

ADP-mediated Dissociation of Stable Complexes of recA Protein and Single-stranded DNA*

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The complete exchange of strands between circular single-stranded and full length linear duplex DNAs promoted by the recA protein of *Escherichia coli* is dependent upon the hydrolysis of ATP and is strongly stimulated by the single-stranded DNA binding protein (SSB). In the presence of SSB, stable complexes of recA protein and single-stranded DNA are formed as an early step in the reaction. These complexes dissociate when the ADP/ATP ratio approaches a value of 0.6-1.5, depending upon reaction conditions. Thus, ATP hydrolysis never proceeds to completion but stops when 40-60% of the input ATP has undergone hydrolysis. recA protein can participate in a second round of strand exchange upon regeneration of the ATP. While 100-200 mol of ATP are hydrolyzed/mol of heteroduplex base pair formed under standard reaction conditions in the presence of SSB, this value is reduced to 16 at levels of ADP lower than that required to dissociate the complexes. ATP hydrolysis appears to be completely irreversible since efforts to detect exchange reactions using ¹⁸O probes have been unsuccessful.

The recA protein of *Escherichia coli* promotes DNA strand exchange reactions that can take a variety of forms (1-4). A particularly informative reaction is the exchange of strands between circular ϕ X ssDNA¹ and full length linear ϕ X duplex DNA, which produces a replicative form II-like product and a displaced linear (+) single strand (3, 5, 6). The reaction can be divided kinetically into two phases, an initial pairing to form a short region of heteroduplex followed by branch migration to extend the heteroduplex. These processes reflect, *in vitro*, the steps in homologous recombination in which recA protein is believed to participate *in vivo* (1). In addition to its importance in understanding recombination, the branch mi-

gration phase of the reaction exhibits a number of characteristics of fundamental biochemical interest. Most important among these are the continual requirement for ATP hydrolysis (3) and a unique polarity (5, 7-9). These properties define a classical "coupled vectorial process" (10). With this consideration in mind, we have undertaken a detailed analysis of the mechanism of recA protein-promoted strand exchange.

Both the rate and extent of strand exchange are greatly stimulated by the single-stranded DNA-binding protein of *E. coli* (3, 11, 12). Moreover, SSB is required for the formation of stable complexes of recA protein and ssDNA (6). Formation of these complexes, which contain up to 1 recA protein monomer/2 nucleotides of ssDNA, is a prerequisite for efficient strand exchange. recA protein does not dissociate from these complexes at any stage during or subsequent to complete strand exchange. In the cell, however, it is to be expected that a mechanism for recycling recA protein exists. Since, in addition to SSB, ATP is required for formation of the stable complex, our search for such a mechanism led us to examine more closely the properties of recA protein-promoted ATP hydrolysis during strand exchange.

A number of known properties of the DNA-dependent ATPase of recA protein (13, 14) are relevant in this regard. All of the steady state kinetic parameters are highly sensitive to pH and the nature of the DNA substrate. The product, ADP, is a competitive inhibitor of the reaction. There is also a high degree of cooperativity (Hill coefficients of 3 or greater) consistent with the high levels of recA protein required for most recA protein-associated activities. Perhaps the most unusual characteristic of recA protein-promoted ATP hydrolysis is its limited extent in the presence of duplex DNA at pH 6.2 (13). Under these conditions, recA protein catalyzes the hydrolysis of only 60% of the ATP provided in the reaction, an effect that is largely independent of ATP concentration. We have recently noted the same phenomenon at pH 7.2 during recA protein-promoted strand exchange in the presence of SSB. This effect and its relationship to the stability of recA complexes are the subject of this report.

EXPERIMENTAL PROCEDURES

Materials

recA protein was purified to homogeneity as described (15). Its concentration was calculated from the extinction coefficient $E_{280}^{1\%} = 5.9$ (16). SSB was purified by a modification of a published procedure (17). Its concentration was calculated from the extinction coefficient $E_{280}^{1\%} = 6.2$ (18). SSB concentrations determined in this way were 10% lower than those determined by methods described in earlier work (6). Nuclease S1, phosphoenolpyruvate, and pyruvate kinase were purchased from Sigma. The restriction endonuclease *Ava*I was purchased from New England Biolabs. To reduce the salt content of the

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¹ The abbreviations used are: ssDNA, single-stranded DNA; ϕ X, bacteriophage ϕ X174; SSB, *E. coli* single-stranded DNA-binding protein; ATP_γS, adenosine 5'-O-(3-thio)triphosphate.

pyruvate kinase preparation, an aliquot of the ammonium sulfate suspension was centrifuged and the pellet was resuspended in reaction buffer to the original volume.

Linear duplex and labeled or unlabeled ϕ X ssDNAs were prepared, and their concentration was determined as described (3, 6). ^3H -labeled ϕ X ssDNA preparations had specific radioactivities $\geq 100,000$ cpm/ μg (nucleotide). DNA concentrations are given in nucleotides.

