Three-strand Exchange by the *Escherichia coli* RecA Protein Using ITP as a Nucleotide Cofactor

MECHANISTIC PARALLELS WITH THE ATP-DEPENDENT REACTION OF THE RecA PROTEIN FROM *STREPTOCOCCUS PNEUMONIAE*

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The RecA protein from *Escherichia coli* promotes an ATP-dependent three-strand exchange reaction between a circular single-stranded DNA (ssDNA) and a homologous linear double-stranded (dsDNA). We have now found that under certain conditions, the RecA protein is also able to promote the three-strand exchange reaction using the structurally related nucleoside triphosphate, ITP, as the nucleotide cofactor. However, although both reactions are stimulated by single-stranded DNA-binding (SSB) protein, the ITP-dependent reaction differs from the ATP-dependent reaction in that it is observed only at low SSB protein concentrations, whereas the ATP-dependent reaction proceeds efficiently even at high SSB protein concentrations. Moreover, the circular ssDNA-dependent ITP hydrolysis activity of the RecA protein is strongly inhibited by SSB protein (suggesting that SSB protein displaces RecA protein from ssDNA when ITP is present), whereas the ATP hydrolysis activity is uninhibited even at high SSB protein concentrations (because RecA protein is resistant to displacement by SSB protein when ATP is present). These results suggest that SSB protein does not stimulate the ITP-dependent strand exchange reaction presynaptically (by facilitating the binding of RecA protein to the circular ssDNA substrate) but may act postsynaptically (by binding to the displaced strand that is generated when the circular ssDNA invades the linear dsDNA substrate). Interestingly, the mechanistic characteristics of the ITP-dependent strand exchange reaction of the *E. coli* RecA protein are similar to those of the ATP-dependent strand exchange reaction of the RecA protein from *Streptococcus pneumoniae*. These findings are discussed in terms of the relationship between the dynamic state of the RecA-ssDNA filament and the mechanism of the SSB protein-stimulated three-strand exchange reaction.

The RecA protein of *Escherichia coli* (Mr, 37,842; 352 amino acids) is essential for homologous genetic recombination and recombinational DNA repair. The purified RecA protein binds cooperatively to ssDNA, forming a helical filament-like structure with 1 RecA monomer/3 nucleotides of ssDNA and 6 RecA monomers/filament turn. This RecA-ssDNA complex catalyzes the hydrolysis of ATP to ADP and P\textsubscript{i}. In addition, the RecA protein is able to promote a variety of ATP-dependent DNA pairing reactions that reflect *in vivo* recombination functions. The most extensively studied is the three-strand exchange reaction, in which a circular ssDNA and a homologous linear dsDNA are recombined to form a nicked circular dsDNA and a linear ssDNA. This reaction proceeds in three phases. In the first phase, the circular ssDNA is covered with a continuous filament of RecA protein, forming a structure known as the presynaptic complex. In the second phase, the presynaptic complex interacts with a homologous linear dsDNA, and pairing between the circular ssDNA and the complementary strand from the linear dsDNA is initiated. In the third phase, the complementary linear strand is completely transferred to the circular ssDNA by unidirectional branch migration to yield the nicked circular dsDNA and displaced linear ssDNA products. The *E. coli* SSB protein, which stimulates the strand exchange reaction both presynaptically (by melting out secondary structure in the circular ssDNA substrate, which otherwise impedes RecA protein binding) and postsynaptically (by binding to the displaced strand that is generated when the ssDNA substrate invades the homologous dsDNA), is routinely included as an accessory factor in strand exchange assays (1, 2).

In earlier studies, we found that the RecA protein has a curious nucleotide cofactor specificity. For example, the structurally related nucleoside triphosphates ATP and ITP are hydrolyzed by the RecA protein with identical turnover numbers (20 min\textsuperscript{-1}). However, ATP (S\textsubscript{0.5} = 50 μM) functions as a cofactor for the three-strand exchange reaction, whereas ITP (S\textsubscript{0.5} = 500 μM) is inactive as a strand exchange cofactor under standard reaction conditions (pH 7.5, 37 °C) (3). We have now found that although ITP is ineffective at pH 7.5, the RecA protein is able to promote the three-strand exchange reaction using ITP as the nucleotide cofactor at pH 6.5. The mechanistic characteristics of the ITP-dependent strand exchange reaction, however, differ significantly from the ATP-dependent reaction, especially in terms of the dependence on SSB protein. These findings provide new insight into the relationship between the dynamic state of the RecA-ssDNA filament and the mechanism
of the SSB protein-stimulated three-strand exchange reaction and are described in this report.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wild type RecA protein (5) and [D100N]RecA protein (6) were prepared as described. *E. coli* SSB protein was from Promega. ATP, ITP, and [α-32P]ATP were from Amersham Biosciences. [γ-32P]ITP was prepared as described (3). Circular φX ssDNA (+ strand) and circular φX dsDNA were from New England Biolabs. Linear φX dsDNA was prepared from circular φX dsDNA by PstI digestion as described (7). All DNA concentrations are expressed as total nucleotides.

