Inhibition of RecA Protein Promoted ATP Hydrolysis. 2. Longitudinal Assembly and Disassembly of RecA Protein Filaments Mediated by ATP and ADP†

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ABSTRACT: There are at least two major conformations of recA nucleoprotein filaments formed on poly(deoxythymidylic acid) [poly(dT)], one stabilized by ATP [or adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS)] and one stabilized by ADP. Assembly of filaments in the ATP conformation is much faster than assembly in the ADP conformation. A third conformation may be present in the absence of nucleotides. The ATP and ADP conformations are mutually exclusive. When a mixture of ATP and ADP is present, recA protein binding is a function of the ADP/ATP ratio. Complete dissociation is observed when the ratio becomes 1.0–1.5. When a mixture of ATP and ADP is present at the beginning of a reaction, a transient phase lasting several minutes is observed in which the system approaches the state characteristic of the new ADP/ATP ratio. This phase is manifested by a lag in ATP hydrolysis when ATP is added to preformed ADP filaments, and by a burst in ATP hydrolysis in all other cases. More than 15 ATPs are hydrolyzed per bound recA monomer during the burst phase. The transient phase reflects an end-dependent disassembly process propagated longitudinally through the filament, rather than a slow conformation change in individual recA monomers or a slow exchange of one nucleotide for the other. The hysteresis exhibited by the system provides a number of insights relevant to the mechanism of recA-mediated DNA strand exchange.

The recA protein of *Escherichia coli* promotes a DNA strand exchange reaction in vitro that mimics key steps in homologous genetic recombination in vivo (Cox & Lehman, 1987; Kowalczykowski, 1987; Radding, 1988). Under conditions optimal for strand exchange, recA protein exhibits a DNA-dependent ATPase activity (Roberts et al., 1978; Weinstock et al., 1981), and ATP hydrolysis is required for recA-mediated DNA strand exchange (Cox & Lehman, 1981). RecA protein also exhibits a DNA-independent ATPase activity in the presence of high (~2 M) salt concentrations (Pugh & Cox, 1988).

The inhibition of recA-mediated ATP hydrolysis by ADP and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS)[1] is the focus of this and the preceding paper (Lee & Cox, 1990). One goal is to distinguish and characterize different conformations of recA protein that may exist. Two previous observations have indicated that ATP and ADP stabilize different states of recA protein. ATP and ADP increase and decrease, respectively, the affinity of recA protein for DNA (Menetski & Kowalczykowski, 1985; Menetski et al., 1988). ATP also leads to formation of an extended filament conformation (Flory et al., 1984; Chrysogelos et al., 1985; Stasiak & Egelman, 1986; Williams & Spengler, 1986), while ADP results in a "collapsed" conformation as seen in the electron microscope (Stasiak & Egelman, 1988). A similar collapsed conformation is observed in the absence of nucleotide (Flory et al., 1984; Stasiak & Egelman, 1986, 1988; Williams & Spengler, 1986). Both sets of results have been interpreted as manifesting states that are interconverted during the ATP hydrolytic cycle.

The preceding paper (Lee & Cox, 1990) provides evidence consistent with the hypothesis that ATP and ADP stabilize distinct conformations of recA protein, which we refer to as the T and D states, respectively (Lee & Cox, 1990). However, recA filaments may not cycle between these limiting forms during ATP hydrolysis. During ATP hydrolysis, the filament remains largely in the T state as long as ADP levels are kept low via ATP regenereation (Egelman & Stasiak, 1988; Pugh et al., 1989). The states observed when either ATP or ADP is present alone appear to be mutually exclusive, and a mixture of ATP and ADP generally leads to dissociation of recA nucleoprotein filaments. Each of the inhibitors, ATPγS and ADP, antagonize the effects of the other, leading to inhibition kinetic patterns that are complex and informative (Lee & Cox, 1990). The effects of both inhibitors are magnified and propagated through the filaments by cooperative interactions between recA monomers. Neither ATPγS or ADP acts simply as a competitive inhibitor.

One complexity of the inhibition patterns is related to the dissociation of recA protein observed when the ADP/ATP ratio is ~1.5 (Cox et al., 1983; Lee & Cox, 1990). The dissociation results in a complete cessation of ATP hydrolysis at this ADP/ATP ratio. However, when ATP hydrolysis reactions are initiated at this ADP/ATP ratio, the negligible ATP hydrolysis characteristic of the dissociated state is not observed immediately. Instead, a transient phase is observed before the inactive, dissociated state is established (Lee & Cox, 1990). This phase lasts for several minutes and consists of a burst or lag in ATP hydrolysis, depending upon whether ATP and ADP, respectively, is added first.

A time lag exhibited in the change of some kinetic or physical properties of an enzyme in response to a rapid change

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[1] Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SSB, single-stranded binding protein; EDTA, ethylenediaminetetraacetate; DTT, 1,4-dithiothreitol; PEP, phosphoenolpyruvate; etheno-ssDNA, modified ssDNA containing 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine; poly(dT), poly(deoxythymidylic acid); FI, supercoiled closed circular form of a DNA molecule as isolated from *E. coli* cells; FII, nicked circular form of the same DNA molecule; FIII, linear form of the same DNA molecule; FX, closed circular form of the same DNA molecule but underwound by approximately 40% as a result of recA protein binding; PEI, poly(ethylenimine).