H_2^{18}O , 100 atom %, was purchased from KOR Isotopes. $\text{K}_2\text{HP}^{18}\text{O}_4$ was prepared according to a published procedure (19). ^3H -labeled ATP and ADP in 50% ethanol were purchased from Amersham. Ethanol was removed before use.

Methods

The assay for heteroduplex formation, based on the conversion of ^3H -labeled circular ϕ X ssDNA into an S1 nuclease-resistant form, has been described (6). Reaction mixtures contained: 25 mM Tris-HCl, 80% cation (final pH 7.2), 5% (v/v) glycerol, 10 mM MgCl_2 , 1 mM dithiothreitol, and DNA, *recA* protein, SSB, ATP, and ADP as described in the text. In experiments requiring an ATP-regenerating system, the Tris-HCl buffer concentration was decreased to 20 mM and 10 mM KCl was added.

ATP-regenerating systems contained sufficient phosphoenolpyruvate and pyruvate kinase to convert all ADP present to ATP with $t_{1/2}$ of 5–10 s and to maintain a negligible ADP concentration for 60 min. The highest phosphoenolpyruvate concentration used was 2 mM.

Unless otherwise noted, reactions were initiated by the addition of ATP and SSB as a mixture after preincubation of other components. Where measurements of extent are reported, the values represent the average of at least three determinations taken between 40 and 60 min after the start of the reaction. Where both labeled and unlabeled ϕ X ssDNA were present, "per cent heteroduplex" refers to the fraction of ^3H -labeled DNA incorporated into heteroduplex rather than the fraction of the total DNA that had reacted.

DNA-dependent ATPase—This method has been described (3, 13). The parameter measured is the ratio of ADP to ATP at a given time using ^3H -labeled nucleotides. Approximately 0.8–1.5% of the ^3H in all [^3H]ATP preparations was present as ADP. This background was measured independently in each experiment in triplicate. Data were treated by adding counts/min in the ADP and ATP, multiplying the total by the background percentage to determine the total background counts/min in that sample. This value was subtracted from the total counts/min in ADP to obtain a corrected ADP value. This value was divided by the sum of the corrected ADP and total ATP values to obtain "per cent hydrolysis." Initial rate measurements were taken in each case from at least five points in which 15% or less of the ATP had been hydrolyzed.

^{31}P NMR spectra were measured using a Bruker Instruments WM 360 spectrometer operating at 145.8 MHz in the Fourier transform mode. The spectra were obtained using a 3012-Hz spectral width (32K data points; 0.784 Hz/point) and a 45° ($12\ \mu\text{s}$) pulse. Approximately 100 scans were averaged for each spectrum. The spectra were referenced to internal P_i . The samples contained 6 mM nucleotide, 20 mM EDTA, 30% D_2O as well as protein and nucleic acid components.

Sucrose Density Gradients—Ten to 30% linear sucrose gradients were buffered with 25 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol. One hundred-microliter samples were layered onto the tops of the gradient and the gradients were centrifuged in an SW 60 rotor at $65,000 \times g$ and 2°C for 3 h. The gradients were then fractionated by collecting drops from a 21-gauge needle that had been pushed through the bottom of the tube. Aliquots were taken from the fractions and counted in a liquid scintillation counter.

RESULTS

The term *recA* protein complex refers in all instances to the stable complexes of *recA* protein and ssDNA formed in the presence of SSB and ATP (6). Unless noted, all experiments were carried out under conditions in which these complexes were formed as an early step in the reaction. A detailed description of the role of SSB in *recA* protein-promoted strand exchange appears in the accompanying report (11).

ATP Hydrolysis during Strand Exchange

The progress of ATP hydrolysis during strand exchange under standard reaction conditions is shown in Fig. 1. The most notable feature of the reaction is that the extent of

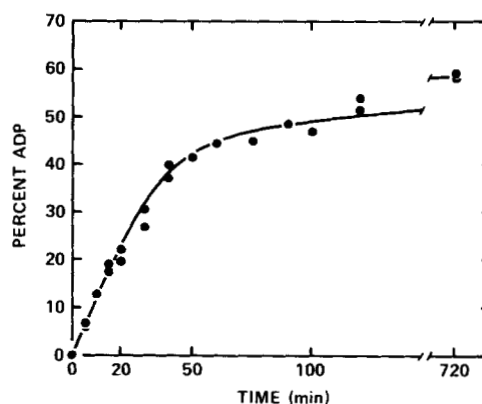


FIG. 1. ATP hydrolysis during *recA* protein-promoted strand exchange. The reaction was carried out as described under "Methods." Reaction mixtures contained $3.3\ \mu\text{M}$ circular ϕ X ssDNA, $5.6\ \mu\text{M}$ linear ϕ X duplex DNA, $0.98\ \mu\text{M}$ *recA* protein, $0.3\ \mu\text{M}$ SSB, $1.0\ \text{mM}$ ATP.

hydrolysis never exceeded 60% of the input ATP. As shown in Table I, this effect was largely independent of the initial concentration of ATP, at least for ATP concentrations above $200\ \mu\text{M}$.

