Reevaluation of the Nucleotide Cofactor Specificity of the RecA Protein from Bacillus subtilis

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The RecA protein from the Gram-positive bacterium, Bacillus subtilis, has been reported to catalyze dATP hydrolysis and to promote strand exchange in the presence of dATP but to have no ATP hydrolysis or ATP-dependent strand exchange activity (Lovett, C. M., Jr., and Roberts, J. W. (1985) J. Biol. Chem. 260, 3305–3313). The well characterized RecA protein from Escherichia coli, in contrast, catalyzes the hydrolysis of ATP and dATP at similar rates and can use either ATP or dATP as a cofactor for the strand exchange reaction. To explore this reported difference in nucleotide cofactor specificity in detail, we developed an overexpression system for the B. subtilis RecA protein and purified the protein to greater than 95% homogeneity. Contrary to the previous report, we find that the B. subtilis RecA protein catalyzes the hydrolysis of both dATP and ATP and can perform strand exchange using either dATP or ATP as a cofactor. Our results suggest that the inability of previous investigators to detect the ATP hydrolysis and ATP-dependent strand exchange activities of the B. subtilis RecA protein may have been due to the particular assay conditions that were used in the earlier study.

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 (1).

The E. coli RecA protein catalyzes the ssDNA-dependent hydrolysis of both ATP and dATP and can use either ATP or dATP as a cofactor for the strand exchange reaction (1–3). Interestingly, the RecA protein from the Gram-positive bacterium, Bacillus subtilis, has been reported to catalyze dATP hydrolysis and to promote strand exchange in the presence of dATP but to have no ATP hydrolysis or ATP-dependent strand exchange activity (4). To study this physiologically and mechanistically intriguing difference in cofactor specificity in detail, we cloned the B. subtilis recA gene from B. subtilis genomic DNA, overexpressed the protein in a recA deletion E. coli strain, and purified the B. subtilis RecA protein to greater than 95% homogeneity. In contrast to the published report, we find that the B. subtilis RecA protein can catalyze the hydrolysis of both ATP and dATP and can promote DNA strand exchange with either of these nucleotides as cofactors.

EXPERIMENTAL PROCEDURES

Materials—E. coli RecA protein was prepared as described previously (5). E. coli SSB was provided by Dr. Roger McMacken (Johns Hopkins University). ATP, dATP, [α-32P]ATP, and [α-32P]dATP were from Amersham Pharmacia Biotech. Circular δX ssDNA (5′-strand) and circular δX dsDNA were from New England Biolabs; linear δX dsDNA was prepared from circular δX dsDNA by PstI digestion as described (6). Single- and double-stranded DNA concentrations were determined by absorbance at 260 nm using the conversion factors 36 and 90 μg/ml/μM, respectively. All DNA concentrations are expressed as total nucleotides.

Cloning of the B. subtilis recA Gene—Genomic DNA was isolated from the rec- B. subtilis strain YB886 (7) using standard procedures (8). The B. subtilis recA gene was amplified from the genomic DNA by polymerase chain reaction using Pfu DNA polymerase as described by the manufacturer (Stratagene). The primers used (5′-GGAGCCATAT- GAGTGATCGTCAGGCAGCC-3′ and 5′-GGAGGCCATTTTAT- TCCTCAATATTGATTTCTTGGTGC-3′) were complementary to the 5′ and 3′ ends of the coding sequence (italics) and also contained “GA clamp” sequences (GGAGG) to facilitate the polymerase chain reaction as well as recognition sequences for the restriction enzymes NdeI and BamHI (underlined). The blunt-ended DNA product that was obtained from the polymerase chain reaction was ligated into pCR-Blunt (Invitrogen) and transformed into E. coli strain JM107. Plasmid DNA containing the desired insert was isolated, and the insert DNA was excised by NdeI/BamHI digestion. The insert was ligated into pET-21a (Novagen) to give the final construct, pETRecA(BS). The coding region of the insert was sequenced by the Johns Hopkins University DNA Sequencing Facility and was found to be identical to that reported previously for the B. subtilis recA gene by Stranathan et al. (9).