**NTP Hydrolysis Assay**—ATP and ITP hydrolysis reactions were analyzed using a thin layer chromatography method as previously described (8). All DNA concentrations are expressed as total nucleotides.

**Circular ssDNA** was prepared as described (3). Circular ssDNA was prepared from circular φX dsDNA by PstI digestion as described (7). The specific conditions that were used for each set of reactions are given in the relevant figure legends.

**Three-strand Exchange Assay**—Three-strand exchange reactions were analyzed using an agarose gel electrophoresis method as previously described (7). Aliquots (20 μl) were removed from the reaction solutions and quenched with SDS (final concentration, 1%) and EDTA (final concentration, 15 mM). The quenched aliquots were analyzed by ethidium bromide staining. The specific conditions that were used for each set of reactions are given in the relevant figure legends.

**RESULTS**

**ATP and ITP-dependent Three-strand Exchange Reactions**—The RecA protein was analyzed for three-strand exchange activity at pH 7.5 and 6.5, using either ATP or ITP as the nucleotide cofactor. In the three-strand exchange assay, a circular φX ssDNA molecule (5386 nucleotides) and a homologous linear φX dsDNA molecule (5386 base pairs) are recombined to form a nicked circular φX dsDNA molecule and a linear φX ssDNA molecule. The substrates and products of this reaction are readily monitored by agarose gel electrophoresis (7).

**Circular ssDNA** was provided as the nucleotide cofactor, partially exchanged DNA intermediates were visible within 10 min, and essentially all of the circular ssDNA substrate was converted into the completely exchanged nicked circular dsDNA product within 30 min. In contrast, there was no detectable strand exchange activity at pH 7.5 when ITP was provided as the nucleotide cofactor, even after a prolonged reaction period (Fig. 1). These results are consistent with our previously reported results (3).

The strand exchange reactions that were promoted by the RecA protein at pH 7.5 are shown in Fig. 1. When ATP was provided as the nucleotide cofactor, partially exchanged DNA intermediates were visible within 10 min, and essentially all of the circular ssDNA substrate was converted into the completely exchanged nicked circular dsDNA product within 30 min. In contrast, there was no detectable strand exchange activity at pH 7.5 when ITP was provided as the nucleotide cofactor, even after a prolonged reaction period (Fig. 1). These results are consistent with our previously reported results (3).

The strand exchange reactions that were promoted by the RecA protein at pH 6.5 are also shown in Fig. 1. The reaction that was observed with ATP as the nucleotide cofactor was similar to that at pH 7.5, with nearly all of the circular ssDNA being converted into the fully exchanged nicked circular dsDNA product within 30 min. In contrast to the results at pH 7.5, however, the RecA protein also exhibited strand exchange activity at pH 6.5 with ITP as the nucleotide cofactor (Fig. 1).

The yield of fully exchanged products was somewhat lower in the ITP-dependent reaction than in the ATP-dependent reaction, these results clearly demonstrate that ITP is able to function as a cofactor for the three-strand exchange reaction.

**Dependence of ATP and ITP-dependent Strand Exchange Reactions on SSB Protein Concentration**—To compare the ATP- and ITP-dependent strand exchange reactions in more detail, additional sets of reactions were carried out at pH 7.5 and 6.5 in which the concentrations of circular φX ssDNA (5 μM), linear φX dsDNA (15 μM), NTP (3 mM), and RecA protein (6 μM) were kept constant, and the concentration of SSB protein was varied from 0 to 0.5 μM (the concentration of SSB protein required to saturate the φX ssDNA (5 μM) under our reaction conditions was 0.15–0.3 μM). As shown in Fig. 2, a low level of strand exchange was observed at pH 7.5 in the absence of SSB protein when ATP was provided as the nucleotide cofactor. The efficiency of the ATP-dependent strand exchange reaction increased significantly, however, as the concentration of SSB protein was increased to 0.05 μM (the concentration used in the reaction shown in Fig. 1), with nearly all of the circular ssDNA being converted into the nicked circular dsDNA product at this SSB protein concentration. Moreover, the ATP-dependent reaction remained undiminished even when the SSB protein concentration was increased to 0.5 μM. In contrast, no strand exchange was observed at pH 7.5 when ITP was provided as the nucleotide cofactor, at any of the SSB protein concentrations tested (Fig. 2).