As shown in Table II, ATP hydrolysis during strand exchange was inhibited by ADP. It was, however, unaffected by P_i .² As found in earlier studies (14), the inhibition of the initial rate of ATP hydrolysis by ADP appeared to be competitive. When ADP was added to an ongoing reaction, the expected degree of inhibition of ATP hydrolysis occurred immediately (not shown). The effect of ADP on heteroduplex formation is shown in Fig. 2. Addition of sufficient ADP to bring the ADP/ATP ratio ≥ 1.5 at $t = 0$ prevented heteroduplex formation. Addition of similar concentrations of ADP to an ongoing reaction resulted in an immediate cessation of heteroduplex formation. As shown below, lower concentrations of ADP, while inhibiting ATP hydrolysis, had little effect on heteroduplex formation and in some cases even stimulated it.

The Effect of SSB

Both the rate and final extent of ssDNA-dependent ATP hydrolysis at pH 7.2 were affected by SSB (Fig. 3). These experiments were carried out in the presence of sufficient *recA* protein to saturate the ssDNA (1 monomer/3.5 nucleotides) and thus do not reflect SSB binding to stretches of DNA free of *recA* protein.

With increasing concentrations of SSB, the final extent of ATP hydrolysis declined. The decrease in extent had three features: (i) it exhibited an apparent cooperativity with respect to SSB concentration; (ii) it reached a maximum at concentrations of SSB sufficient to saturate the ssDNA (1 SSB monomer/9 nucleotides) (17, 18, 20); and (iii) it was only minimally affected by the addition of duplex DNA. The effect of SSB on the initial rate of ATP hydrolysis exhibited the same three characteristics except that the rate increased rather than decreased with increasing SSB concentrations, and the rate of ATP hydrolysis continued to increase without reaching saturation unless duplex DNA was present. Although the concentration of *recA* protein (1 monomer/3.5 nucleotides) used in these experiments was sufficient to saturate most of the reactions promoted by *recA* protein (21, 22), greater amounts of *recA* protein could be incorporated into stable complexes when sufficient SSB was added (6). Experiments in which the ratio of *recA* protein to ssDNA was increased by a factor of 2 yielded results similar to those

² M. M. Cox and I. R. Lehman, unpublished results.

TABLE I

Extent of ATP hydrolysis during DNA strand exchange

Reactions were carried out as described under "Methods." Reaction mixtures contained 5.6 μM linear ϕX duplex DNA, 3.3 μM circular ϕX ssDNA, 2.0 μM recA protein, 0.3 μM SSB. The values shown are an average of two determinations.

[ATP]	ADP present after 16 h
mM	%
0.2	60.1
1.0	61.5
2.0	59.7
6.0	57.1

TABLE II

Steady state kinetic parameters for ATP hydrolysis during DNA strand exchange

Reactions were carried out as described under "Methods." At least eight ATP concentrations were used for each determination. At each concentration, the velocity was measured from time points taken before 15% of the ATP had been hydrolyzed. All constants were calculated from an Eadie-Hofstee plot (26). Reaction mixtures contained 0.98 μM recA protein, 3.3 μM ϕX ssDNA, 5.6 μM linear ϕX duplex DNA, 0.3 μM SSB as indicated.

	+SSB	-SSB
K_M (μM)	88	38
K_I (ADP) (μM)	18	23
V_{max} (min^{-1})	16.3	8.4

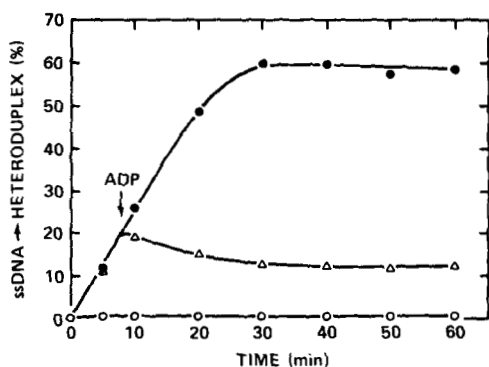


FIG. 2. The effect of high concentrations of ADP on heteroduplex formation. Reactions were performed as described under "Methods." Reaction mixtures contained DNA as described in Fig. 1, 1.9 μM recA protein, 0.23 μM SSB, 0.8 mM ATP. ●, no addition of ADP; ○, ADP to 1.2 μM added at $t = 0$; △, ADP to 1.2 mM added at $t = 8$ min (arrow).

described above. SSB did not cause the large inhibition of ATP hydrolysis observed in earlier work (23) because of the order of addition of reaction components (SSB added last) (6). The effects of SSB on the steady state kinetic parameters of the reaction under these conditions are indicated in Table II. The measurement of these parameters is complicated somewhat by the positive cooperativity noted in previous work (14).

