Effect of Nucleotide Cofactor Structure on RecA Protein-Promoted DNA Pairing. 1. Three-Strand Exchange Reaction†

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ABSTRACT: The structurally related nucleoside triphosphates, adenosine triphosphate (ATP), purine riboside triphosphate (PTP), inosine triphosphate (ITP), and guanosine triphosphate (GTP), are all hydrolyzed by the recA protein with the same turnover number (17.5 min^{-1}). The $S_{0.5}$ values for these nucleotides increase progressively in the order ATP (45 μM), PTP (100 μM), ITP (300 μM), and GTP (750 μM). PTP, ITP, and GTP are each competitive inhibitors of recA protein-catalyzed ssDNA-dependent ATP hydrolysis, indicating that these nucleotides all compete for the same catalytic site on the recA protein. Despite these similarities, ATP and PTP function as cofactors for the recA protein-promoted three-strand exchange reaction, whereas ITP and GTP are inactive as cofactors. The strand exchange activity of the various nucleotides correlates directly with their ability to support the isomerization of the recA protein to a strand exchange-active conformational state. The mechanistic deficiency of ITP and GTP appears to arise as a consequence of the hydrolysis of these nucleotides to the corresponding nucleoside diphosphates, IDP and GDP. We speculate that nucleoside triphosphates with $S_{0.5}$ values greater than 100 μM will be intrinsically unable to sustain the strand exchange-active conformational state of the recA protein during ongoing NTP hydrolysis and will therefore be inactive as cofactors for the strand exchange reaction.

The recA protein of *Escherichia coli* (M_r 37 852) is essential for homologous genetic recombination and for the postreplicative repair of damaged DNA. The purified recA protein binds cooperatively to ssDNA,[1] forming a polymeric filament-like structure that catalyzes the hydrolysis of ATP to ADP and inorganic phosphate. In addition, the recA protein will promote a variety of ATP-dependent DNA pairing reactions that presumably reflect in vivo recombination functions. The most extensively investigated DNA pairing reaction is the three-strand exchange reaction, in which a circular ssDNA molecule and a homologous linear dsDNA molecule are recombined by the recA protein to yield a nicked circular dsDNA molecule and a linear ssDNA molecule. This reaction is dependent on ATP and is strongly stimulated by the *E. coli* SSB protein. Although the molecular mechanism of the three-strand exchange reaction is not well understood, the reaction is known to proceed in three phases. In the first phase, the circular ssDNA substrate is coated with recA protein to form a presynaptic complex; SSB aids in the formation of the presynaptic complex by melting out regions of secondary structure in the ssDNA that otherwise impede the binding of recA protein. In the second phase, the presynaptic complex interacts with a dsDNA molecule, the homologous sequences are brought into register, and pairing between the circular ssDNA and the complementary strand from the 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 (Radding, 1982; Griffith & Harris, 1988; Roca & Cox, 1990; Kowalczykowski, 1991).

In an effort to determine the mechanistic role of ATP binding and hydrolysis in recA protein-promoted DNA pairing reactions, we have been investigating the activity of the recA protein with alternate nucleoside triphosphate cofactors. We previously demonstrated that although GTP is hydrolyzed by the recA protein with the same turnover number as ATP, GTP does not function as a cofactor in the three-strand exchange reaction. To account for this result, we proposed that ATP induces a specific isomerization of the recA protein that is required on the strand exchange reaction pathway and that this isomerization does not occur with GTP (Menge & Bryant, 1988). To more precisely identify the structural features that are required in order for a nucleoside triphosphate to serve as a cofactor for the strand exchange reaction, we have examined the ssDNA-dependent hydrolysis and DNA strand exchange properties of two additional nucleoside triphosphates with structures intermediate between ATP and GTP: inosine triphosphate (ITP), which has a 6-keto group but lacks the 2-amino group found in GTP, and purine riboside triphosphate (PTP), which has an unsubstituted purine ring (Figure 1). The results of these studies are described in this report.

Experimental Procedures

Materials

E. coli recA protein was purified as previously described (Cotterill et al., 1982). Adenosine, inosine, and guanosine diphosphates and triphosphates were from Sigma. ATPγS and GTPγS were from Boehringer-Mannheim. *E. coli* SSB protein was from Dr. Kathy Stephens (Johns Hopkins University) or Pharmacia. [γ-^{32}P]ATP and [^{3}H]ATP were from ICN. All reagents used in the synthesis of purine riboside monophosphate, diphosphate, and triphosphate were from Aldrich.

Purine riboside 5′-diphosphate (PDP) and purine riboside 5′-triphosphate (PTP) were prepared from purine riboside 5′-monophosphate (PMP) using diphenyl phosphoro-

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ϕX, bacteriophage ϕX174; SSB, *E. coli* single-stranded DNA binding protein; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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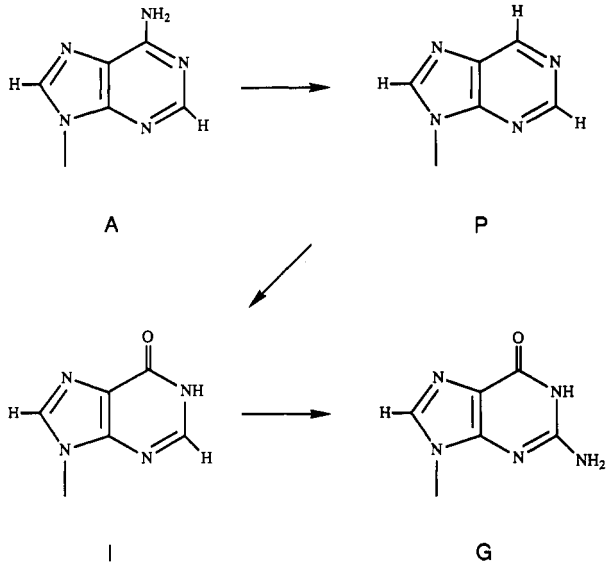