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in concentration of substrates that affect that property has been referred to as hysteresis (Frieden, 1970). As defined by Frieden, hysteresis can be a manifestation of three processes: (a) a slow interconversion between two conformational states that differ with respect to the property being measured, (b) a slow exchange of one bound ligand for a new one, or (c) a slow assembly or disassembly that interconverts oligomeric forms of the enzyme with different properties.

In this report, the hysteresis observed in recA-mediated ATP hydrolysis in the presence of substantial concentrations of ADP is characterized. The results offer insights into the assembly, disassembly, and interconversion of the major ATP and ADP conformations of recA filaments. We also provide evidence that the ADP conformation is not identical with that present in the absence of nucleotide, suggesting yet a third conformation.

Materials and Methods

All reagents, methods, and reaction conditions are described in the preceding paper (Lee & Cox, 1990), with one exception. Oligo(deoxythymidylic acid), 60 bases in length [oligo(dT)$_{60}$], was prepared by the DNA Synthesis Facility at the University of Wisconsin Biotechnology Center. This material was characterized and stored, and its concentration was determined in the same manner as the poly(dT) (average length 650 bases) described in the preceding report (Lee & Cox, 1990). The oligo(dT)$_{60}$ preparation was 70% pure, and 99.5% of the oligomers were 58–60 bases in length. As in the preceding report, DNA concentrations are given in terms of total nucleotides.

Because ADP was present in all experiments in this report, the thin-layer chromatographic method with ^{3}H-labeled ATP (Lee & Cox, 1990) was used to measure ATP hydrolysis exclusively.

The hysteretic effects described in this report were somewhat variable. All data presented in a figure panel were carried out on the same day in all cases, but different panels of a figure in some cases represent experiments carried out several months apart. The variability noted in the text for identical experiments carried out on different days represents normal experimental error. Most experiments were repeated several times, and trends noted in figure panels were highly reproducible.

Results

Hysteresis in the Response of RecA-Mediated ATP Hydrolysis to Added ADP. DNA-dependent ATP hydrolysis mediated by recA protein ceases when 60–65% of the available ATP is converted to ADP (Cox et al., 1983; Lee & Cox, 1990). This is a stable reaction end point that is not exceeded even in overnight incubations. In the preceding paper (Lee & Cox, 1990), experiments were carried out demonstrating that poly(dT)-dependent ATP hydrolysis reactions initiated at various ADP/ATP ratios ($\leq$1.5) proceeded to the same end point. At ADP/ATP ratios approaching 1.5, however, the kinetics of ATP hydrolysis were nonlinear. A transient phase lasting several minutes preceded the establishment of the steady-state rate of ATP hydrolysis characteristic of certain ADP and ATP concentrations (Figure 1).

The observed effect depends on the order of addition of ATP and ADP. When ATP was added first (not shown) or ATP and ADP were added together (Figure 1A) after preincubation of recA protein and poly(dT), a burst in ATP hydrolysis was observed. The effect is observed when ADP is over 30% of the total nucleotide and is most evident at ADP/ATP ratios of 1.0–1.5. In the experiment of Figure 1A, about 4% of the

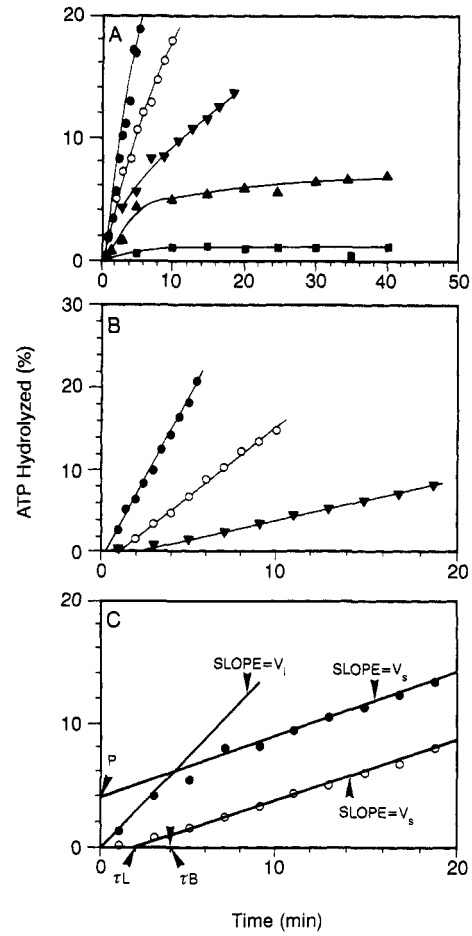

FIGURE 1: Effect of ADP on the kinetics of poly(dT)-dependent ATP hydrolysis. The amount of ATP hydrolyzed at different times was measured in the presence of ADP with two different orders of addition. The concentrations of recA protein, poly(dT), and ATP are 5 μM, 10 μM, and 2 mM, respectively. (A) RecA protein and poly(dT) were preincubated for 10 min, and then the reaction was initiated with a mixture of ATP and ADP. Concentrations of ADP were as follows: 0 (●), 1 (○), 2 (▼), 3 (▲), and 4 (■) mM. (B) RecA protein, poly(dT), and ADP were preincubated for 10 min, and then ATP was added to initiate the reaction. Concentrations of ADP were as follows: 0 (●), 1 (○), and 2 (▼) mM. (C) Comparison of burst and lag type kinetics at an ADP/ATP ratio = 1.0. The variables used are as follows: the time intercept (τ), the amplitude of the burst (P), the initial velocity (V_i), and the steady-state velocity (V_s). Conditions are identical with those in panels A and B, except that ATP and ADP are both 2 mM. Lines defined by (●) and (○) reflect the protocols of panels A and B, respectively.