A low level of strand exchange was also observed in the absence of SSB protein at pH 6.5 when ATP was provided as the nucleotide cofactor (Fig. 2). The efficiency of the ATP-dependent strand exchange reaction increased significantly as the concentration of SSB protein was increased to 0.05 μM (the concentration used in the reaction shown in Fig. 1) and remained undiminished even when the SSB protein concentration was increased to 0.5 μM. These results are similar to those at pH 7.5 (Fig. 2). In contrast to the results at pH 7.5, however, the RecA protein also exhibited a low level of strand exchange activity at pH 6.5.
in absence of SSB protein when ITP was provided as the nucleotide cofactor (Fig. 2). Moreover, the efficiency of the ITP-dependent strand exchange reaction increased as the concentration of SSB protein was increased to 0.05 \( \mu M \) (the concentration used in the reaction shown in Fig. 1). Unlike the ATP-dependent reaction, however, the level of ITP-dependent strand exchange decreased markedly as the SSB protein concentration was increased above 0.05 \( \mu M \), with no strand exchange being detectable with ITP at 0.5 \( \mu M \) SSB protein. These results indicate that the ITP-dependent strand exchange activity of the RecA protein is stimulated optimally by low concentrations of SSB protein and that higher concentrations of SSB protein act to counter this stimulatory effect.

**Effect of SSB Protein on the ssDNA-dependent ATP and ITP Hydrolysis Reactions**—SSB protein has been shown to stimulate the ATP-dependent strand exchange reaction, at least in part, by facilitating the binding of RecA protein to the circular ssDNA substrate. This facilitated binding is thought to occur in two steps. First, SSB protein binds to the circular ssDNA and melts out regions of secondary structure that otherwise impede the binding of RecA protein. RecA protein then displaces the SSB protein from the ssDNA, leading to the formation of a presynaptic complex in which the circular ssDNA is covered by a continuous filament of RecA protein. Although this facilitated binding mechanism is well established for the ATP-dependent strand exchange reaction, the ITP-dependent strand exchange reaction showed a more complex dependence on SSB protein concentration (Fig. 2). Thus, it was not clear that the ITP-dependent reaction was stimulated by SSB protein in the same manner as the ATP-dependent reaction. An experimental consequence of the SSB protein-mediated increase in RecA protein binding that occurs in the presence of ATP is that the observed rate of ssDNA-dependent ATP hydrolysis increases when SSB protein is added to the reaction solution (9, 10). Therefore, to investigate the role of SSB protein in the ITP-dependent strand exchange reaction, the effect of SSB protein on the \( \delta X \) ssDNA-

**Fig. 2. Dependence of ATP- and ITP-dependent three-strand exchange reactions on SSB protein concentration.** The reaction solutions contained 25 mM Tris-HCl (pH 7.5) or BisTris-HCl (pH 6.5), 5% glycerol, 1 mM DTT, 10 mM MgCl\(_2\), 5 \( \mu M \) circular \( \delta X \) ssDNA, 15 \( \mu M \) linear \( \delta X \) dsDNA, 6 \( \mu M \) RecA protein, 3 mM ATP or ITP, and the indicated concentrations of SSB protein (from 0 to 0.5 \( \mu M \)). The reactions were initiated by the simultaneous addition of SSB protein and either ATP or ITP. The final reaction solutions were incubated at 37 °C. The aliquots were removed after 180 min and analyzed by agarose gel electrophoresis as described under “Experimental Procedures.” Under these conditions, the concentration of circular ssDNA was limiting relative to the linear dsDNA, and the maximum amount of the linear dsDNA that could be converted to nicked linear dsDNA product was 67%. S, linear dsDNA substrate; P, partially exchanged reaction intermediates; F, fully exchanged nicked circular dsDNA product; ss, single-stranded DNA.

**Fig. 3. Dependence of \( \delta X \) ssDNA-dependent ATP and ITP hydrolysis on RecA protein concentration.** The reaction solutions contained 25 mM Tris-HCl (pH 7.5) or 25 mM BisTris-HCl (pH 6.5), 5% glycerol, 1 mM DTT, 10 mM MgCl\(_2\), 5 \( \mu M \) circular \( \delta X \) ssDNA, 3 \( \mu M \) [\( \gamma^{32}P \)]ATP or [\( \gamma^{32}P \)]ITP, and the indicated concentrations of RecA protein. The reactions were initiated by the addition of RecA protein. The final reaction solutions were incubated at 37 °C. The points represent the initial rates of ATP hydrolysis (closed symbols) or ITP hydrolysis (open symbols) that were measured at pH 7.5 (squares) or pH 6.5 (circles).