The Effect of ADP on the Stability of recA Protein-ssDNA Complexes

Competitive inhibition by ADP cannot in itself explain the limitation of the extent of ATP hydrolysis. As described previously (6), addition of ATP and SSB to recA protein and ssDNA leads to the formation of stable complexes from which recA protein does not dissociate. Experiments were therefore designed to determine whether ADP affected the stability of recA protein-ssDNA complexes.

As in the earlier study (6), the basic experiment involves

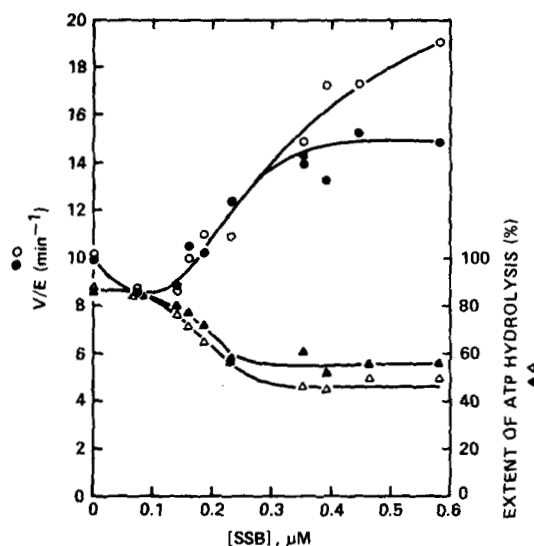


FIG. 3. The effect of SSB on recA protein-catalyzed ATP hydrolysis. Reactions were carried out as described under "Methods." Reaction mixtures contained 3.3 μM circular ϕX ssDNA, 5.6 μM linear ϕX duplex DNA as indicated, 0.98 μM recA protein, 1 mM ATP, and the indicated concentrations of SSB. Filled symbols are reaction mixtures containing duplex DNA; open symbols are reaction mixtures lacking duplex DNA. Circles denote initial rates and triangles are the final extent of ATP hydrolysis. All extents were measured 9–12 h after $t = 0$.

competition between two sets of ssDNAs for available recA protein with only one of the two ssDNAs labeled. recA protein is added in sufficient concentration to provide nearly a maximal extent of heteroduplex formation for one set of substrates; however, a submaximal reaction occurs if both sets of substrates participate in the reaction. Based on previous data (6), it is assumed that addition of an ATP-regenerating system in the presence of SSB is sufficient to prevent release of recA protein from the ssDNA.

Complexes were formed in the presence of 0.4 mM ATP. Additional ATP was added subsequently to provide appropriate controls for the experiments in which ADP was added. As shown in Table III, the complexes reacted readily with homologous duplex DNA added together with an ATP-regenerating system (experiment 1). Additional ssDNA, added with the duplex, was excluded from the reaction (experiment 2) despite the fact that some ATP hydrolysis had occurred prior to the addition of duplex DNA and the ATP regeneration system. When the complexes were challenged with a 2-fold excess of ADP, instead of ATP, the added ssDNA participated in heteroduplex formation (experiment 3). The extent of heteroduplex formation was almost as great as that observed when stable complexes were not permitted to form (SSB omitted) before addition of duplex DNA and the ATP-regenerating system (experiment 4). In this last case, the recA protein should be equally distributed between the two sets of ssDNAs. This experiment indicates that the stable complexes of recA protein are disrupted upon addition of high levels of ADP, and that recA protein can be recycled in a form in which it can react with ssDNA added subsequently if an ATP regeneration system is provided.

The final extent of ATP hydrolysis was less in the absence of duplex DNA than in its presence (Figs. 3 and 4). Coupled to the relatively high rate of ATP hydrolysis when saturating levels of SSB were present, ATP hydrolysis reached a limit after only 30 min. The finding that ATP hydrolysis ceases rather abruptly under these conditions provided an opportunity to examine more closely the relationship between the

TABLE III

ADP-mediated recycling of recA protein

Reactions were carried out as described under "Methods." Final concentrations resulting from the indicated additions are: unlabeled circular ϕ X ssDNA (SS), 3.3 μ M; 3 H-labeled circular ϕ X ssDNA (3 H]SS), 3.3 μ M; full length linear ϕ X duplex DNA (DS), 5.6 μ M; recA protein (recA), 0.9 μ M; SSB, 0.3 μ M; ATP or ADP, 0.4 mM. Addition of an ATP-regenerating system (phosphoenolpyruvate and pyruvate kinase) is indicated by the designation ATP regen. Where a number precedes the indicated addition (*i.e.* 2SSB), the final concentration resulting from that addition should be increased by that factor (to 0.6 μ M in the case above). Components added at the same time were added as a mixture. Where an addition appears more than once in the same experiment, the full amount indicated was added each time. Reaction mixtures were kept at 37 °C for 5 min before the first addition.