FIGURE 1: Purine ring structure of alternate nucleoside triphosphates. The structures correspond to the purine rings of adenosine triphosphate (A), purine riboside triphosphate (P), inosine triphosphate (I), and guanosine triphosphate (G).

chloridate/pyridine and orthophosphate or pyrophosphate, respectively (Eckstein & Goody, 1976). PMP was prepared from purine riboside (Sigma) using phosphoryl chloride/triethyl phosphate (Freist & Cramer, 1978).

[γ-^{32}P]-Labeled GTP, ITP, and PTP were prepared from the corresponding nucleoside diphosphates using [γ-^{32}P]ATP and nucleoside diphosphate kinase (Sigma). The reaction solutions (50 μL) contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 150 μM ATP, 50 μCi of [γ-^{32}P]ATP (10 Ci/mmol), 750 μM PDP, IDP, or GDP, and 0.14 unit of nucleoside diphosphate kinase. The reaction mixtures were incubated for 10–20 min at 37 °C, resulting in exchange of approximately 80% of the total radioactivity from [γ-^{32}P]ATP into PTP, ITP, or GTP. The [γ-^{32}P]-labeled NTPs were purified by DEAE Sephadex-A25 chromatography.

Circular ϕX ssDNA and linear ϕX dsDNA were prepared as previously described (Cox & Lehman, 1981). DNA concentrations were calculated by using A_{260} of 1 as equivalent to 36 μg/mL ssDNA or 50 μg/mL dsDNA and are expressed as total nucleotides.

Methods

NTP Hydrolysis Assay. The hydrolysis of [γ-^{32}P]-labeled nucleoside triphosphates was measured using a thin-layer chromatography system as previously described (Weinstock et al., 1979). The reaction mixtures contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, and the concentrations of recA protein, ϕX ssDNA, and [γ-^{32}P]NTP indicated in the figure legends.

Three-Strand Exchange Reactions. Standard three-strand exchange reactions were carried out as described by Cox and Lehman (1981). The reaction mixtures (50 μL) contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 3.3 μM circular ϕX ssDNA, 6.6 μM linear ϕX dsDNA, 0.3 μM SSB (tetramer concentration), 2 μM recA protein, and the indicated concentrations of ATP, PTP, ITP, or GTP. Reactions were initiated by the simultaneous addition of SSB and NTP after preincubation of all other components for 10 min at 37 °C. After 60 min at 37 °C, the reactions were quenched with SDS (1%) and EDTA (15 mM). The samples were then subjected to electrophoresis on 0.8% agarose gels (containing 0.5 μg/mL ethidium bromide) using a Tris–acetate–EDTA buffer system (Maniatis et al., 1982). The gels were photographed under UV irradiation with Polaroid Type 57 film.

SSB-independent strand exchange reactions were carried out as described by Munniyappa et al. (1984). Reaction mixtures (50 μL) contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 3.3 μM ϕX ssDNA, 6.6 μM linear ϕX dsDNA, 4 μM recA protein, and the indicated concentrations of ATP, PTP, ITP, or GTP. RecA protein and circular ϕX ssDNA were first incubated with 1 mM $MgCl_2$ for 10 min at 37 °C. The reactions were then initiated by the addition of ϕX dsDNA and $MgCl_2$ (final concentration 10 mM). After 60 min at 37 °C, the reactions were quenched with SDS (1%) and EDTA (15 mM) and analyzed by agarose gel electrophoresis as described above.

ATPγS- and GTPγS-mediated strand exchange reactions were carried out as described by Menetski et al. (1990). Reaction mixtures (25 μL) contained 25 mM Tris–acetate (pH 7.5), 1 mM DTT, 4 mM magnesium acetate, 10 μM circular ϕX ssDNA, 10 μM linear ϕX dsDNA, 6 μM recA protein, 0.22 μM SSB (tetramer concentration), and 1 mM ATPγS or GTPγS. SSB and ϕX ssDNA were first incubated for 10 min at 37 °C. RecA protein and NTPγS were added, and the incubation was continued an additional 10 min. The reactions were then initiated by the addition of ϕX dsDNA. After 30 min at 37 °C, the reactions were quenched with SDS (1%) and EDTA (50 mM) and incubated at 37 °C for 10 min. The products were then analyzed by agarose gel electrophoresis as described above.

RESULTS

Kinetics of ssDNA-Dependent Hydrolysis of PTP and ITP by RecA Protein. The kinetics of the ssDNA-dependent hydrolysis of PTP and ITP by the recA protein were analyzed at pH 7.5 and 37 °C. The reaction solutions contained 30 μM circular ϕX ssDNA and 1 μM recA protein; these conditions ensure that there is sufficient ssDNA to bind all the recA protein present (Bryant et al., 1985).

The dependence of recA protein-catalyzed PTP hydrolysis on PTP concentration is shown in Figure 2 as a double-reciprocal plot. The turnover number ($V_{max}/[E_t]$) for PTP hydrolysis was 17.5 min^{-1}, and the $S_{0.5}$(PTP) was 100 μM.[2] The double-reciprocal plot is concave upward, indicating positive cooperativity for PTP hydrolysis with respect to PTP concentration, and a Hill coefficient of 2.6 was determined at PTP concentrations near $S_{0.5}$(PTP) (50–150 μM).

