-state ATP hydrolysis rate is not achieved. At 37°C, the lag time is decreased to 176 sec and, at 45°C, it is only 60 sec (recA protein is stable to at least 50°C under these conditions). Since the reciprocal of the lag time represents a quantity that is proportional to an apparent rate constant, a plot of the logarithm of the reciprocal lag time versus $1/T$ (K) yields the activation energy of the kinetic process that underlies the lag phase. Such a plot yields an activation energy of ≈27 kcal/mol (1 kcal = 4.184 kJ) for these data. The activation energy associated with the local melting of poly(dA-dT) has been measured to be 29 kcal/mol (22). Thus, though not proof, the large activation energy associated with the lag phase is also consistent with the interpretation that the kinetic pathway for this process involves a transient DNA denaturation event.

Effect of ATP Concentration on the Lag Phase. Since the binding of ATP to recA protein induces a conformation of the protein that has a high affinity for ssDNA (21), it is reasonable to suspect that this form of recA protein may act as a DNA melting protein. ATP was found to *decrease* the lag phase and, in Fig. 3, the lag time (circles) is plotted as a function of

Table 1. Effects of experimental variables on the lag phase

Variable	Lag time,* sec
$MgCl_2$† (mM)	
0.1	26
1.0	70
4.0	107
12.0	158
NaCl (mM)	
0	176
100	227
200	504 [28, 16]
Temperature (°C)	
25	>1350 [45, 16]
37	176
45	60
recA protein‡ (μM)	
0.1	213
0.25	213
0.38	220
0.5	247
1.0	313
2.0	280
3.0	276
DNA§ (μM)	
2.0	488
4.0	419
8.0	326
10	289
12	262
16	241
20	219
25	176
30	176

Unless otherwise indicated by footnote, the experimental conditions were 70 μM poly(dA-dT) and 4 μM recA protein in standard buffer at 37°C.

*Lag times were determined as described in the text and are generally reproducible to ≈20% of the listed value; whenever a non-zero value for the lag time of a ssDNA-dependent reaction is observed, the values for M13 ssDNA and poly(dT), respectively, are listed in brackets.

†Data obtained at 45°C in standard buffer containing 0.1 mM ATP.

‡Data obtained using 10 μM poly(dA-dT).

§Data obtained using 2 μM recA protein.

ATP concentration. A plot of the reciprocal of the lag time (not shown) exhibits a hyperbolic dependence on the ATP concentration with an apparent K_m of ≈0.7 mM and a saturation plateau at 38 sec. Since the effect of ATP can be only through an interaction with recA protein, this result indicates that ATP binding to recA protein has the consequence of decreasing the lag time. Thus, the molecular species involved in the lag phase of the reaction must involve ATP, recA protein, and native dsDNA.

To ascertain whether the presence of ATP is an essential component of the complex present in the lag phase of the reaction, dsDNA was preincubated with recA protein for 60 min in the absence of ATP. When ATP was added, the observed lag time was ≈80% the length of the lag time obtained without the preincubation (not shown). This preincubation time was much longer than the actual duration of the lag phase (2.5 min) and resulted in only a 20% reduction in the lag time. Thus, although the requirement for ATP in this phase of the reaction may not be absolute, its absence has a severe impact on the lag phase. Therefore, an ATP–recA protein complex must play a crucial role during this part of the reaction.

The steady-state phase of the dsDNA-dependent ATPase activity also exhibits a dependence on the ATP concentration (Fig. 3, triangles). The behavior is sigmoid shaped with an apparent K_m of ≈70 μM. A similar sigmoid dependence is observed in the ssDNA-dependent reaction, with K_m values typically ranging from 50 to 100 μM (14, 18, 23). This observation is consistent with our proposal that the steady-state phase of dsDNA-dependent ATP hydrolysis actually occurs by way of a ssDNA intermediate.

Effects of RecA Protein and DNA Concentration. The assembly of many self-associating systems is characterized by a kinetic lag phase that is highly dependent on the protein concentration (24). Since recA protein is known to self-aggregate (25), the lag phase might show a strong dependence on protein concentration. When the protein concentration was varied, there was relatively little effect on the duration of the lag phase (Table 1). However, the terminal steady-state ATP hydrolysis rate did increase with recA protein concentration until an apparent saturation of the dsDNA had occurred (Fig. 4). The apparent stoichiometry obtained from these data is 5 ± 0.5 base pairs (or 10 nucleotides) of DNA per recA protein monomer.