The \( \delta X \) ssDNA-dependent ATP and ITP hydrolysis reactions were first compared in the absence of SSB protein. In these reactions, the concentrations of \( \delta X \) ssDNA (5 \( \mu M \)) and NTP (3 \( \mu M \)) were fixed, and the concentration of RecA protein was varied (0–10 \( \mu M \)). As shown in Fig. 3, the observed rate of ATP hydrolysis at pH 7.5 increased with increasing RecA protein concentration until reaching a maximal value of \( \sim 15 \) \( \mu M \) min\(^{-1}\) at RecA protein concentrations above 2 \( \mu M \). Similar results were obtained for the ATP hydrolysis reaction (Fig. 3). Because the turnover number for ssDNA-dependent ATP and ITP hydrolysis is \( \sim 20 \) min\(^{-1}\) at pH 7.5 (Ref. 3 and data not shown), the maximal rate of ATP or ITP hydrolysis that would be expected under the conditions of the reactions shown in Fig. 3 (with 5 \( \mu M \) \( \delta X \) ssDNA) would be \( \sim 34 \) \( \mu M \) min\(^{-1}\) (1.7 \( \mu M \) RecA protein bound, assuming a maximum binding stoichiometry of 1 RecA monomer/3 nucleotides ssDNA; Refs. 1 and 2). Therefore, the observed rate of 15 \( \mu M \) min\(^{-1}\) indicates that only about one-half of the \( \delta X \) ssDNA was covered with RecA protein at pH 7.5 in the presence of either ATP or ITP, even when the concentration of RecA protein was in stoichiometric excess, relative to the \( \delta X \) ssDNA. These results suggest that approximately one-half of the \( \delta X \) ssDNA was inaccessible to the RecA protein under these conditions, presumably because of the existence of the secondary structure that impedes RecA protein binding (9, 10). By comparison, when the reactions were carried out at pH 6.5, the observed rates of ATP and ITP hydrolysis reached values of \( \sim 36 \) \( \mu M \) min\(^{-1}\), at RecA protein concentrations above 4 \( \mu M \) (Fig. 3). Because the turnover number for ATP and ITP hydrolysis is \( \sim 24 \) min\(^{-1}\) at pH 6.5 (data not shown), the maximal rate of hydrolysis that would be expected under these reaction conditions would be \( \sim 41 \) \( \mu M \) min\(^{-1}\). Therefore, the observed rate of \( \sim 36 \) \( \mu M \) min\(^{-1}\) indicates that the \( \delta X \) ssDNA was almost completely covered by RecA protein at pH 6.5, in the presence of either ATP or ITP.

The \( \delta X \) ssDNA-dependent ATP and ITP hydrolysis reactions were next examined in the presence of SSB protein. In these
and RecA protein (6 μM) were included in the reaction solution, the rate of the ATP hydrolysis reaction increased with increasing SSB protein concentration, until reaching a value of ~40 μM min⁻¹ at SSB protein concentrations above 0.1 μM. This rate is comparable with the expected maximal value of 41 μM min⁻¹ (see above) and indicates that SSB protein is able to promote the binding of additional RecA protein to the φX ssDNA at pH 6.5, when ATP is provided as the nucleotide cofactor. As found at pH 7.5, however, the rate of the ITP hydrolysis reaction decreased with increasing SSB concentration, with ITP hydrolysis being completely inhibited at SSB concentrations above 0.3 μM (Fig. 4). The elimination of ITP hydrolysis activity suggests that SSB protein is able to displace RecA protein from φX ssDNA at pH 6.5, when ITP is provided as the nucleotide cofactor.

To further explore the inhibitory effect of SSB protein on the ITP hydrolysis reaction, a set of reactions was carried out in which SSB protein was added to an ongoing ITP hydrolysis reaction. The reaction solutions contained φX ssDNA (5 μM), NTP (3 mM), RecA protein (6 μM), and a concentration of SSB protein (0.5 μM; added at 12 min) that was sufficient to give complete inhibition of the ITP hydrolysis reaction (Fig. 4). As shown in Fig. 5, the rate of ITP hydrolysis at pH 6.5, before the addition of SSB protein, was 30 μM min⁻¹ (consistent with the results in Figs. 3 and 4). When SSB protein was added to the reaction solution (at 12 min), however, the ITP hydrolysis reaction was completely inhibited. Moreover, the amount of ITP hydrolysis that was measured immediately before the addition of SSB protein (12 min) was indistinguishable from that measured after the inhibition was complete (15 min), indicating that the onset of inhibition was too fast to be resolved by our experimental protocol. A rapid inhibition of ITP hydrolysis was also observed at pH 7.5 when SSB protein was added to the reaction solution (data not shown). By comparison, when SSB protein was added to an ongoing ATP hydrolysis reaction at pH 6.5, the rate of ATP hydrolysis immediately increased from 30 to 40 μM min⁻¹, consistent with facilitated binding of additional RecA protein to the φX ssDNA (Fig. 5). A rapid enhancement of ATP hydrolysis was also observed at pH 7.5 when SSB protein was added to the reaction solution (data not shown). These results suggest that RecA protein is readily displaced from φX ssDNA by SSB protein at either pH 7.5 or 6.5 in the presence of ITP but is resistant to displacement in the presence of ATP.