2 mM available ATP is hydrolyzed in this transient phase, or 80 μM, when the ADP/ATP ratio was 1.5. This corresponds to 16 ATPs for every recA monomer present. RecA protein is present in excess relative to poly(dT) in this experiment, and the number of ATPs hydrolyzed in the burst phase by bound and active recA monomers is probably higher.

When ADP was preincubated with recA protein and poly(dT), and the reaction was initiated with ATP, the result was quite different (Figure 1B). If the ADP concentration (after all additions) exceeded 30% of the total nucleotide, a lag in ATP hydrolysis was observed that lasted about 2 min at the higher ADP concentrations. This lag was not observed unless both poly(dT) and recA protein were present with ADP in the preincubation. All other protocols resulted in burst type kinetics. Preincubation of recA protein with ADP, followed by addition of poly(dT) and ATP, produced a burst (data not shown). This indicates that ADP had little effect on free recA protein and implies that recA protein binds to poly(dT) in the presence of ADP.

Table I: Effect of ADP on Hysteresis

ADP (mM)	τ (min)		P (μM)	
	B[a]	L[a]	B	P/recA[c]
0	0	0	0	0
0.4	0	0	0	0
0.8	0	0	0	0
1.0	2.0	1.4	46	9.2
1.5	2.4	1.9	46	9.2
1.75	2.4		40	8.0
2.0	4.0	2.5	80	16
2.5	3.5		60	12
3.0	5.0		90	18
4.0	ND[b]		84	16.8

[a] B and L indicate burst and lag type kinetics, respectively. [b] ND indicates that the value cannot be determined. [c] Number of ATPs hydrolyzed per recA monomer during the burst phase. The concentrations of recA protein and poly(dT) are 5 and 10 μM, respectively.

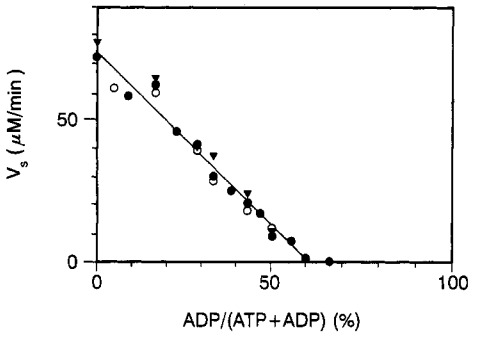

FIGURE 2: Effect of ADP on steady-state rates of poly(dT)-dependent ATP hydrolysis. Rates were taken from time courses such as those in Figure 1 and reflect either the initial rate of the reaction (at low ADP concentration) or an estimate of the rate observed after any transient phase was completed (at ADP/ATP ratios >0.4). RecA, poly(dT), and ATP concentrations were as in Figure 1. Different symbols reflect different protocols: (●) protocol of Figure 1A, (▼) protocol of Figure 1B, and (○) protocol of Figure 1A except that ADP was added 1 min after addition of ATP.

The steady-state rate of ATP hydrolysis observed is independent of the order of addition of ATP and ADP. Reaction protocols resulting in either a burst or lag are compared in Figure 1C under identical reaction conditions and an ADP/ATP ratio of 1.0. Several parameters defining the transient phases are noted (Neet & Ainslie, 1980). These are the initial velocity (V_i), the steady-state velocity (V_s), the length of the transient phase (τ), and the amplitude of the transient (P). τ and P have different meanings for a burst vs a lag. For a burst, τ corresponds to the intersection of asymptotic lines defined by V_i and V_s. P corresponds to the amount of excess ATP hydrolyzed in the transient phase (i.e., the y intercept of the line defined by V_s). For a lag, τ corresponds to the intersection of the asymptotic line defining V_s with the x axis. Values of τ determined at a variety of ADP/ATP ratios with both burst and lag protocols ranged from 2 to 5 min for bursts and 1.4 to 2.5 min for lags (Table I). There was an apparent gradual increase in the measured τ as ADP concentration increased. The amplitude of the bursts ranged from 40 to 90 μM ATP hydrolyzed, corresponding to 8–18 ATPs per recA protein monomer present.

The steady-state rate of ATP hydrolysis (V_s) is plotted vs the concentration of ADP as percent of total nucleotide in Figure 2. Data are derived from Figure 1 and additional results not shown. As indicated above, V_s is identical under both burst and lag type conditions. The dependence on % ADP is linear and intersects with the x axis where ADP is just over 60% of the total nucleotide, consistent with observations noted above.