Expression and Purification of B. subtilis RecA Protein—The B. sub-
B. subtilis RecA Protein

Preparation of the B. subtilis RecA Protein—In the report from Lovett and Roberts (4), the B. subtilis RecA protein was purified directly from B. subtilis cells. Although mitomycin C was added to the cell culture to induce expression of the protein, the B. subtilis RecA protein represented only 0.05% of the total protein in the crude cell extract, and we were able to obtain 13 mg of highly purified protein from 16 g of cells (Fig. 1). Although the molecular weight of the B. subtilis RecA protein calculated from the gene sequence (M, 38,059; 348 amino acids) is similar to that of the E. coli RecA protein (M, 37,842; 352 amino acids), the mobility of the B. subtilis RecA protein was lower than that of the E. coli RecA protein during polyacrylamide gel electrophoresis (Fig. 1). This difference in mobility was also noted by Lovett and Roberts (4), who estimated the molecular weight of the B. subtilis RecA protein to be 42,000 (this estimate was made before the gene sequence had been determined). Mass spectroscopic analysis of our purified B. subtilis RecA protein, however, yielded a molecular weight of 38,029 ± 38, in excellent agreement with the molecular weight of 38,059 that was calculated from the gene sequence. Amino-terminal protein sequencing confirmed that our preparation corresponded to the B. subtilis RecA protein.

ssDNA-dependent NTP Hydrolysis Activity—The B. subtilis RecA protein was analyzed for ssDNA-dependent ATP and dATP hydrolysis activity at pH 7.5 and 37 °C. The reaction solutions contained 1 μM B. subtilis RecA protein and 30 μM ssDNA; these conditions ensured that there was sufficient ssDNA to bind all of the B. subtilis RecA protein present, assuming that the ssDNA binding stoichiometry of the B. subtilis protein is similar to that of the E. coli protein (1 RecA monomer/3 nucleotides of ssDNA (1)). The dependence of the rate of ssDNA-dependent ATP and dATP hydrolysis on NTP concentration is shown in Fig. 2, and the kinetic parameters for the hydrolysis of each NTP by the B. subtilis RecA protein (as well as those for the E. coli RecA protein) are presented in Table I.

As shown in Fig. 2, our preparation of the B. subtilis RecA protein catalyzed the hydrolysis of both dATP and ATP. The turnover numbers (Vₐ₉₀/Vₑ₉₀) for hydrolysis were 10 min⁻¹ for ATP and 14 min⁻¹ for dATP, and the Sₐ₉₀ values were 50 and 40 μM for ATP and dATP, respectively. Under the same conditions, the E. coli RecA protein catalyzes ATP and dATP hydrolysis with turnover numbers of 18 min⁻¹ for ATP and 22

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RESULTS

Preparation of the B. subtilis RecA Protein—E. coli strain BLR(DE3)pLysS (Novagen). Competent BLR pLysS cells were transformed with pETRecA(Bl) and selected for growth on LB/carbenicillin plates. Individual BLR(DE3)pLysS/pETRecA(Bl) colonies were used to inoculate LB broth (3 liters) containing carbenicillin (50 μg/ml). The cells were grown at 37 °C to an A₆₀₀ of 0.6, and then isopropyl-1-thio-
β-D-galactopyranoside (1 mM) was added to induce expression of the B. subtilis RecA protein. After 3 h at 37 °C, the cells (16 g) were collected by centrifugation, suspended in 16 ml of 250 mM Tris-HCl (pH 8.1), 25% sucrose, 1 mM EDTA, and frozen in liquid nitrogen. The following protein purification steps were then carried out at 4 °C. The frozen cell suspension was thawed on ice, and 14 ml of lysosome (2.5 mg/ml) was added. After 30 min, 14 ml of EDTA (25 mM) was added and the incubation was continued. After 15 min, 54 ml of 50 mM Tris-HCl (pH 8.1), 1% Brij 35, 1 mM DTT was added, and the incubation was continued for 45 min. The mixture was then centrifuged at 100,000 × g for 60 min. The pellet was discarded and 1.9 ml of Polymin-P (10%), pH 7.9 was added to the supernatant. After 1 h, the suspension was centrifuged at 12,000 × g for 15 min. The supernatant was discarded, and the pel-}