To investigate the SSB protein-mediated inhibition of the ITP hydrolysis reaction more closely, a set of reactions was carried out in which SSB protein was added to an ongoing ITP hydrolysis reaction. The reaction solutions contained φX ssDNA (5 μM), NTP (3 mM), and RecA protein (6 μM) were fixed, and the concentration of SSB protein was varied (0–0.5 μM). Because the binding of RecA protein and SSB protein to ssDNA is competitive and mutually exclusive, the maximal rates of ATP or ITP hydrolysis will be observed when the φX ssDNA is completely covered by RecA protein, and no ATP or ITP hydrolysis will be observed if the φX ssDNA is completely covered by SSB protein (9, 10).

As shown in Fig. 4, the rates of ATP and ITP hydrolysis were similar at pH 7.5 in the absence of SSB protein (10–12 μM min⁻¹) and were consistent with a partial coverage of the φX ssDNA by RecA protein at this pH. When SSB protein was included in the reaction solution, the rate of the ATP hydrolysis reaction increased with increasing SSB concentration until reaching a value of ~30 μM min⁻¹ at SSB concentrations above 0.1 μM (Fig. 4). This rate is close to the expected maximal value of 34 μM min⁻¹ (see above) and indicates that SSB protein is able to promote the binding of additional RecA protein to the φX ssDNA at pH 7.5 when ATP is provided as a nucleotide cofactor. In contrast, the rate of the ITP hydrolysis reaction decreased with increasing SSB concentration, with ITP hydrolysis being completely inhibited at SSB concentrations above 0.1 μM (Fig. 4). The complete elimination of ITP hydrolysis activity suggests that SSB protein acts not to facilitate RecA protein binding but rather to displace RecA protein from the φX ssDNA by RecA protein at pH 7.5 (Fig. 4). When SSB protein was included in the reaction solution, the rate of the ATP hydrolysis reaction increased with increasing SSB protein concentration, until reaching a value of ~40 μM min⁻¹ at SSB protein concentrations above 0.1 μM. This rate is comparable with the expected maximal value of 41 μM min⁻¹ (see above) and indicates that SSB protein is able to promote the binding of additional RecA protein to the φX ssDNA at pH 7.5, when ATP is provided as the nucleotide cofactor.
RecA protein is able to displace SSB protein from the \( \phi X \) ssDNA at either pH 7.5 or 6.5 in the presence of ATP but is unable to do so in the presence of ITP. The inhibitory effect of SSB protein on the \( \phi X \) ssDNA-dependent ITP hydrolysis reaction is intriguing inasmuch as the results in Fig. 2 show that the ITP-dependent strand exchange reaction (pH 6.5) is strongly stimulated by SSB protein. A comparison of the results in Figs. 2 and 4, however, shows that the low concentration of SSB protein (0.05 \( \mu M \)) that was found to stimulate the ITP-dependent strand exchange reaction (Fig. 2) does not result in a significant inhibition of the ITP hydrolysis reaction (Fig. 4). This indicates that this concentration of SSB protein (which is not sufficient to cover the circular \( \phi X \) ssDNA) does not displace a significant amount of RecA protein from the circular ssDNA substrate and therefore would not be expected to inhibit the strand exchange reaction. When SSB protein is present at higher concentrations (that are sufficient to cover the circular \( \phi X \) ssDNA), however, the ITP hydrolysis activity is strongly inhibited, suggesting that the RecA protein has been displaced from the ssDNA (Fig. 4). Correspondingly, ITP-dependent strand exchange activity is not observed at these higher concentrations of SSB protein (Fig. 2).