The dependence of recA protein-catalyzed ITP hydrolysis on ITP concentration is also shown in Figure 2. The turnover number for ITP hydrolysis was 17.5 min^{-1}, and the $S_{0.5}$(ITP) was 300 μM. ITP hydrolysis exhibited positive cooperativity with respect to ITP concentration, with a Hill coefficient of 1.4 determined at ITP concentrations near $S_{0.5}$(ITP) (250–550 μM).

The kinetic parameters for PTP and ITP hydrolysis, as well as those previously determined for ATP and GTP hydrolysis (Menge & Bryant, 1988), are summarized in Table I. All four nucleotides are hydrolyzed by the recA protein with the same turnover number, but differ in their individual $S_{0.5}$ values. Assuming the $S_{0.5}$ values reflect the relative affinities of the recA protein for NTP, removal of the 6-amino group of ATP results in a 2-fold decrease in apparent affinity (PTP), whereas replacement of the 6-amino group with a keto group results in a 6-fold decrease in apparent affinity (ITP). The combi-

[2] $S_{0.5}$ is the substrate concentration required for half-maximal velocity.

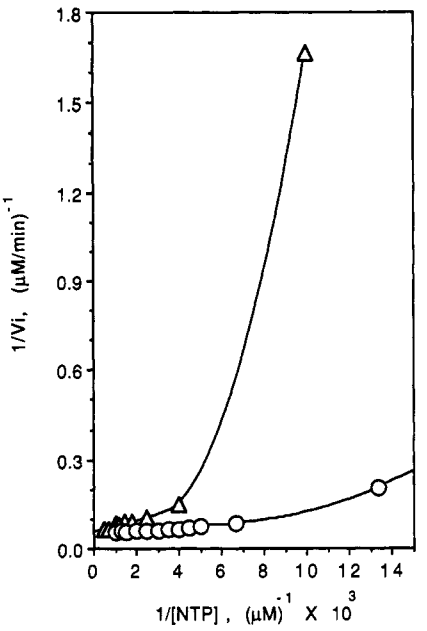

FIGURE 2: Dependence of PTP and ITP hydrolysis on nucleotide concentration. Reaction solutions contained 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 30 μM φX ssDNA, 1 μM recA protein, and the indicated concentration of either [γ-^{32}P]PTP or [γ-^{32}P]ITP. The reactions were initiated by the addition of recA protein after incubation of all other components for 10 min at 37 °C. The points represent the initial rates of PTP hydrolysis (triangles) and ITP hydrolysis (circles) at 37 °C. The PTP hydrolysis curve also includes additional points at 20, 30, and 50 μM PTP (points not shown).

Table I: Kinetic Parameters for RecA Protein-Catalyzed NTP Hydrolysis

NTP	pH	$V_{max}/[E_t]$ (min^{-1})	$S_{0.5}$ (μM)
ATP[a]	7.5	17.5	45
PTP	7.5	17.5	100
ITP	7.5	17.5	300
GTP[a]	7.5	17.5	750
ATP[a]	6.2	17	40
ITP	6.2	19	110
GTP[a]	6.2	17	150

[a] Menge and Bryant (1988).

nation of a 6-keto group and an exocyclic 2-amino group (GTP) leads to an overall 17-fold reduction in apparent affinity, relative to ATP.

Effect of ITP and PTP on RecA Protein-Catalyzed ATP Hydrolysis. The dependence of recA protein-catalyzed ATP hydrolysis on ATP concentration was determined in the presence of various fixed concentrations of PTP or ITP. As shown in Figure 3, the plots of the apparent $S_{0.5}$(ATP) versus PTP or ITP concentration are linear, indicating that both act as competitive inhibitors of ATP hydrolysis. The apparent K_i values for the inhibition of ATP hydrolysis were 75 μM for PTP and 115 μM for ITP.

We note that the K_i values for PTP and ITP are lower than the $S_{0.5}$ values that were measured for these nucleotides in the ssDNA-dependent hydrolysis reaction. We have encountered this phenomenon previously with GTP, which has an $S_{0.5}$ of 750 μM for ssDNA-dependent hydrolysis, but an apparent K_i of 150 μM for the competitive inhibition of ATP hydrolysis. To account for this discrepancy, we proposed a kinetic model in which ATP can act as a positive effector for the binding of other nucleoside triphosphates to the recA protein [see Menge and Bryant (1988)]. Thus, the $S_{0.5}$ values for the various alternate nucleotides (which were determined in the absence of ATP) are higher than the K_i values (which were necessarily measured in the presence of ATP).

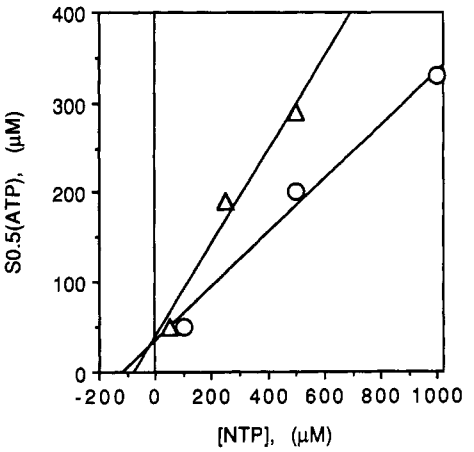

FIGURE 3: Dependence of $S_{0.5}$(ATP) on PTP and ITP concentration. Reaction solutions contained 30 μM φX ssDNA, 1 μM recA protein, various concentrations of ATP, and the indicated concentrations of either PTP or ITP. The apparent values of $S_{0.5}$(ATP) at each concentration of PTP or ITP were determined from double-reciprocal plots (not shown) and are plotted as a function of ITP concentration (circles) or PTP concentration (triangles). The intercepts on the [NTP] axis correspond to a K_i of 75 μM for PTP and 115 μM for ITP for the competitive inhibition of ATP hydrolysis.