When the DNA concentration is varied at a fixed recA protein concentration, the lag time decreases with increasing DNA concentration (Table 1). In addition, the steady-state phase of the reaction displays an increase in the ATP hydrolysis rate with increasing DNA concentration (not shown). A plot of the observed steady-state rate versus the DNA concentration shows saturation behavior similar to that seen in Fig. 4, yielding an apparent stoichiometry of 4.5 base pairs (or 9 nucleotides) per recA protein monomer at a plateau hydrolysis rate of ≈20 mol of ATP per mol of recA protein per min.

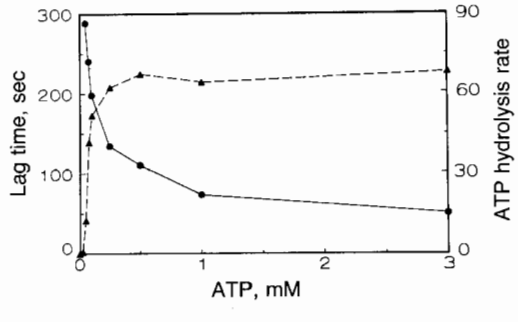

FIG. 3. Dependence of the lag time and the steady-state hydrolysis rate on ATP concentration. Reactions were carried out in standard buffer containing the ATP concentrations indicated at 37°C using 4 μM recA protein and 70 μM poly(dA-dT): lag time (circles) and steady-state ATP hydrolysis rate in μM/min (triangles).

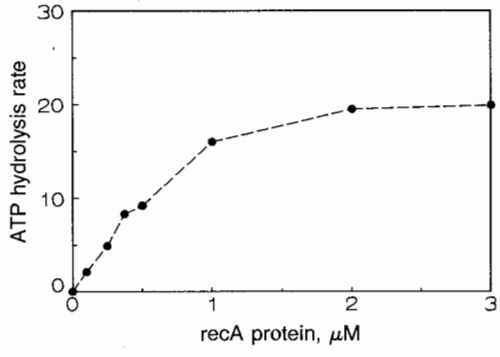

FIG. 4. Dependence of the steady-state rate of ATP hydrolysis on recA protein concentration. Reactions were carried out in standard buffer at 37°C using 10 μM poly(dA-dT) and the recA protein concentrations indicated.

DISCUSSION

We have shown that the properties of the dsDNA-dependent ATPase activity of recA protein are different from those of the ssDNA-dependent activity. The time course of the dsDNA-dependent reaction displays a lag phase during which there is little or no ATP hydrolysis. The duration of the lag phase is increased by factors that increase the stability of the dsDNA (increasing $MgCl_2$ and NaCl concentration) and is decreased by a factor that decreases the stability of duplex DNA (increasing temperature).

Though any one of these observations could be interpreted as being mediated through an effect on the affinity of the recA protein–DNA complex, when considered as a whole, the relationship between t_m and the lag time is quite evident. Although the affinity of the recA protein–DNA complex is an essential component of this reaction, the strong dependence of the lag time on the t_m of the DNA substrates implies that a structural transition in the dsDNA is involved as the rate-limiting step. This structural alteration must represent a significant conformational change that involves the free energy of DNA melting and a variety of melting transitions are possible (see ref. 26). We propose that this rate-limiting structural transition involves the local transient denaturation of dsDNA resulting in ssDNA that can support the ATPase activity of recA protein. The observation that the lag is shorter by a factor of 2 for λ dsDNA containing ssDNA tails compared to λ dsDNA without the ssDNA tails is also consistent and suggests that these ssDNA sites may serve as entry points for recA protein (unpublished observations). Thus, the ATPase activity of recA protein observed with dsDNA is actually an activity mediated by way of the transiently denatured single strands of duplex DNA.

We have also studied the effects of ATP, recA protein, and DNA concentration on the lag phase. Since ATP binding is known to induce a high ssDNA-binding affinity form of recA protein (21), the ATP-bound form of recA protein might be expected to act as a DNA melting protein if no equivalent increase in dsDNA affinity is induced. The observed decrease in the lag time with increasing ATP concentrations is consistent with this expectation. The reciprocal of the lag time displays a hyperbolic dependence on ATP concentration with an apparent K_m of ≈0.7 mM. This behavior is quite distinct from that of the steady-state phase, which displays a sigmoid dependence on ATP concentration with an apparent K_m of ≈70 μM. Thus, the ATP concentration dependence readily distinguishes the lag and steady-state phases as kinetically distinct steps. From these results, we conclude that the *ATP bound* form of recA protein is responsible for binding to the locally denatured form of the dsDNA and that *ATP hydrolysis* is a *consequence* of the transient denaturation.