**Fig. 5.** Effect of addition of SSB protein to an ongoing \( \phi X \) ssDNA-dependent ATP or ITP hydrolysis reaction. The initial reaction solutions contained 25 mM BisTris-HCl (pH 6.5), 5% glycerol, 1 mM DTT, 10 mM MgCl\(_2\), 5 \( \mu M \) circular \( \phi X \) ssDNA, 6 \( \mu M \) RecA protein, and 3 mM \([\gamma^{32}P]ATP \) or \([\gamma^{32}P]ITP \). The reactions were initiated by the addition of RecA protein, and the reaction solutions were incubated at 37 °C. After 12 min (indicated by arrow), either reaction buffer (closed circles) or SSB protein (final concentration, 0.5 \( \mu M \); open circles) was added to the reaction solution, and the incubation was continued at 37 °C. The points represent the amounts of ATP or ITP hydrolysis that were measured at the indicated times.

**Fig. 6.** Effect of addition of SSB protein prior to RecA protein on the \( \phi X \) ssDNA-dependent ATP and ITP hydrolysis reactions. The initial reaction solutions contained 25 mM BisTris-HCl (pH 6.5), 5% glycerol, 1 mM DTT, 10 mM MgCl\(_2\), 5 \( \mu M \) circular \( \phi X \) ssDNA, 3 mM \([\gamma^{32}P]ATP \) or \([\gamma^{32}P]ITP \), and either 0 \( \mu M \) SSB protein (closed circles) or 0.5 \( \mu M \) SSB protein (open circles). The reaction solutions were incubated at 37 °C. After 12 min (indicated by arrow), RecA protein (final concentration, 6 \( \mu M \)) was added, and the incubation was continued at 37 °C. The points represent the amounts of ATP or ITP hydrolysis that were measured at the indicated times.
RecA protein is 6-amino group of ATP (11). This same Asp\(^{100}\) side chain presumably interacts unfavorably with the 6-carbonyl group of ITP, and this unfavorable interaction is likely responsible for the elevated \(S_0.5\) value of ITP relative to that of ATP. In the [D100N]RecA protein, however, the Asp\(^{100}\) group has been replaced with an uncharged Asn residue. This mutation has no effect on the turnover number for ITP hydrolysis but does lower the \(S_0.5\) value for ITP (\(S_0.5 = 90 \mu M\)). Moreover, unlike the wild type protein, the [D100N]RecA protein is able to promote the three-strand exchange reaction under standard conditions (pH 7.5, 37 °C) with ITP as a cofactor.

The 6X ssDNA-dependent ITP hydrolysis activity of the [D100N]RecA protein was first examined under standard reaction conditions in the absence of SSB protein. As shown in Fig. 7A, the observed rate of ITP hydrolysis increased with increasing [D100N]RecA protein concentration until reaching a value of \(\sim 30 \mu M\) min\(^{-1}\) at [D100N]RecA protein concentrations above 2 \(\mu M\). Because the turnover number for ITP hydrolysis by the [D100N]RecA protein is \(-22 \mu M\) min\(^{-1}\) at pH 7.5 (data not shown), the maximal rate of ITP hydrolysis that would be expected under these conditions would be \(\sim 37 \mu M\) min\(^{-1}\) (1.7 \(\mu M\) [D100N]RecA protein bound, assuming a maximum binding stoichiometry of 1 [D100N]RecA monomer/3 nucleotides ssDNA). Therefore, the observed maximal rate of 30 \(\mu M\) min\(^{-1}\) indicates that the 6X ssDNA was extensively covered by [D100N]RecA protein in the presence of ITP.

The 6X ssDNA-dependent ITP hydrolysis reaction of the [D100N]RecA protein was next examined under standard reaction conditions in the presence of SSB protein. As shown in Fig. 7B, the rate of ITP hydrolysis in the absence of SSB protein was 32 \(\mu M\) min\(^{-1}\) and increased further with increasing SSB protein concentration, until reaching a maximal value of \(-42 \mu M\) min\(^{-1}\) at SSB protein concentrations above 0.1 \(\mu M\). This rate is comparable with the expected maximal value of 37 \(\mu M\) min\(^{-1}\) and indicates that SSB protein is able to promote the binding of [D100N]RecA protein to 6X ssDNA at pH 7.5 in the presence of ITP. These results are in direct contrast to the strong inhibitory effect of SSB protein on the ITP hydrolysis activity of the wild type RecA protein (Fig. 4) and demonstrate that the [D100N]RecA protein is resistant to displacement from 6X ssDNA by SSB protein when ITP is provided as the nucleotide cofactor. Accordingly, the [D100N]RecA protein is able to promote an ITP-dependent strand exchange reaction, even at pH 7.5 and in the presence of a concentration of SSB protein (0.5 \(\mu M\)) that is completely inhibitory for the ITP-dependent strand exchange activity of the wild type RecA protein (Fig. 8).