Alternate Nucleoside Triphosphates as Cofactors for the RecA Protein-Promoted Three-Strand Exchange Reaction. The three-strand exchange reaction was employed to determine whether PTP, ITP, or GTP could function as a cofactor in a recA protein-promoted DNA pairing reaction. In the three-strand exchange reaction, a circular φX ssDNA molecule and a homologous linear φX dsDNA molecule are recombined by the recA protein to form a nicked circular dsDNA molecule and a linear ssDNA molecule. This reaction is dependent on ATP and is strongly stimulated by SSB (Cox & Lehman, 1981). The reaction solutions contained 3.3 μM circular φX ssDNA, 6.6 μM linear φX dsDNA, 2 μM recA protein, and 0.33 μM SSB. Each nucleoside triphosphate was examined for activity over a concentration range of 0–5000 μM in order to allow for the variations in the individual $S_{0.5}$ values and ensure saturating conditions.

The dependence of the three-strand exchange reaction on ATP concentration is shown in Figure 4A. Strand exchange was detected at 50 μM ATP and increased in extent with increasing ATP concentration, proceeding to completion at ATP concentrations above 1 mM; no reaction occurred in the absence of ATP. As shown in Figure 4B, PTP also functioned as a cofactor for strand exchange, with strand exchange detectable at 250 μM PTP, and proceeding to a maximal extent at PTP concentrations above 1 mM. The reduced levels of strand exchange that were observed at the lower concentrations of ATP and PTP were likely due either to depletion of NTP or to inhibition by the NDP formed by the NTP hydrolysis reaction. In contrast to the results that were obtained with ATP and PTP, neither GTP (Figure 4C) nor ITP (data not shown) functioned as cofactors for the three-strand exchange reaction, even at the highest concentrations examined (5 mM).

It has been shown that the three-strand exchange reaction is dependent on Mg^{2+}, with optimal activity occurring at 10 mM $MgCl_2$ (Cox & Lehman, 1982). Since ITP and GTP have relatively high $S_{0.5}$ values in the ssDNA-dependent hydrolysis reaction (Table I), we considered the possibility that these nucleotides might be active in strand exchange at high concentrations if the $MgCl_2$ concentration was increased to offset the depletion of free Mg^{2+} which occurs as a result of complexation by the nucleotides. As shown in Figure 5A,

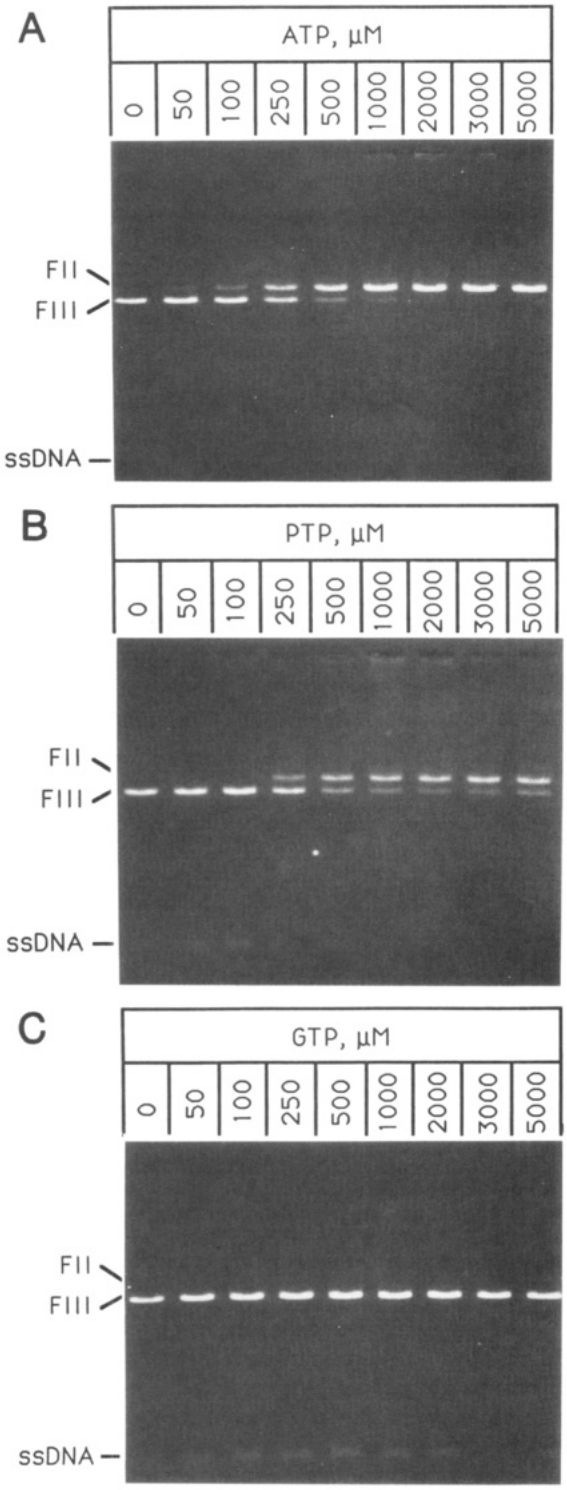

FIGURE 4: Alternate nucleoside triphosphates as cofactors in three-strand exchange reaction. Reaction solutions contained 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 3.3 μM circular ϕX ssDNA, 6.6 μM linear ϕX dsDNA, 2 μM recA protein, 0.33 μM SSB (tetramer concentration), and the indicated concentrations of ATP (panel A), PTP (panel B), and GTP (panel C). Reactions were carried out for 60 min at 37 °C and analyzed by electrophoresis on a 0.8% agarose gel. The positions of FII DNA (circular duplex DNA containing a nick), FIII DNA (linear duplex DNA), and ssDNA (single-stranded DNA) are indicated.