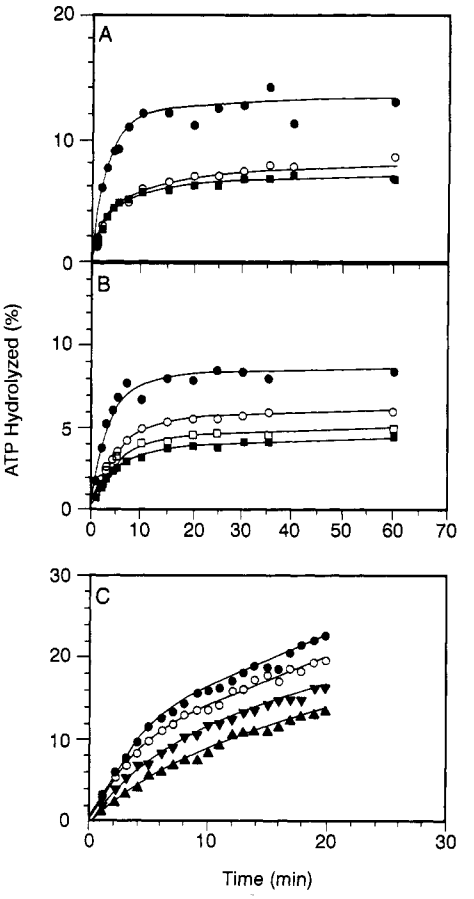

FIGURE 3: Characterization of burst kinetics: (A) Effect of recA protein and poly(dT) concentration on the amplitude of the burst at ADP/ATP = 1.5. The concentrations of ATP and ADP were 2 and 3 mM, respectively, and the order of addition protocol of Figure 1A was used. The concentrations of poly(dT) and recA protein, respectively, were as follows: (■) 10 μM, 5 μM; (○) 10 μM, 10 μM; (●) 20 μM, 10 μM. (B) Effect of order of addition on burst amplitude. RecA protein, poly(dT), ATP, and ADP concentrations were 5 μM, 10 μM, 2 mM, and 3 mM, respectively. Preincubations were for 10 min at 37 °C with components designated "pre", before initiation with components designated "I": (●) (pre) poly(dT) and recA, (I) ATP and ADP; (○) (pre) recA and ATP, (I) poly(dT) and ADP; (□) (pre) recA, (I) poly(dT), ATP, and ADP; (■) (pre) recA and ATP and ADP, (I) poly(dT). (C) Effect of recA protein concentration on burst kinetics at 2 (▲), 5 (▼), 10 (○), and 20 (●) μM recA protein and at an ADP/ATP ratio of 1.0. Concentrations of poly(dT), ATP, and ADP are 10 μM, 2 mM, and 2 mM, respectively, and the order of addition protocol is that of Figure 1A.

Characterization of Burst Kinetics. The burst is observed when ATP is added first or when ATP and ADP are added together and is most distinctive at an ADP/ATP ratio of 1.5, where the steady-state rate is negligible and the difference between V_i and V_s is greatest. The effects of poly(dT) and recA protein concentrations on τ and P at this ADP/ATP ratio are shown in Figure 3A. Doubling the recA protein concentration has virtually no effect on the amplitude or length of the burst, but doubling the concentrations of both recA and poly(dT) results in nearly a doubling of the burst amplitude (but not τ). This indicates that under these conditions the burst is a phenomenon that is limited to the recA protein bound to poly(dT), and free (excess) recA protein has little or no effect.

The amplitude of the burst is also altered by changes in the order of addition protocol. In particular, the amplitude is enhanced somewhat if recA protein is preincubated with poly(dT) prior to addition of ATP and ADP (Figure 3B). A slight enhancement is also observed if recA protein is preincubated with ATP prior to addition of poly(dT) and ADP

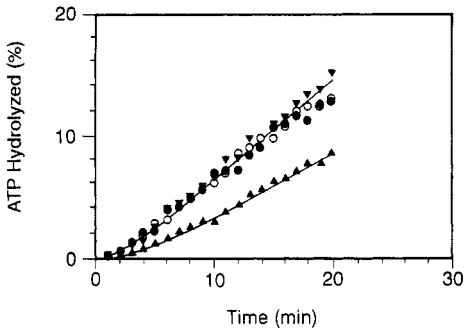

FIGURE 4: Effect of recA protein concentration on lag type kinetics. Poly(dT), ATP, and ADP concentrations were 10 μM, 2 mM, and 2 mM, respectively. The protocol of Figure 1B was used. RecA protein concentrations were as follows: (▲) 2 μM, (●) 5 μM, (○) 10 μM, and (▼) 20 μM.

(Figure 3B). Note that an experiment in Figure 3B (●) is essentially identical with experiments in Figures 3A (■) and 1A (▲). These were all carried out on different days, and the burst amplitude seen in Figure 3B was the highest observed in many trials of this experiment. The normal burst amplitude here was 6–7%, and the effect seen here may be somewhat overestimated.

At a lower ADP/ATP ratio (1.0), recA protein concentration has a significant effect on V_i, but a minimal effect on τ or V_s. The amplitude of the burst (P) therefore increases with recA protein concentration even when recA protein is present in excess (Figure 3C). This suggests that the transient phase reflects a dissociative or associative process [recA and poly(dT)] that is affected by free recA protein when the ADP levels are not too high. This hypothesis is supported and expanded by results described below.

Characterization of Lag Kinetics. As noted above, the observation of a lag requires preincubation of poly(dT), recA, and ADP prior to addition of ATP. This suggests that recA protein binds poly(dT) in the presence of ADP. This is substantiated in the experiments described below.

The effect of recA protein concentration is shown in Figure 4. V_s increases in this experiment with recA protein concentration until enough recA protein is present to bind all of the poly(dT) present. Additional recA protein has little or no effect on τ or V_s, again suggesting that only bound recA protein participates in the transient phase. This indicates that a bound recA protein–poly(dT) complex assembles in the presence of ADP during the preincubation and that some change must occur in this complex before ATP hydrolysis can commence. Note that one experiment in Figure 4 (●) is essentially identical with an experiment described in Figure 1B (▼). These were carried out on different days. The very slow rate of ATP hydrolysis observed after the lag in this experiment was very sensitive to minute changes in reaction conditions, and these two experiments reflect the extremes of the observed variability in this particular experiment.