There was little effect of recA protein concentration on the lag phase. This result eliminates the possibility that the lag phase is due to a nucleation event highly dependent on protein concentration (24). It also suggests that the complex formed during the lag phase is essentially saturated with regard to protein concentration. A decrease in the lag time is observed with increasing DNA concentration, suggesting that the sites for recA protein binding during the lag phase are limiting. Perhaps these sites are the ends of dsDNA or loop structures in the poly(dA-dT). Thus, when taken together with the ATP concentration dependence data, these results indicate that the complex formed during the lag phase consists of recA protein, dsDNA, and ATP (Fig. 5).

The second stage of the dsDNA-dependent ATP hydrolysis reaction is the steady-state phase that follows the lag phase. The rate of ATP hydrolysis during this steady-state phase approaches the hydrolysis rate observed with ssDNA, when the DNA concentration is in stoichiometric excess relative to the protein concentration. In addition, the observed steady-state hydrolysis rate is dependent on recA protein and DNA concentration and saturates at a stoichiometry of ≈5 base pairs per recA protein monomer. The simplest interpretation of this reaction phase is that it represents ATP hydrolysis by the recA protein–*ssDNA* complex that forms as a consequence of transient dsDNA denaturation after the lag phase.

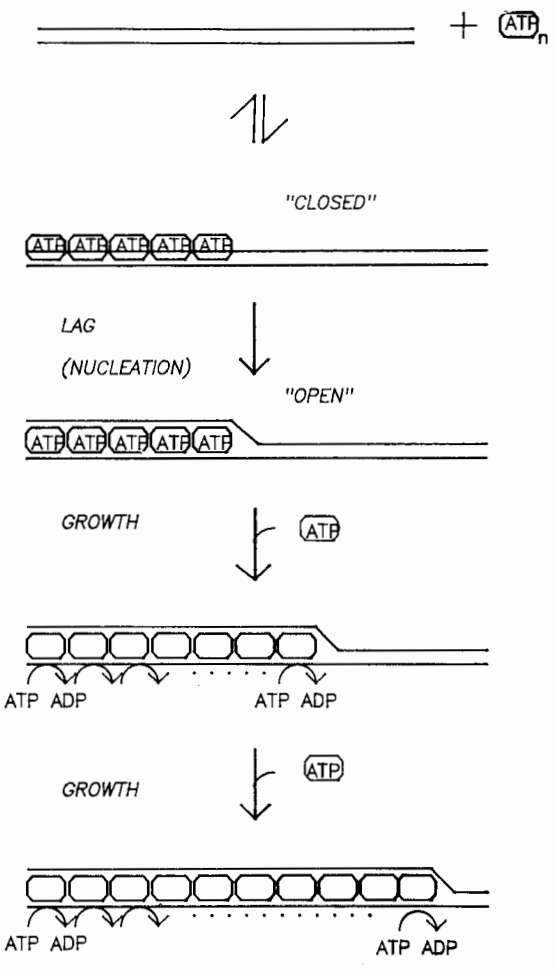

FIG. 5. Model for the dsDNA-dependent ATPase activity of recA protein.

The measured stoichiometry for the ssDNA-dependent ATPase activity (using DNA devoid of secondary structure) is 6 ± 1 nucleotides per recA protein monomer (18). Since the stoichiometry for the dsDNA-dependent reaction is 10 nucleotides or 5 base pairs per protein monomer, this implies that approximately one-half of the dsDNA is available as a substrate. This can mean that only one strand of the entire duplex DNA molecule is bound by recA protein or that both strands are being utilized but only one-half of the dsDNA molecule is transiently denatured at any given time. Though we currently cannot discriminate between these two extremes, we favor the former for the following reasons. (*i*) The binding of recA protein to dsDNA yields a stoichiometry of 5 ± 1 base pairs per recA protein monomer (S.C.K., unpublished data). (*ii*) When poly(dA) and poly(dT) renature in the presence of excess recA protein, the ATP hydrolysis rate of the resultant poly(dA)·poly(dT) duplex is only one-half the sum of the individual ssDNA-dependent hydrolysis rates (18). (*iii*) Nuclease protection experiments of the recA protein–DNA complex formed during the strand-exchange reaction show that only one strand of the dsDNA is protected by recA protein (27).