however, when the $MgCl_2$ concentration was increased from 10 to 15 mM, ITP and GTP (5 mM) were still unable to support strand exchange. ATP, in contrast, was fully active in strand exchange at both $MgCl_2$ concentrations.

Although strand exchange reactions are generally carried out in a Tris-HCl buffer system, it has been noted that the rate of strand exchange is approximately 3–4-fold higher in a Tris–acetate buffer system (Roman & Kowalczykowski, 1986). We therefore examined the various nucleoside triphosphates for strand exchange activity in Tris–acetate buffer containing either 10 or 15 mM $Mg(OAc)_2$. As shown in Figure 5B, strand exchange proceeded to completion at both $Mg(OAc)_2$ concentrations when ATP was included as a cofactor, whereas no strand exchange occurred with either ITP or GTP under these conditions.

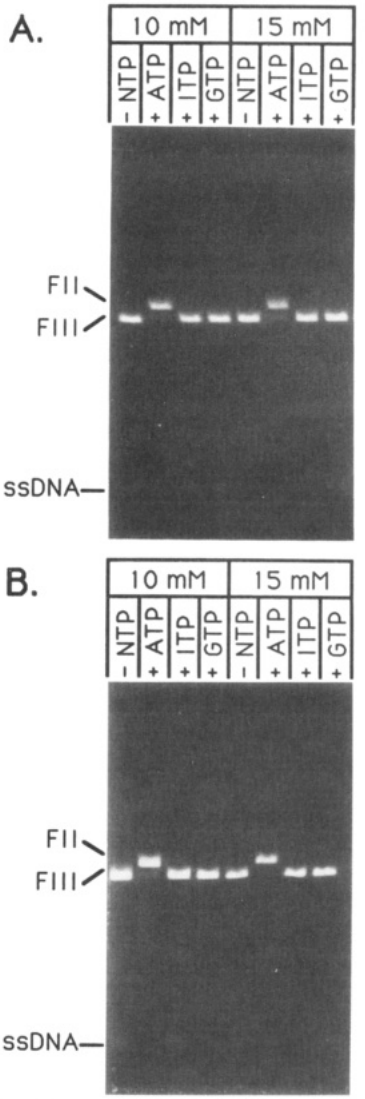

FIGURE 5: Effect of magnesium chloride and magnesium acetate on three-strand exchange reaction. Reactions were carried out in 25 mM Tris-HCl (panel A) or 25 mM Tris–acetate (panel B). Reaction solutions contained 3.3 μM circular ϕX ssDNA, 6.6 μM linear ϕX dsDNA, 2 μM recA protein, 0.3 μM SSB (tetramer concentration), 5 mM NTP, and 10 or 15 mM $MgCl_2$ (panel A) or $Mg(OAc)_2$ (panel B). Reactions were carried out for 60 min at 37 °C and analyzed by electrophoresis on a 0.8% agarose gel.

Effect of SSB on RecA Protein-Catalyzed NTP Hydrolysis. To explore the inability of ITP and GTP to support strand exchange, PTP, ITP, and GTP hydrolysis was measured under strand exchange conditions in the presence and absence of SSB. The reaction solutions contained 3.3 μM circular ϕX ssDNA, 6.6 μM linear ϕX dsDNA, 2 μM recA protein, 1 mM NTP, and 0.33 μM SSB where indicated. Under these conditions, there is a sufficient amount of both recA protein and SSB to completely cover the ssDNA present; maximal NTP hydrolysis will be observed when the ssDNA is completely coated by recA protein (note: recA protein does not bind to dsDNA under these conditions). In this section, observed rates of NTP

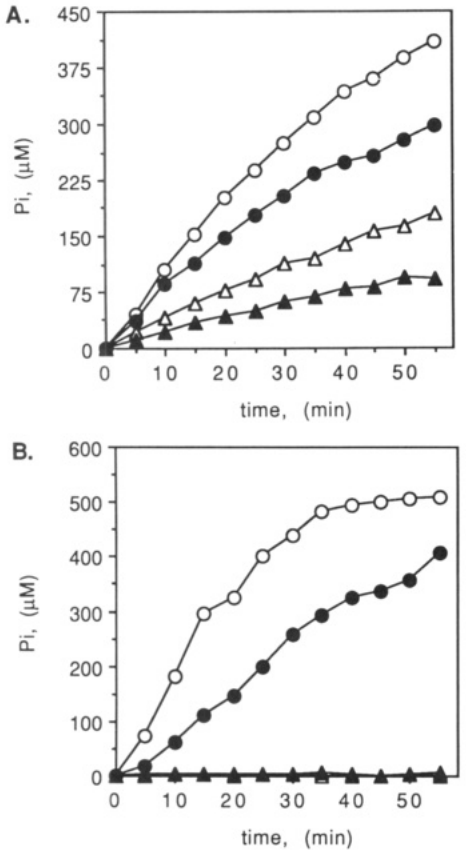

FIGURE 6: Effect of SSB on recA protein-catalyzed NTP hydrolysis. Reactions were carried out in the absence of SSB (panel A) or presence of SSB (panel B). Reaction solutions contained 3.3 μM circular ϕX ssDNA, 6.6 μM linear ϕX dsDNA, 2 μM recA protein, 0 or 0.33 μM SSB (tetramer concentration), and 1 mM [γ-^{32}P]-labeled ATP (open circles), PTP (closed circles), ITP (open triangles), or GTP (closed triangles).