To determine the length of time required for assembly of this ADP-mediated complex, the preincubation time with ADP was varied (Figure 5). With no preincubation, a burst is observed. Complete suppression of the burst requires a preincubation of about 5 min with ADP. The ADP complex therefore requires about 5 min for assembly, with a half-time of 1–2 min.

ADP in the preincubation mix has no effect on free recA protein even when some bound recA protein is present. If excess recA protein is present [relative to the available poly(dT)] in the preincubation, and additional poly(dT) is added with the ATP used to start the reaction, a mixed result is

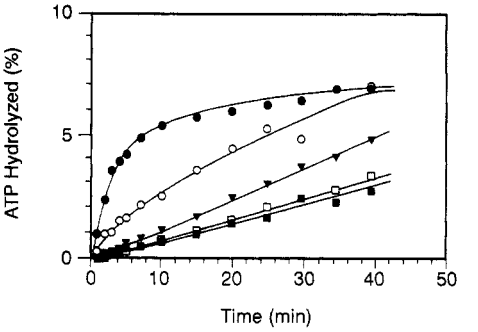

FIGURE 5: Effect of preincubation time in the presence of ADP on lag type kinetics. The concentrations of recA protein, poly(dT), ATP, and ADP are 5 μM, 10 μM, 2 mM, and 3 mM, respectively. RecA protein was preincubated with poly(dT) for 10 min before ADP was added. After incubation times of 0 (●), 1 (○), 5 (▼), 10 (□), and 30 (■) min, ATP was added to initiate the reaction.

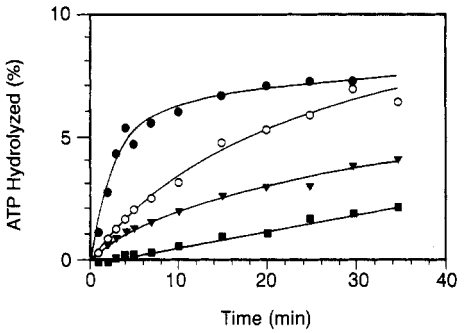

FIGURE 6: Effect of additional poly(dT) on the transient kinetic phase. Poly(dT) (10 μM) and 2 (■), 5 (▼), or 10 (○) μM recA protein were preincubated with ADP (3 mM). After 10 min, more poly(dT) (10 μM) and ATP (2 mM) were added to start the reaction. As a control to illustrate burst type kinetics for comparison purposes, poly(dT) (10 μM) and recA protein (5 μM) were preincubated, and then a mixture of ATP (2 mM) and ADP (3 mM) was added to start the reaction (●).

obtained (Figure 6). The free recA protein binds the second aliquot of poly(dT) to give a burst that partially cancels out the lag in ATP hydrolysis produced by the recA protein bound to poly(dT) in the preincubation.

One experiment in Figure 6 (●) is identical with experiments in Figure 1A (▲) and Figure 5 (●). These were carried out on different days, and the small differences in the results reflect experimental error.

The Transient Phase Reflects a Slow Process Propagated Longitudinally through the Filament. Several experiments were done to distinguish between the several possible causes of the transient phase. If the slow step is a polar assembly or disassembly of the filament, or some other process propagated lengthwise through the filament, the length of the transient phase should be proportional to the length of the filament. If the slow step is a conformation change or exchange of bound ligand occurring simultaneously throughout the filament, then the length of the transient phase should be unaffected by filament length.

A shorter dT oligomer, $(dT)_{60}$, was used in these experiments. This is 10-fold shorter than the average length of the poly(dT) used above, but long enough to provide nearly optimal binding of recA protein (Brenner et al., 1987). The apparent k_{cat} for ATP hydrolysis and the binding stoichiometry for recA protein were affected minimally by the substitution (data not shown). As shown in Figure 7, use of the $(dT)_{60}$ largely eliminates the transient phase, with protocols that produce either bursts or lags with the longer poly(dT). Rates of ATP hydrolysis decrease as ADP is added, but the order of addition of ATP and ADP has little effect on the observed

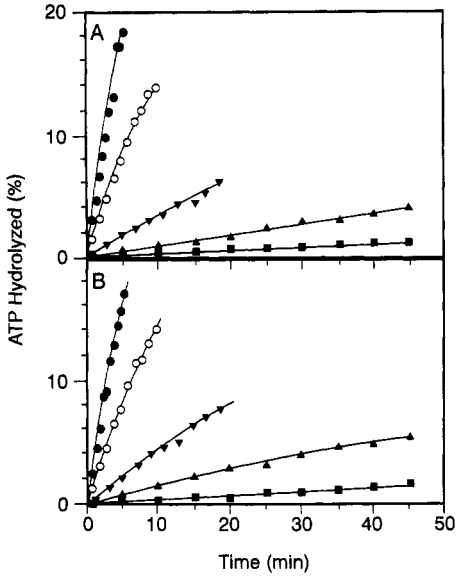

FIGURE 7: Effect of the length of poly(dT) on the transient phase. Shorter DNA [$(dT)_{60}$] was used. Concentrations of recA protein, $(dT)_{60}$, and ATP are 6 μM, 10 μM, and 2 mM, respectively. (A) RecA protein was preincubated with $(dT)_{60}$. After 10 min, a mixture of ATP and ADP was added to initiate reaction. (B) RecA protein was preincubated with $(dT)_{60}$ and ADP. After 10 min, ATP was added. In both cases, ADP concentrations were 0 (●), 1 (○), 2 (▼), 3 (▲), and 4 (■) mM ADP.