Our interpretation of the molecular mechanism of the recA protein dsDNA-dependent ATPase activity is summarized schematically in Fig. 5. The first line shows the binding of an ATP–recA protein complex to native duplex DNA in a rapid preequilibrium step to form an ATP–recA protein–*native* dsDNA complex. By analogy to the RNA polymerase–promoter complex, we refer to this complex as a "closed" complex. This closed complex is unable to catalyze the hydrolysis of ATP.

The next step in the model represents the lag phase. This is a kinetically slow transition (nucleation) to an "open" ternary complex of ATP, recA protein, and locally denatured duplex DNA and is the rate-limiting step. The kinetic mechanism by which the open complex forms probably involves the trapping, by recA protein, of transiently denatured regions of ssDNA that form as a consequence of "breathing" (26). ATP hydrolysis may require a contiguous cluster of at least 15 recA protein molecules (23) and, thus, is shown requiring several contiguously bound recA protein molecules in the open complex form. This would further contribute to the formation of an ATP hydrolysis-free lag phase.

The third step in the mechanism represents the onset of the steady-state phase of the reaction. Here a relatively rapid growth of the open form filament occurs and the rate of ATP hydrolysis accelerates due to elongation of the open region of the recA–dsDNA complex. Because the binding of recA protein to ssDNA has been shown to occur in a 5′ to 3′ polar direction (28), we assume that a similar polarity would be maintained for the binding to an individual single strand of duplex DNA. Growth continues until the final steady-state rate is achieved. We have never observed any dependence of the ATP turnover number on the extent of DNA saturation by recA protein (18), implying that ATP hydrolysis is not limited to the ends of protein clusters; thus, all bound proteins are shown hydrolyzing ATP in the final step. Finally, it should be emphasized that the last two complexes in Fig. 5 are not static and that dissociation of recA protein from ssDNA [most probably from the ends of filaments (21)] occurs after 1–10 ATP hydrolysis events (unpublished observations). This dissociation is the likely explanation for the observation that recA protein is not a helicase (29); dissociation would result in a collapse of the DNA to a double helical form.

Based on the mechanism for the dsDNA-dependent ATP hydrolysis reaction proposed in Fig. 5, it appears that this reaction could play a role in the dsDNA unwinding step that occurs during the branch migration phase of the strand-exchange reaction. Elsewhere, we have shown that the amount of product formed during *in vitro* strand exchange is linearly related to the amount of ATP hydrolyzed in the dsDNA-dependent reaction but not to the ssDNA-dependent reaction (20). Since branch migration is the rate-limiting step for product formation in strand exchange (2), those results imply that the dsDNA-dependent ATP hydrolysis reaction is the rate-limiting step in branch migration (20). It was estimated that, at early times in the reaction, *only* 0.75 ATP molecule is hydrolyzed per base pair branch migrated when only the amount of dsDNA-dependent ATP hydrolysis is considered. This suggests that branch migration is the result of the binding of an ATP–recA protein complex to a growing recA–ssDNA filament within the duplex DNA molecule (the growth phase in Fig. 5). Note that despite similarities (e.g., polar filament growth), our proposed model for branch migration (Fig. 5) is *not* a classical treadmilling model (5), since all of the bound recA protein molecules are capable of hydrolyzing ATP. Only the addition of a new recA protein molecule to the growing filament contributes to branch migration and, as a result, the apparent efficiency of ATP hydrolysis decreases with time (20). For these reasons, we refer to our model simply as a "polar polymerization" model.

Since the recA protein-catalyzed branch migration reaction is polar, the question arises as to how polarity originates on 2-fold symmetric dsDNA. We suggest that polarity is defined with regard to the *ssDNA* substrate molecule and that association of the recA protein–ssDNA complex with dsDNA molecule at the synapsis step defines the polarity of the dsDNA "opening" that is driven by the dsDNA-dependent ATPase activity. This association is envisioned to occur between DNA strands of like polarity in the ternary complex that forms as an early event in the strand-exchange reaction (27, 30, 31). Subsequent strand exchange can occur by a passive mechanism or by a concerted mechanism that may require the energy of ATP hydrolysis. Further studies will be required to define the exact nature of the proposed intermediates.

This work was supported in part by funds from National Institutes of Health Grant AI-18987 and from the Earl M. Bane Biomedical Research Fund and by an American Cancer Society Junior Faculty Fellowship (JFRA-70).

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