hydrolysis are reported (rather than turnover numbers) since the exact number of recA monomers that are bound to ssDNA (and thus activated for NTP hydrolysis) under each set of conditions is not known.

The time courses for NTP hydrolysis in the absence of SSB are shown in Figure 6A. Under these conditions, ATP, PTP, ITP, and GTP were hydrolyzed at initial rates of 10 μM min^{-1}, 7 μM min^{-1}, 4 μM min^{-1}, and 2 μM min^{-1}, respectively. Similar rates were obtained when the NTP concentration was increased from 1 to 5 mM (data not shown). Since these NTPs are hydrolyzed with identical turnover numbers when ssDNA is in excess relative to recA protein (Table I), the variations in hydrolysis rates that are seen in Figure 6A (with limiting ssDNA) likely reflect differences in the degree to which recA protein can bind to regions of secondary structure in the ssDNA (and thereby be activated for NTP hydrolysis) in the presence of each cofactor.

The time courses for NTP hydrolysis in the presence of SSB are shown in Figure 6B. In the presence of SSB, the rates of hydrolysis of ATP and PTP increased to 16 μM min^{-1} and 9 μM min^{-1}, respectively. In contrast to the stimulatory effect on ATP and PTP hydrolysis, however, SSB completely inhibited the hydrolysis of ITP and GTP. Identical results were obtained when the NTP concentration was increased from 1 to 5 mM (data not shown).

In the absence of ATP, the *E. coli* SSB protein will competitively displace recA protein from ssDNA. In the presence of ATP, however, the recA protein isomerizes to a form that is resistant to displacement from ssDNA by SSB (Tsang et al., 1985; Morrical et al., 1986; Kowalczykowski et al., 1987; Thresher et al., 1988). The stimulation of ssDNA-dependent ATP hydrolysis by SSB has been reported previously and has been attributed to the melting of secondary structure in the ssDNA by SSB, which otherwise impedes the binding of the recA protein; in the presence of ATP, the recA protein can displace the SSB from the ssDNA, and as a result, more recA protein can bind to the ssDNA and observed rate of ATP hydrolysis increases (Kowalczykowski & Krupp, 1987). The stimulation of PTP hydrolysis by SSB indicates that PTP also is able to induce the SSB-resistant conformational state of the recA protein. However, the inhibition of ITP and GTP hydrolysis by SSB suggests that the recA protein is ineffective in isomerizing to an SSB-resistant state in the presence of ITP or GTP and, as a result, is displaced from ssDNA by SSB, leading to the loss of NTPase activity.

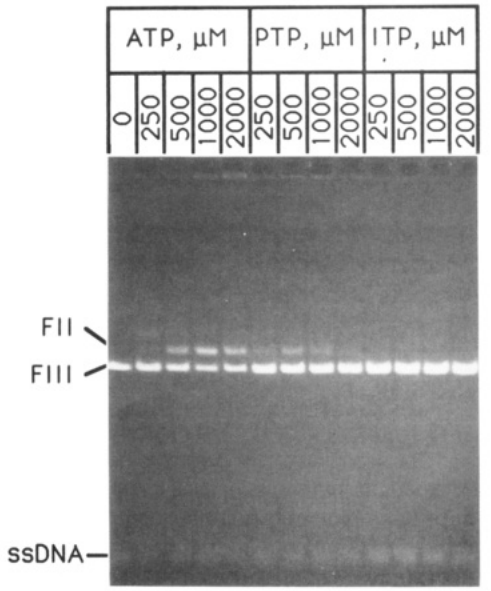

FIGURE 7: SSB-independent three-strand exchange reaction. Reaction solutions contained 3.3 μM circular ϕX ssDNA, 6.6 μM linear ϕX dsDNA, 4 μM recA protein, and the indicated concentrations of ATP, PTP, or ITP. The first lane contained all components except NTP. Reactions were carried out for 60 min at 37 °C and analyzed by electrophoresis on a 0.8% agarose gel. The positions of FII DNA (circular duplex DNA containing a nick), FIII DNA (linear duplex DNA), and ssDNA (single-stranded DNA) are indicated.

ITP and GTP as Cofactors for SSB-Independent Three-Strand Exchange Reaction. To investigate the relationship between the suppression of ITP and GTP hydrolysis by SSB and the inability of these nucleotides to support strand exchange, we carried out strand exchange reactions using a magnesium shift protocol that bypasses the requirement for SSB (Munniyappa et al., 1984). In this procedure, recA–ssDNA complexes are initially formed at 1 mM $MgCl_2$; the low Mg^{2+} concentration disfavors the formation of secondary structure in ssDNA and allows recA protein to bind more efficiently to the ssDNA. The homologous linear dsDNA is then added, and the $MgCl_2$ concentration is increased to 10 mM in order to allow optimal strand exchange to occur. As shown in Figure 7, the recA protein promoted substantial SSB-independent strand exchange in the presence of ATP and PTP under these conditions. However, no strand exchange occurred with ITP (Figure 7) or GTP (data not shown). These results demonstrate that SSB suppression of ITP and GTP hydrolysis is not, in itself, the reason these nucleotides fail to function as cofactors in the strand exchange reaction.