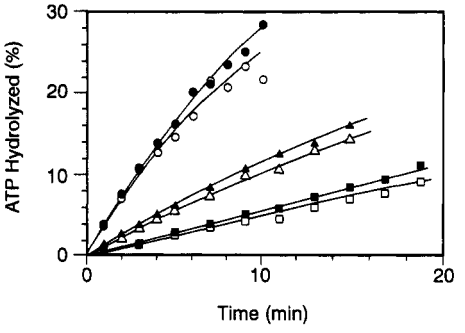

FIGURE 8: Effect of ADP on high-salt-activated (DNA-independent) ATP hydrolysis. ATP hydrolysis was measured in the presence of ADP with two different orders of addition of nucleotides. First, recA protein was preincubated for 10 min, and then a mixture of ATP and 1 (○), 3 (△), or 6 (□) mM ADP was added to initiate the reaction. Second, recA protein and 1 (●), 3 (▲), or 6 (■) mM ADP were preincubated for 10 min, and then ATP was added to initiate the reaction. The concentrations of recA protein and ATP were 5 μM and 2 mM, respectively.

course of ATP hydrolysis. DNA-independent ATP hydrolysis (in the presence of high salt concentrations) was also examined with respect to these ADP effects, and no hysteresis was observed under any circumstances (Figure 8, and Lee and Cox, unpublished data). This indicates that the length of the transient phase depends upon the length of the individual filaments. The lack of a transient phase with short dT oligomers or in high salt indicates that conformation changes in individual recA monomers and exchange of one bound nucleotide for another are not rate-limiting processes on this time scale. Note that some of the data in Figure 8 were used in generating Figure 1 in the preceding paper (Lee & Cox, 1990).

Slow Response (Hysteresis) to ADP Accumulation in an Ongoing ATP Hydrolysis Reaction. Is hysteresis in ADP inhibition kinetics relevant to the course of normal recA-mediated ATP hydrolysis? The k_{cat} for ATP hydrolysis by recA protein is relative low ($\sim$25 min^{-1}), and a typical reaction time course can span 30 min or more. A transient phase in the response to ADP accumulation that lasts a few minutes

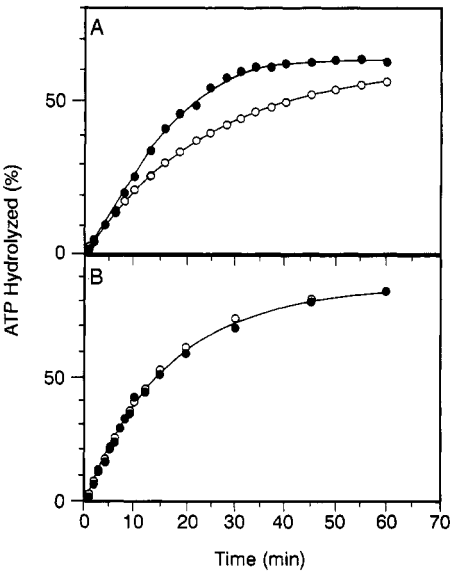

FIGURE 9: Comparison of experimental theoretical values for ATP hydrolyzed (%) at different times. (A) Poly(dT)-dependent ATP hydrolysis: Experimental values (●) were obtained by preincubating 5 μM recA protein and 10 μM poly(dT) for 10 min at 37 °C, and then initiating the reaction by addition of 3 mM ATP. The theoretical values (○) were obtained by using rates from data such as that in Figure 2, but in which the total nucleotide concentration (ATP + ADP) was kept constant at 3 mM. Calculations are described in the supplementary material. (B) High-salt-activated (DNA-independent) ATP hydrolysis: Experimental values (●) were obtained by preincubating recA protein (5 μM) for 10 min at 37 °C, and then adding 2 mM ATP to initiate the reaction. Theoretical values (○) were calculated by using rates provided in Figure 10 and following the method described in the supplementary material.

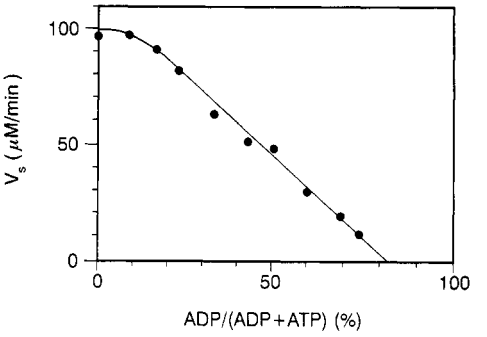

FIGURE 10: Effect of ADP on steady-state rates of high-salt-activated (DNA-independent) ATP hydrolysis. RecA protein and ATP concentrations were 5 μM and 2 mM, respectively. Data shown were obtained by preincubating recA protein for 10 min, and then initiating the reaction with a mixture of ATP and ADP.

may not have a significant impact on the overall kinetics. Given the change in steady-state rate of ATP hydrolysis expected as a function of the fraction of total nucleotide present as ADP (Figure 2), an expected time course for ATP hydrolysis can be calculated (see the supplementary material). As seen in Figure 9A, the observed hydrolysis of ATP significantly exceeds the calculated rate over most of the time course. Instead of the expected hyperbolic curve, the reaction rate is essentially linear for 10–15 min before any curvature due to the accumulation of ADP is evident.