Experiment No.	Additions						Extent of heteroduplex formation %
	-24 min	-18 min	-12 min	-6 min	0 min	1.5 min	
1	3 H]SS	recA	ATP SSB	2ATP	DS, SS ATP regen.	SSB	53.4
2	SS	recA	ATP SSB	2ATP	DS, 3 H]SS ATP regen.	SSB	3.2
3	SS	recA	ATP SSB	2ADP	DS, 3 H]SS ATP regen.	SSB	26.7
4	SS	recA		2ADP	ATP, DS ATP regen., 3 H]SS	2SSB	33.3

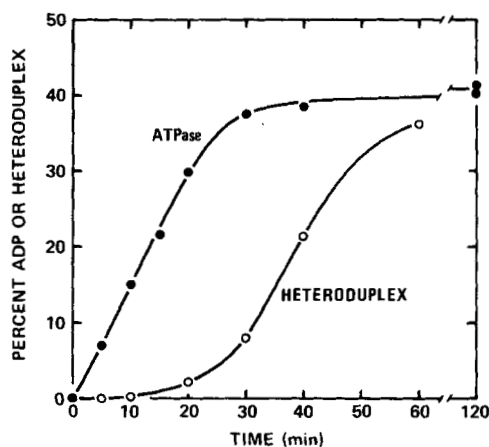


FIG. 4. Recycling of recA protein by the net accumulation of ADP. Reactions were carried out as described under "Methods" and in the text. Reaction mixtures contained 0.98 μ M recA protein, 3.3 μ M circular ϕ X ssDNA, 0.3 μ M SSB, 1.0 mM ATP. Challenge mixtures contained 3.3 μ M 3 H-labeled circular ϕ X ssDNA, 5.6 μ M linear ϕ X duplex DNA, and an ATP-regenerating system. SSB (an additional 0.3 μ M) was added 30 s after the challenge in each case. ●, time course of ATP hydrolysis in an unchallenged reaction; ○, final extent of heteroduplex formation of labeled ssDNA in a reaction challenged at the indicated time. Extents of heteroduplex formation were measured 40–60 min after the challenge.

point at which ATP hydrolysis ceases and the dissociation of recA protein from stable complexes. Several identical reaction mixtures were set up. ATP hydrolysis was measured in one, while the others were challenged at various times with a mixture containing a second equivalent of 3 H-labeled ssDNA, duplex DNA, and an ATP-regenerating system. Additional SSB was provided after the challenge to maximize any reaction of the 3 H-labeled DNA. If the complexes formed at the beginning of the reaction are intact, the labeled DNA in the challenge should be excluded from heteroduplex formation. However, if the complexes dissociate as ADP accumulates, free recA protein will interact with and promote heteroduplex formation with the labeled DNA upon addition of an ATP-regenerating system. The fate of the labeled DNA, measured 40–60 min after the challenge, should thus reflect the state of complex at the time of the challenge. As shown in Fig. 4, the complexes did not dissociate significantly until ATP hydroly-

ysis approached its limit. The limited extent of ATP hydrolysis is thus related to dissociation of the recA protein-containing complexes to a form in which the recA protein is inactive unless the ADP is removed by addition of an ATP-regenerating system. The structural stability of the complexes therefore appears to be controlled by the ADP/ATP ratio. Moreover, the ratio required for dissociation depends upon the concentration of SSB and the presence or absence of duplex DNA.

An additional experiment was performed to determine the rate at which the complexes dissociate when challenged with high levels of ADP. In this case, complexes were formed as in the previous experiment in the presence of 1 mM ATP. The reaction was then challenged with 1.6 mM ADP after 5 min. The point at which ADP was added defines $t = 0$ for dissociation. At various times, a mixture again containing 3 H-labeled ssDNA, duplex DNA, and an ATP-regenerating system was added. SSB was provided 30 s later to maximize any reaction of the challenging DNA. The final extent of heteroduplex formation involving the labeled DNA was measured 40–60 min after the time of addition of 3 H-labeled ssDNA. When no ADP was added, and the challenging DNA and ATP-regenerating mixture were added 5 min after complex formation, no reaction of the 3 H-labeled ssDNA occurred ($t = 0$) (Fig. 5). When a similar reaction was performed in which ssDNA in the complex was labeled instead of the challenging DNA, there was an efficient strand exchange reaction, again consistent with previous results. After 5 min, a maximal reaction was observed with the challenging DNA, while the DNA present in the original complex appeared to be inert. Values at intermediate times indicated that ADP addition was followed by a lag of approximately 24 s, whereupon the complexes dissociated with a half-time of 35–40 s. These results probably reflect a cooperativity in the dissociation of recA protein. They also suggest that, upon dissociation of recA protein from the complex, SSB remains associated with the ssDNA. Previous results (6) showed that recA protein will interact more readily with free than with SSB-bound ssDNA. In this experiment, the fully dissociated recA protein present 5 min after ADP addition might be expected to partition equally between the ssDNA within the complex and the challenging DNA once the ATP-regenerating system is added. Thus, the finding that the challenging DNA reacted almost exclusively with the recA protein must reflect the fact that the SSB remained associated