ATPγS and GTPγS as Cofactors for Three-Strand Exchange Reaction. It has been shown that, under certain conditions, the recA protein can promote the formation of

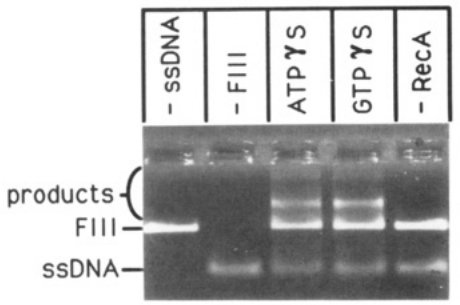

FIGURE 8: ATPγS and GTPγS as cofactors in three-strand exchange reaction. Reaction solutions contained 10 μM circular φX ssDNA, 10 μM linear φX dsDNA, 6 μM recA protein, 0.22 μM SSB (tetramer concentration), and 1 mM ATPγS or 1 mM GTPγS where indicated. Lane 1, plus ATPγS, minus ssDNA; lane 2, plus ATPγS, minus dsDNA; lane 3, plus ATPγS; lane 4, plus GTPγS; lane 5, plus ATPγS, minus recA protein. Reactions were carried out for 30 min at 37 °C and analyzed by electrophoresis on a 0.8% agarose gel. The positions of FIII DNA (linear duplex DNA), ssDNA (single-stranded DNA), and heteroduplex products are indicated.

heteroduplex DNA strand exchange products in the presence of the nonhydrolyzable ATP analog, ATPγS, indicating that ATP hydrolysis is not absolutely required for this reaction (Menetski et al., 1990). In order to evaluate further the mechanistic deficiency of GTP (and ITP), the nonhydrolyzable GTP analog, GTPγS, was examined as a cofactor for the three-strand exchange reaction using the protocol described previously for the ATPγS reaction (Menetski et al., 1990). The reaction solutions contained 10 μM circular φX ssDNA, 10 μM linear φX dsDNA, 6 μM recA protein, 0.22 μM SSB, and 1 mM ATPγS or GTPγS.

As shown in Figure 8, the recA protein formed heteroduplex strand exchange products both in the presence of ATPγS and in the presence of GTPγS. These products were not formed in the absence of recA protein, or in the presence of dsDNA alone or ssDNA alone. Furthermore, the products were not formed when the φX ssDNA was replaced with M13mp9 ssDNA, indicating that homology between the DNA substrates is required (gel not shown). Thus, although GTP does not function as a cofactor for strand exchange, GTPγS can support recA protein-promoted heteroduplex formation.

Properties of ITP and GTP at pH 6.2. The ssDNA-dependent hydrolysis and strand exchange properties of ITP and GTP were also examined at pH 6.2 and 37 °C (all preceding experiments were at pH 7.5). At pH 6.2, the turnover numbers for ITP hydrolysis (19 min^{-1}) and GTP hydrolysis (17 min^{-1}) were similar to those measured at pH 7.5. The $S_{0.5}$ values for ITP (110 μM) and GTP (150 μM), however, were significantly lower than those determined at pH 7.5 (Table I). Nevertheless, the ssDNA-dependent hydrolysis of ITP and GTP was completely inhibited by SSB at pH 6.2, and neither nucleotide was active in strand exchange at this pH (data not shown). We have previously shown that strand exchange does occur with ATP at pH 6.2 (Muench & Bryant, 1990).

DISCUSSION

The structurally related nucleoside triphosphates, ATP, PTP, ITP, and GTP, are all hydrolyzed by the recA protein with the same turnover number (17.5 min^{-1}). Furthermore, PTP, ITP, and GTP are each competitive inhibitors of recA protein-catalyzed ssDNA-dependent ATP hydrolysis, indicating that these nucleotides all compete for the same catalytic site on the recA protein. However, ATP and PTP function as cofactors for the recA protein-promoted three-strand exchange reaction, whereas ITP and GTP do not. The strand exchange activity of the various nucleotides appears to correlate with their ability to support an isomerization of the recA protein to a state in which the NTP hydrolysis activity is resistant to inhibition by SSB: ITP hydrolysis and GTP hydrolysis are inhibited by SSB, whereas ATP hydrolysis and PTP hydrolysis are not.

When the recA protein binds to ssDNA, it forms a right-handed helical filament with approximately six recA monomers per helical turn. The structure of the recA–ssDNA filament varies depending on the nucleotide cofactor present. In the absence of nucleotide cofactor, or in the presence of ADP, the recA protein binds to ssDNA in a "closed" conformation with a helical pitch of about 64 Å. In the closed conformation, the recA protein is inactive in strand exchange and is displaced from ssDNA by SSB. In the presence of ATP, in contrast, the recA protein binds to ssDNA in an "open" conformation with a helical pitch of about 95 Å. In the open conformation, the recA protein is active in strand exchange and is not displaced from ssDNA by SSB (Stasiak & Egelman, 1986). We assume that the closed conformation corresponds to the state in which NTP hydrolysis is inhibited by SSB and that the open conformation corresponds to the state in which the NTP hydrolysis in resistant to SSB inhibition (Muench & Bryant, 1990). Thus, our results with alternate nucleoside triphosphates indicate that PTP is able to support isomerization of the recA protein to the open conformational state, but that ITP and GTP are ineffective in isomerization.