The same experiment was conducted for the DNA-independent reaction in high salt, where the response to ADP is much faster. Here the calculated and observed time courses for ATP hydrolysis coincide quite well (Figure 9B). The steady-state rates of ATP hydrolysis on which the calculated curve in Figure 9B is based are shown in Figure 10 and are taken from results in Figure 8 and similar data not shown. The

DNA-independent reaction is relatively insensitive to ADP when the fraction of total nucleotide as ADP is less than 10%. The rate is then a linear decreasing function of increasing ADP/(ATP + ADP).

Discussion

Our principal conclusion is that recA protein exhibits hysteresis in its response to ADP (or to ATP when ADP is present first). The slow process involves only recA monomers that are bound to DNA, and it is mediated longitudinally through the filament. The properties of the transient phases provide evidence for two major and mutually exclusive conformations stabilized respectively by ATP and ADP and suggest a third distinct conformation is present in the absence of nucleotide. The transient phases reflect filament disassembly processes that proceed from one or both filament ends.

The observation of an extended recA nucleoprotein filament in the presence of ATP and a "collapsed" filament in the presence of ADP has led to the suggestion that these two filament states are interconverted during the ATP hydrolytic cycle (Stasiak & Egelman, 1988; Heuser & Griffith, 1989). This would require more or less simultaneous ATP hydrolysis throughout the filament and produce an accordion-like filament motion. The results presented here and in the preceding paper argue against this hypothesis. The T and D states appear to be mutually exclusive rather than related by interconversion, and the hysteresis described here indicates a filament in one state must be disassembled before a filament in the other state is formed. These results complement electron microscopy (Egelman & Stasiak, 1988) and DNA underwinding (Pugh et al., 1989) studies that indicate no collapse of the filament to anything approaching the D state occurs during normal hydrolysis of ATP. Apparently, ATP hydrolysis does not occur simultaneously in all filament monomers, and the presence of some monomers with bound ATP may maintain the entire filament in a form resembling the T state.

The results can be brought together with the aid of the model presented in Figure 11. In the presence of either ATP alone or ADP alone, a recA filament forms on poly(dT) in either the T state or D state, respectively. Only the bound recA protein participates in the transient kinetic effects observed if the second nucleotide is added subsequently as indicated by the data in Figures 3–6. All of the observed effects exhibit an absolute requirement for the DNA cofactor [in this case poly(dT)]. As shown in previous work (Cox et al., 1983; Lee & Cox, 1990), a nucleotide mixture containing 60% ADP and 40% ATP results in a state in which recA protein is largely dissociated from the DNA. Since it occurs only with a mixture of ATP and ADP, the dissociated state does not correspond to either the T or D state, but most likely to an incompatible mixture of the two. The transient phase observed when ADP is added to an ATP filament and vice versa is best interpreted as a disassembly process leading to this dissociated state (Figure 11). Disassembly must be propagated longitudinally through the filament, since this phase clearly depends on the length of the filament. In other words, disassembly occurs at one or both ends rather than at random throughout the filament. ATP is being hydrolyzed throughout the filament during this process, hence the 15 or more ATPs hydrolyzed by each bound recA monomer during the burst phase. The key evidence for a longitudinal disassembly is that hysteresis is largely abolished when $(dT)_{60}$ replaces poly(dT). Hysteresis is also not observed in the DNA-independent reaction in high salt. This indicates that events that might take place synchronously in monomers throughout the filament, such as a conformation change or the exchange of one nucleotide ligand for another, cannot explain the transient phase.

The existence of the transient phase indicates that recA monomers must go through a bound form before the dissociated state present at ADP/ATP = 1.5 is established. Assembly of filaments in the ATP conformation is much faster (≥10-fold) than assembly of ADP filaments (see Table I and Figure 5). This leads to a burst rather than a lag when both nucleotides are added together. It also indicates that binding of recA protein to DNA in the ATP form is significantly faster than the ADP-mediated process that subsequently results in dissociation. Presumably, however, some ADP-mediated dissociation occurs before assembly of the ATP filaments is complete when both nucleotides are added at the same time as either the DNA or recA protein. When recA and poly(dT) were preincubated long enough for binding to occur before the ATP and ADP were added, the amplitude of the burst was enhanced (Figures 3B and 11).

These experiments do not provide information as to whether filament disassembly involves one end or both, but recent experiments provide clues. In the presence of ATP, filament assembly occurs 5′ → 3′ along ssDNA (Register & Griffith, 1985). Under some conditions recA protein will dissociate from linear duplex DNA in the presence of ATP, and this dissociation occurs primarily at the end opposite to that at which assembly occurs (Lindsley & Cox, 1990). RecA filaments therefore have some capacity to treadmill. It is likely that ADP would affect this process and that the present results are related to the association/dissociation processes described previously (Lindsley & Cox, 1990), but further work is required to determine if the reactions described here have an inherent polarity.

The conclusion that poly(dT) is bound by recA protein in the presence of ADP is consistent with results reported by Bryant et al. (1985) and Menetski and Kowalczykowski (1985). This does not conflict with the dissociation of recA protein at high ADP/ATP ratios, a phenomenon that evidently requires a mixture of the two nucleotides. The best explanation for the dissociation, based on available data, is that the conformations stabilized by each nucleotide are incompatible and mutually exclusive. In this scenario, each nucleotide antagonizes the formation of filaments in the form stabilized by the other [see Lee and Cox (1990)].