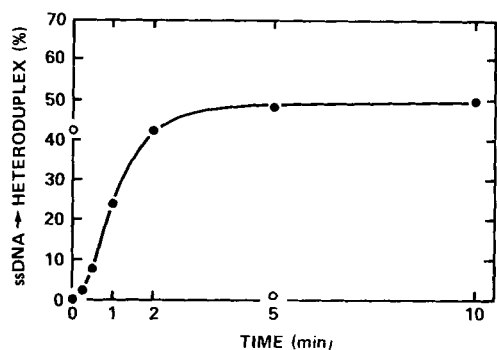


FIG. 5. Time course of ADP-mediated dissociation of *recA* protein from stable *recA* protein complexes. Reactions were carried out as described under "Methods" and elsewhere in the text. Reaction mixtures contained $0.98 \mu\text{M}$ *recA* protein, $3.3 \mu\text{M}$ circular ϕX ssDNA, 1.0 mM ATP, $0.3 \mu\text{M}$ SSB. After 5 min, reactions were challenged with 1.5 mM ADP ($t = 0$). At the times indicated, a second challenge was added containing $3.3 \mu\text{M}$ circular ϕX ssDNA, $5.6 \mu\text{M}$ linear ϕX duplex DNA, and an ATP-regenerating system. In addition, $0.3 \mu\text{M}$ SSB was added 30 s after the second challenge. ●, ^3H -label in the challenge ssDNA: final extent of heteroduplex formation of the challenge ssDNA; ○, ^3H -label in the original reaction mixture ssDNA: final extent of heteroduplex formation with this DNA. Extents of heteroduplex formation were measured 40–60 min after the second challenge.

with the ssDNA initially present in the complex.

Although these findings suggest that *recA* protein dissociates from the complex completely, the possibility remains that it is not released, but rather converted from a form that is unable to interact with the challenging ssDNA to one that can. Dissociation is supported, however, by a sucrose density gradient analysis (Fig. 6). Complexes were formed and then treated with ATP γS to stabilize *recA* protein-DNA interactions (21, 24). The labeled DNA migrated to a position in the gradient corresponding to a sedimentation constant of approximately 85 S (Fig. 6). When ADP and excess unlabeled ssDNA were added prior to the ATP γS , the label appeared at the top of the gradient at a position corresponding to free ssDNA. An experiment using ^{35}S -labeled *recA* protein produced similar results. A detailed analysis of the structure of the *recA* protein complexes and the effect of ADP on their stability will appear elsewhere.³

ATP-related Exchange Reactions

In an attempt to determine the mechanism of ATP hydrolysis, a number of potential ATP-related exchange reactions were measured. The following measurements were performed.

^3H ADP \rightleftharpoons ATP Exchange—Reactions were carried out as in Fig. 1 except that labeled nucleotide was not present. A small amount (final concentration $< 0.05 \mu\text{M}$) of ^3H -labeled ADP was added to the reaction at either 12 or 32 min to monitor any ATP formation that might have occurred. To be detected, more than 1% of the ADP would have to be converted to ATP, or the net $\text{ADP} \rightarrow \text{ATP}$ conversion should have exceeded 10 cycles/*recA* protein monomer. No exchange was detected after 60 min (data not shown).

Intermediate $\text{HPO}_4^- \rightleftharpoons \text{H}_2^{18}\text{O}$ Exchange—Reaction mixtures containing $3.3 \mu\text{M}$ ssDNA, $2.0 \mu\text{M}$ *recA* protein, 6 mM ATP were permitted to react for 9 h in 20 atom % H_2^{18}O , in the presence or absence of $0.3 \mu\text{M}$ SSB. NMR measurements indicated that 61.8% of the ATP had been hydrolyzed in the absence of SSB, and 45.6% in the presence of SSB. In both instances, 20–24% of the phosphate produced had incorpo-