The recA protein also binds to ssDNA in an open conformation in the presence of the nonhydrolyzable ATP analog, ATPγS (Stasiak & Egelman, 1986), and, under certain conditions, is able to promote heteroduplex DNA formation in the presence of this analog (Menetski et al., 1990). This indicates that it is ATP binding, rather than ATP hydrolysis, that induces isomerization of the recA protein. Our finding that the recA protein is also able to promote heteroduplex DNA formation in the presence of GTPγS suggests that GTPγS is similarly able to induce the open conformational state of the recA protein. It therefore seems likely that GTP may actually be able to induce isomerization upon binding to the recA protein and that the mechanistic deficiency of GTP and ITP arises as a consequence of the hydrolysis of these nucleotides to the corresponding nucleoside diphosphates, GDP and IDP.

It has been shown that the ATP and ADP states of the recA–ssDNA filament are mutually exclusive such that when a high concentration of ADP accumulates in solution over the course of an ATP hydrolysis reaction (greater than 50% hydrolysis), the incompatibility of the two states leads to filament disruption and dissociation of the recA protein from the ssDNA. Interestingly, dissociation of recA–ssDNA filaments is generally not observed during the initial phase of ATP hydrolysis (before a significant level of ADP accumulates), even though some of the recA monomers in the filament at any instant must be bound to ADP molecules being generated in the ATPase active site. To account for this observation, it has been suggested that the subset of recA monomers that are bound to unhydrolyzed ATP are somehow able to stabilize the entire filament in the ATP-induced conformational state (Lee & Cox, 1990a,b).

Following this interpretation, we propose that the recA protein may be able to isomerize to the open conformational state upon binding of GTP and ITP (on the basis of the GTPγS results), but may be unable to sustain the open conformational state during ongoing GTP or ITP hydrolysis. Instead, during GTP or ITP hydrolysis, the recA protein may collapse to a state functionally equivalent to the closed con-

formational state, in which it is susceptible to displacement by SSB and inactive in strand exchange. It may be significant that although the four nucleoside triphosphates we have examined are hydrolyzed by the recA protein with the same turnover number, the variations in the structure of the purine ring do affect the $S_{0.5}$ values for NTP hydrolysis, which increase progressively in the order ATP (45 μM), PTP (100 μM), ITP (300 μM), and GTP (750 μM). Thus, those nucleotides which support isomerization and strand exchange (ATP and PTP) have $S_{0.5}$ values that are significantly lower than those which do not function as cofactors (ITP and GTP).

The findings described in this paper closely parallel our recent studies on the recombination-deficient mutant [G160N]recA and [H163A]recA proteins (Bryant, 1988; Muench & Bryant, 1990; Muench & Bryant, 1991). These mutant proteins catalyze ATP hydrolysis with turnover numbers that are similar to that for the wild-type protein, but are unable to promote the ATP-dependent three-strand exchange reaction at pH 7.5. The ssDNA-dependent ATPase activities of the mutant proteins are strongly suppressed by SSB at pH 7.5, indicating that the mutant proteins are unable to isomerize efficiently to the open conformational state at this pH. Furthermore, the mutant proteins are able to promote heteroduplex formation in the presence of ATPγS, indicating that the mechanistic defect occurs at the level of ATP hydrolysis and not ATP binding (Menge and Bryant, unpublished results). Interestingly, the mutant proteins are able to carry out ATP-dependent strand exchange at pH 6.0–6.8, and with both mutants, the induction of strand exchange activity at lower pH correlates directly with the activation of ATP-dependent isomerization of the mutant protein to the SSB-resistant conformational state (Muench & Bryant, 1990, 1991).

Although the mechanistic basis for the pH-dependent isomerization behavior of the mutant proteins is not yet clear, we have noted that the $S_{0.5}$(ATP) values for the [G160N]recA protein (150 μM) and the [H163A]recA protein (150 μM) are 4-fold higher than that for the wild-type protein (35 μM) at pH 7.5, whereas the $S_{0.5}$(ATP) values for the wild-type and mutant proteins are similar (20–30 μM) at pH 6.2 (Bryant, 1988; Muench & Bryant, 1991). Thus, the decreases in $S_{0.5}$(ATP) values for the mutant proteins may be responsible for their activation in strand exchange at the lower pH. In this paper, we find a similar pH-dependent decrease in the $S_{0.5}$ values for the ssDNA-dependent hydrolysis of the alternate nucleotides by the wild-type protein. Although the $S_{0.5}$ values for ITP and GTP decrease to 110 μM and 150 μM, respectively, at pH 6.2, both nucleotides are still inactive in strand exchange at this pH.

Taken together, our studies of altered nucleoside triphosphate cofactors and of mutant recA proteins suggest that NTPs with $S_{0.5}$ values greater than 100 μM may be intrinsically unable to sustain the recA filament in the open conformational state and are therefore nonfunctional in strand exchange. Possibly, those NTPs with higher $S_{0.5}$ values have altered rates of NTP association/dissociation which disrupt the balance between conformational states and leave the filament susceptible to destabilization by the NDPs that are generated in the NTPase active sites. In the accompanying paper, we examine the effects of the alternate NTPs on the DNA renaturation activity of the recA protein and find that those NTPs which are inactive in strand exchange indeed behave in a manner that is functionally equivalent to the corresponding NDPs (Menge & Bryant, 1992).

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