RecA protein also binds to poly(dT) in the absence of nucleotide, but this state is not equivalent to the ADP-bound conformation even though the two appear similar in the electron microscope (Stasiak & Egelman, 1988). Adding ATP to filaments in the ADP conformation results in a lag, but no lag is observed when ATP is added to recA protein preincubated with poly(dT) alone. When ATP and ADP are added together, the amplitude of the observed burst is actually increased somewhat if recA protein is first preincubated with poly(dT) (Figure 3B). This suggests a third distinct conformation is present when recA protein binds to poly(dT) in the absence of nucleotide.

ADP has a variety of effects on recA-mediated strand exchange. Low levels of ADP stimulate the initial rate of strand exchange (Cox et al., 1983; Kahn & Radding, 1984). The pairing or synapsis phase of the reaction, however, is more sensitive to ADP inhibition than the branch migration phase (Kahn & Radding, 1984). We cannot yet explain all of these effects, but the relative insensitivity of the branch migration phase to ADP may be due, in part, to the slow response of this system to ADP when it is added after a reaction is initiated and filaments have formed. At steady state, the amount of recA protein bound to DNA is decreased by ADP (Lee & Cox,

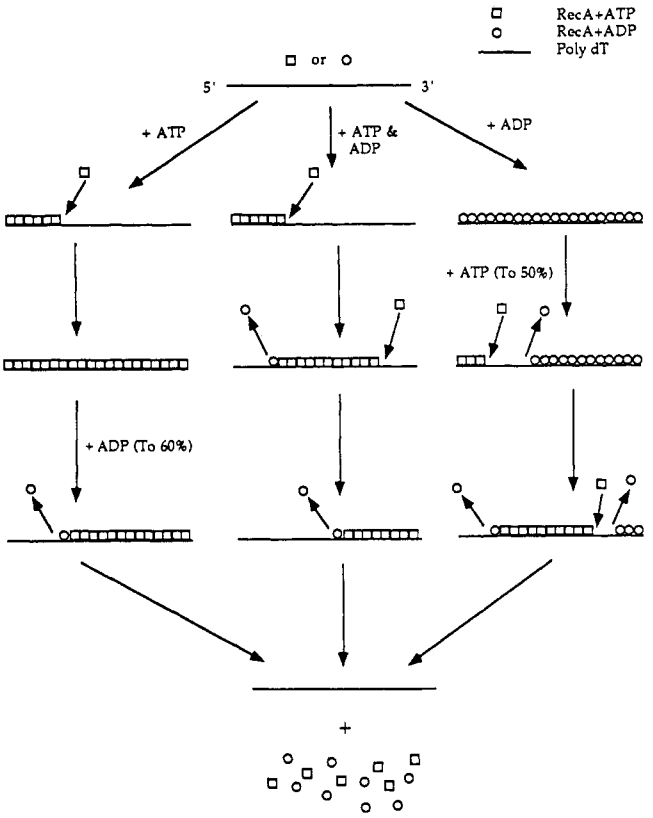

FIGURE 11: A model for recA filament assembly and disassembly mediated by ATP and ADP. The ATP- and ADP-stabilized conformations of recA protein are denoted by squares and circles, respectively. The polarity of filament assembly in the presence of ATP is that observed by Register and Griffith (1985). The polarity of assembly in the presence of ADP (if any) is unknown. The illustrated polarities of disassembly are hypothetical.

1990), but this partially or completely disassembled state is approached very slowly in a long filament and may not be observed until many minutes after ADP is added.

There is clearly a structural polarity in the filament (Stasiak et al., 1988) and a kinetic polarity to the assembly and disassembly processes as characterized in the literature to date (Register & Griffith, 1985; Lindsley & Cox, 1990). The transient phases we have characterized here clearly reflect conformation changes at the ends that are propagated longitudinally through the filament. It can be hypothesized that the change occurring in the monomer at the end is communicated to its neighbor via cooperative interactions of the kind evident in the preceding paper (Lee & Cox, 1990). In other words, dissociation of one monomer may be tightly coupled to dissociation of the next, leading to a coordinated wave of dissociation proceeding unidirectionally from one end. However, while assembly and disassembly (where they occur) are limited to filament ends, ATP hydrolysis is occurring throughout the filament. It is interesting to speculate that the processes evident at the ends reflect microscopic conformation changes that are occurring throughout the filament, resulting in dissociation only when a monomer is present on a particular filament end. In this scenario, internal ATP hydrolysis is also organized so that the ATP hydrolytic cycle of each recA monomer is coupled to those of its neighbors in a polar manner, with cooperative waves of ATP hydrolysis propagating unidirectionally through the filaments (Cox, 1989). Some polar and cooperative organization of this kind is required to explain the unidirectional nature of the strand exchange reaction driven by ATP hydrolysis in this system. An understanding of ATP hydrolysis and the molecular events associated with it remains a key to understanding the mechanism by which recA protein promotes DNA strand exchange.

Supplementary Material Available

Description of the calculations used in generating the theoretical curves in Figure 9 (3 pages). Ordering information is given on any current masthead page.

Registry No. 5'-ATP, 56-65-5; 5'-ADP, 58-64-0; poly(dT), 25086-81-1.

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