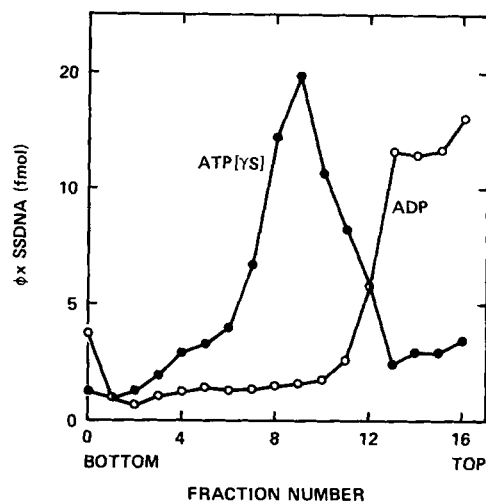


FIG. 6. ADP-mediated dissociation of *recA* protein complexes. Sucrose density gradients were run and the reaction was carried out as described under "Methods." The reaction mixture contained $1.9 \mu\text{M}$ *recA* protein, $0.61 \mu\text{M}$ SSB, $4.6 \mu\text{M}$ circular ^3H - ϕX ssDNA, $7.0 \mu\text{M}$ linear ϕX duplex DNA, 2.7 mM ATP. After incubation at 37°C for 30 min, $100\text{-}\mu\text{l}$ aliquots were removed. ATP γS was added to one aliquot at a final concentration of $200 \mu\text{M}$. To another aliquot, ADP and ϕX ssDNA were added at final concentrations of 2.1 mM and $22 \mu\text{M}$, respectively, 5 min before addition of ATP γS . The two aliquots were incubated for 5 min at 37°C before loading onto the sucrose gradients.

rated one ^{18}O ; however, no $\text{HP}^{16}\text{O}_2^{18}\text{O}_2$ could be detected. Thus, only one ^{18}O was incorporated per enzymatic turnover.

A similar reaction (in the absence of SSB) was performed except that 6 mM ADP and HKPO_4 were substituted for ATP. After a 9-h incubation, no ^{18}O could be detected in the phosphate. When complex formation was permitted by adding 1 mM ATP and SSB 10 min before addition of 6 mM ADP, 20% of the phosphate from the ATP hydrolysis which preceded ADP addition was present as $\text{HP}^{16}\text{O}_3^{18}\text{O}_1$. No ^{18}O was detected in the ATP.

Medium $\text{HP}^{18}\text{O}_4 \rightleftharpoons \text{H}_2\text{O}$ Exchange—A reaction containing $3.3 \mu\text{M}$ ϕX ssDNA, $5.6 \mu\text{M}$ linear ϕX duplex DNA, $2.0 \mu\text{M}$ *recA* protein was initiated with 1 mM ATP, $0.3 \mu\text{M}$ SSB. After 10 min, ADP and $\text{K}_2\text{HP}^{18}\text{O}_4$ were added to final concentrations of 6 mM . After 9 h, no loss of ^{18}O could be detected in the phosphate spectrum. A small amount of P^{18}O_4 was present, a product of the 10 min of ATP hydrolysis preceding addition of the ^{18}O -labeled phosphate. This result was unaffected by omission of SSB.

Effect of ADP on the Efficiency of Strand Exchange

As judged by ATP hydrolysis, strand exchange is an exceedingly inefficient process. Thus, based on the data of Fig. 2 and experiments published previously (3, 6), more than 1000 ATPs are hydrolyzed per base pair of heteroduplex formed in the absence of SSB. The addition of SSB improves the efficiency to 100–200 ATPs hydrolyzed/heteroduplex base pair. SSB may thus be considered a "coupling factor," although the reaction remains inefficient.

The effect of ADP on the structure of *recA* protein-containing complexes suggested that the concentration of ADP may influence the efficiency of the reaction. As shown in Fig. 7, under standard reaction conditions with 1 mM ATP, 152 ATPs were hydrolyzed per base pair of heteroduplex formed, as judged by initial rate measurements. As expected, the initial rate of ATP hydrolysis decreased with increasing ADP concentration; on the other hand, the initial rate of heteroduplex formation was stimulated by low concentrations of ADP. The

³ D. A. Soltis and I. R. Lehman, in preparation.

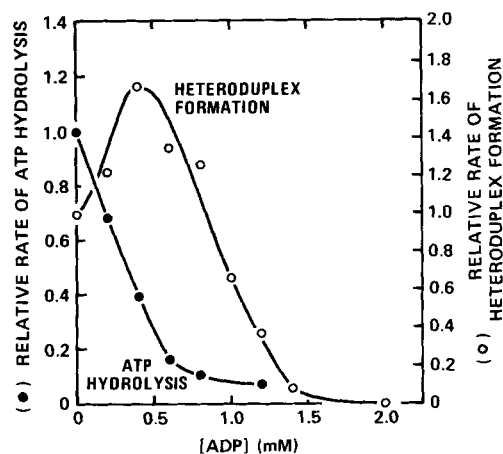


FIG. 7. Effect of ADP on the efficiency of strand exchange. Reactions were carried out as described under "Methods." Reaction mixtures contained $0.98 \mu\text{M}$ recA protein, $0.3 \mu\text{M}$ SSB, $3.3 \mu\text{M}$ circular ϕX ssDNA, $5.6 \mu\text{M}$ linear ϕX duplex DNA, 1.0 mM ATP, and the indicated concentration of ADP. ●, initial rate of ATP hydrolysis; ○, initial rate of heteroduplex formation. Initial rates of heteroduplex formation were determined from at least five time points taken before 15% of the [^3H]ssDNA had been incorporated into heteroduplex.

reaction was, however, inhibited at higher concentrations of ADP. As a consequence, the efficiency of strand exchange increased with increasing concentrations of ADP. Maximum efficiency was reached at 0.6 mM ADP, where 16 ATPs were hydrolyzed per base pair of heteroduplex formed. Thus at least 90% of the ATP utilized under normal reaction conditions is wasted. Indeed, recA protein shows potent ATPase activity under conditions in which no base pairing or branch migration can take place, *e.g.* in the presence of ssDNA alone (13, 14).