**DISCUSSION**

The RecA protein is able to promote the three-strand exchange reaction at pH 6.5, using either ATP or ITP as the nucleotide cofactor. However, although both reactions are stimulated by SSB protein, the ITP-dependent reaction differs from the ATP-dependent reaction in that it is only observed at low SSB protein concentrations, whereas the ATP-dependent reaction proceeds optimally even at high SSB protein concentrations. Moreover, the 6X ssDNA-dependent ITP hydrolysis activity of the RecA protein is strongly inhibited by SSB protein (suggesting that SSB protein displaces RecA protein from ssDNA when ITP is present), whereas the ATP hydrolysis activity is uninhibited even at high SSB concentrations (because RecA protein is resistant to displacement from ssDNA by SSB protein when ATP is present). Taken together, these results indicate that the stimulatory effect of SSB protein on the ITP-dependent strand exchange reaction must be due to a mechanism other than the facilitation of presynaptic complex formation between RecA protein and the circular 6X ssDNA substrate.

In addition to enhancing presynaptic complex formation, it has been reported that SSB protein also stimulates the three-strand exchange reaction postsynaptically by binding to the partially displaced linear single strand that is generated when the circular ssDNA invades the homologous linear dsDNA substrate. This binding may stabilize the initial DNA pairing
intermediates, prevent secondary DNA pairing reactions, and drive the reaction forward to the formation of the completely exchanged circular dsDNA product (12, 13). Thus, it is likely that the stimulatory effect of SSB protein on the ITP-dependent strand exchange reaction is due to this postsynaptic mechanism. This idea is consistent with the observation that the ITP-dependent strand exchange reaction is only observed at SSB protein concentrations below that which would be required to cover the circular $\phi X$ ssDNA. At low (subsaturation) concentrations, the SSB protein may bind to the partially displaced linear ssDNA rather than displace RecA protein from the circular $\phi X$ ssDNA substrate and would therefore serve to stimulate the ITP-dependent strand exchange reaction. At higher concentrations, however, SSB protein may displace the RecA protein from the circular $\phi X$ ssDNA substrate, resulting in the elimination of the ITP-dependent strand exchange activity. Even at the optimum SSB protein concentration, the competing displacement of RecA protein from the circular ssDNA substrate may reduce the efficiency of the ITP-dependent strand exchange reaction, relative to the ATP-dependent reaction (Fig. 2). By comparison, because RecA protein is not displaced from $\phi X$ ssDNA by SSB protein when ATP is present as the nucleotide cofactor, the ATP-dependent strand exchange reaction proceeds with high efficiency, even in the presence of an excess concentration of SSB protein.

The results discussed above establish that ITP is able to induce the strand exchange active state of the RecA-ssDNA filament. This raises the question of why the ITP-dependent strand exchange reaction was observed at pH 6.5, but not at pH 7.5. Because it is generally believed that the ssDNA substrate has to be covered with a continuous filament of RecA protein before strand exchange can occur (1, 2), the reason for the pH dependence may be that the circular $\phi X$ ssDNA substrate can be extensively covered by RecA protein at pH 6.5 in the presence of ITP, without the assistance of SSB protein. In fact, ITP-dependent strand exchange activity was detected in the absence of SSB protein at pH 6.5. At pH 7.5, on the other hand, the coverage of $\phi X$ ssDNA by RecA protein is much lower (presumably because RecA protein is more impeded by regions of secondary structure), and the level of coverage cannot be increased by including SSB protein in the reaction solution when ITP is present as the nucleotide cofactor. These findings may account for the apparent absence of ITP-dependent strand exchange activity at pH 7.5, either in the absence or presence of SSB protein. However, there also appears to be a low level of coverage of the $\phi X$ ssDNA at pH 7.5 when ATP is provided as the nucleotide cofactor, and yet a limited amount of ATP-dependent strand exchange is observed in the absence of SSB protein at this pH. These results suggest that (i) although only about one-half of the $\phi X$ ssDNA, on average, is covered by RecA protein at pH 7.5, there may be some $\phi X$ ssDNA molecules that are much more extensively covered by RecA protein, and (ii) these RecA-$\phi X$ ssDNA complexes may be intrinsically less active in strand exchange with ITP than with ATP, even when SSB protein is not present to displace RecA protein from the ssDNA.