DISCUSSION

Our principal conclusion is that the stability of the recA protein-ssDNA complexes formed in the presence of SSB and ATP is governed by the ADP/ATP ratio. Hydrolysis of ATP halts before completion of the reaction because the complexes dissociate and the recA protein is unable to catalyze further ATP hydrolysis. This dissociation is not a consequence of the completion of strand exchange but is simply a response to the ADP/ATP ratio. It does, however, permit the recA protein to be recycled, *i.e.* to participate in another round of strand exchange, once the ATP has been regenerated. Dissociation of the complex explains the initial decrease in heteroduplex formation when an ongoing reaction was challenged with ADP (Fig. 2). At the time of challenge, most of the DNA molecules had not yet been converted to product replicative form II, but were still in the D-loop phase (3, 5). Dissociation of the recA protein leaves these molecules open to random branch migration, which in this case produces a transient decrease in total heteroduplex. We do not yet understand the basis for the ADP-mediated dissociation. The ADP may function at the primary ATP-binding site, where it acts as a competitive inhibitor. Alternatively, it may produce an effect separate from the competitive inhibition, possibly through a secondary ADP-binding site. At present, there is no firm evidence for a secondary ADP-binding site, but this possibility has not been ruled out.

Our findings can be summarized in the following way. In the presence of ATP and SSB, recA protein is incorporated into a stable complex with ssDNA. This complex will react with homologous duplex DNA in a strand exchange reaction and, if an ATP-regenerating system is provided, it is stable for up to 90 min (6). Net hydrolysis of ATP to ADP and P_i has no effect on the stability of the complex until the ADP/

ATP ratio exceeds a threshold level which varies with reaction conditions but is always ≥ 0.2 . Additional ADP causes some dissociation of recA protein from the complexes. At ADP/ATP ratios of 1.5 or more, complete dissociation of recA protein from the complexes occurs but SSB remains associated with the ssDNA. recA protein can then be recycled to begin a new strand exchange reaction if an ATP-regenerating system is provided.

The inability thus far to detect exchange reactions associated with ATP hydrolysis is puzzling. Coupled vectorial systems such as the actin-myosin complex and ion transport systems retain the binding energy of ATP so that at certain steps in their reaction pathways ATP hydrolysis is microscopically reversible on the enzyme (10). The exchange experiments were designed to detect: (a) a classical $\text{ATP} \rightleftharpoons \text{P}_i$ exchange owing to the reversibility of the hydrolysis and the facile dissociation of both substrates from recA protein; (b) incorporation of H_2^{18}O in excess of a single hydrolytic event arising from resynthesis of ATP from ADP and P_i , where P_i release is slow relative to the hydrolysis step; and (c) formation of ATP from P_i and ADP under conditions where ATP release is slow relative to ATP synthesis, a condition that would obviate the exchange sought in (a). While the failure to detect ATP synthesis by at least one of these three experiments supports an irreversible ATP hydrolysis, there can be several reasons for the failure to observe an exchange reaction. First, no such exchange may occur. Secondly, the conditions used to observe the exchange might not be appropriate, or alternatively, a factor necessary to properly couple ATP hydrolysis to strand exchange is lacking. Finally, the extensive formation of ADP through an uncoupled hydrolysis could overwhelm the ability to detect exchange arising from a coupled component.

Excessive hydrolysis of ATP is a characteristic of a number of DNA-dependent ATPases (25). In many instances, the excess may reflect the absence of an effector. In the case of recA protein, some fraction of the ATP hydrolysis must be coupled to branch migration since it exhibits a constant requirement for ATP hydrolysis. Any treatment that interferes with ATP hydrolysis terminates branch migration (3), although the inhibition of branch migration (for example, by placing a heterologous region of DNA in the path of the migrating branch) has little effect on the rate of ATP hydrolysis.²

The stimulatory effect of low concentrations of ADP on heteroduplex formation was unexpected. Since high levels of ADP have an obvious structural effect on recA protein complexes, it is, however, reasonable to assume that ADP may influence the structure of the complexes at any concentration. As noted previously (6), studies of the stable recA protein complexes formed in the presence of ATP and SSB are not inconsistent with a treadmilling reaction similar to that observed with actin filaments (26). recA protein released in such a reaction should show a marked preference for binding recA protein filaments over binding free DNA to be consistent with the earlier results (6). ADP might accelerate such a treadmilling reaction at low concentrations while disrupting the complexes at high concentrations. The answers to this and other questions raised by these experiments await completion of the more detailed kinetic and structural studies now in progress.

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