It has been shown that the binding of RecA protein and SSB protein to ssDNA is competitive and mutually exclusive (9, 10). Therefore, for SSB protein to displace RecA protein from $\phi X$ ssDNA, the RecA protein presumably must first dissociate from the ssDNA before the SSB protein can bind to the vacated site (9, 10). It has recently been shown that RecA protein dissociates uniquely from the 5′ end of the polymeric RecA-ssDNA filament and that the rate of filament disassembly is dependent on the nucleotide cofactor that is present. For example, although RecA-ssDNA filament disassembly can be detected in the presence of ATP, the disassembly process is completely suppressed when dATP is provided as the nucleotide cofactor (14, 15). Although ATP and dATP are hydrolyzed with similar turnover numbers, the $S_{0.5}$ value for dATP ($S_{0.5} = 20 \mu M$) is lower than that for ATP ($S_{0.5} = 50 \mu M$) (16). Therefore, it is conceivable that filament disassembly is related to the $S_{0.5}$ value of the NTP being hydrolyzed, with those NTPs with higher $S_{0.5}$ values leading to increased rates of disassembly. If this is the case, it would follow that ITP ($S_{0.5} = 500 \mu M$) may induce a rate of filament disassembly than is even greater than that which occurs with ATP. This increased rate of disassembly may, in turn, leave the RecA-ssDNA filament more susceptible to displacement by SSB protein. This proposal is consistent with our results with the mutant [D100N]RecA protein, in which Asp100 in the NTP-binding site of the RecA protein has been replaced by an Asn residue. This mutation lowers the $S_{0.5}$ value for ITP ($S_{0.5} = 90 \mu M$), and accordingly, the [D100N]RecA protein is resistant to displacement from ssDNA by SSB protein when ITP is provided as the nucleotide cofactor. We have also shown that the apparent $S_{0.5}$ value for an NTP can decrease as much as 2–3-fold when the pH of the reaction solution is decreased from pH 7.5 to 6.5 (3). Furthermore, it has been shown that ATP-mediated RecA-ssDNA filament disassembly is strongly suppressed at pH 6.5, relative to that at pH 7.5 (15). Although this pH-mediated stabilization is apparently not sufficient to render the RecA-ssDNA filament resistant to displacement by SSB protein in the presence of ITP ($S_{0.5} = 240 \mu M$ at pH 6.5, data not shown), it may contribute to the activation of ITP-dependent strand exchange activity at pH 6.5.

Intriguingly, the ssDNA-dependent ITP hydrolysis and ITP-dependent strand exchange reactions of the $E. coli$ RecA protein (pH 6.5) are similar to the ssDNA-dependent ATP hydrolysis and ATP-dependent strand exchange activities that we recently described for the RecA protein from Streptococcus pneumoniae (17). The $\phi X$ ssDNA-dependent ATP hydrolysis activity of the $S. pneumoniae$ RecA protein is completely inhibited by SSB protein (at either pH 7.5 or 6.5), apparently because SSB protein displaces $S. pneumoniae$ RecA protein from ssDNA when ATP is present as the nucleotide cofactor. Nevertheless, the ATP-dependent strand exchange activity of the $S. pneumoniae$ RecA protein is strongly stimulated by SSB protein. However, the ATP-dependent strand exchange reaction of the $S. pneumoniae$ RecA protein differs from that of the $E. coli$ RecA protein in that it proceeds optimally at low SSB protein concentrations and is completely inhibited at high SSB protein concentrations.
concentrations. These results indicated that SSB protein does
not stimulate the S. pneumoniae RecA protein-promoted strand exchange reaction by facilitating the formation of a presynaptic complex between the S. pneumoniae RecA protein and the dX ssDNA substrate and that stimulatory effect of SSB protein in this reaction is likely due to the postsynaptic mechanism discussed above (17).

Taken together, these results suggest that the dynamic state of the E. coli RecA-ssDNA filament in the presence of ITP may be similar to that of the S. pneumoniae RecA-ssDNA filament in the presence of ATP (as judged by the susceptibility to displacement by SSB protein). As a result, the E. coli RecA protein can be induced to behave in a manner that is functionally similar to that of the S. pneumoniae RecA protein (in terms of the SSB protein-dependence of the strand exchange reaction), simply by providing ITP in place of ATP as the nucleotide cofactor. More generally, these findings indicate that the dynamic state of the RecA-ssDNA filament will depend strongly on the NTP that is provided as the nucleotide cofactor and can also vary significantly between RecA proteins from different bacterial species. These NTP cofactor and species-dependent variations in RecA-ssDNA filament dynamics can directly affect the nature of the strand exchange reaction and, in particular, the manner in which the reaction is influenced by recombination accessory proteins.

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*J. Biol. Chem.* 2003, 278:35889-35896.
doi: 10.1074/jbc.M305470200 originally published online July 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M305470200

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