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Fig. 1. SDS-polyacrylamide gel electrophoresis of purified B. subtilis RecA protein. The gel lanes contain purified B. subtilis RecA protein, purified E. coli RecA protein, or molecular mass standards (MW) as indicated. The acrylamide concentration was 5% in the stacking gel and 10% in the separating gel. The gel was stained in 0.1% Coomassie Brilliant Blue R-250.

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region of homology between the B. subtilis RecA protein and over 60 other bacterial RecA proteins.

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preparation corresponded to the B. subtilis RecA protein.
Kinetic parameters for E. coli and B. subtilis RecA protein-catalyzed NTP hydrolysis (pH 7.5)

The steady state kinetic parameters for the B. subtilis recA protein were derived from the data presented in Fig. 2. Those for the E. coli recA protein are from Menge and Bryant (2) (ATP) and Nayak and Bryant, unpublished results (dATP). NTP, nucleoside diphosphate.

<table>
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<tr>
<th>NTP</th>
<th>$V_{max}/E_0$</th>
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$V_{max}/E_0$ in min$^{-1}$ for dATP and with $S_{0.5}$ values of 45 and 20 μM for ATP and dATP, respectively (Table 1). There was no detectable hydrolysis of dATP or ATP by either the B. subtilis or the E. coli RecA protein in the absence of ssDNA (time courses not shown).

Although the kinetic parameters that we determined for dATP hydrolysis are similar to those reported by Lovett and Roberts (4), these previous investigators were unable to detect any ssDNA-dependent ATP hydrolysis activity with their preparation of B. subtilis RecA protein. Our standard reaction conditions (50 μM φX ssDNA, 25 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 5% glycerol, and 1 mM DTT), however, differed from those used by Lovett and Roberts (4) (25 μg/ml denatured λ DNA, 12 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 6.5% sucrose, 0.5 mM EDTA, 2.5 mM DTT, 20 mM KCl, 40 mM NaCl, and 1 mM potassium phosphate). In addition, the concentration of the B. subtilis RecA protein in our reactions (1 μM) was higher than that used by Lovett and Roberts (4) (0.4 μM). In an effort to determine the basis for the discrepancy between our results and those of Lovett and Roberts (4), we measured the dATP and ATP hydrolysis activities of the B. subtilis RecA protein over a range of protein concentrations, in both our standard reaction buffer and in the reaction buffer used by Lovett and Roberts (4).

In Fig. 3, the specific activity (defined as the observed rate of NTP hydrolysis/total RecA concentration) for ATP and dATP hydrolysis by the B. subtilis RecA protein is plotted as a function of protein concentration. In our standard reaction buffer, the specific activity of ATP and dATP hydrolysis was only slightly dependent on protein concentration with values of 7–11 min$^{-1}$ (ATP) and 10–14 min$^{-1}$ (dATP) over a range of 0.2 to 2.0 μM B. subtilis RecA protein. In the reaction buffer used by Lovett and Roberts (4) and at B. subtilis RecA protein concentrations of 0.4 μM and higher, the specific activity of ATP and dATP hydrolysis was again only slightly dependent on protein concentration with values of 4–5 min$^{-1}$ (ATP) and 8–9 min$^{-1}$ (dATP) over the range of 0.4 to 2.0 μM B. subtilis RecA protein. At protein concentrations lower than 0.4 μM, however, the specific activity of ATP and dATP hydrolysis in the Lovett and Roberts (4) buffer decreased sharply with protein concentration; at 0.2 μM B. subtilis RecA protein, neither ATP nor dATP hydrolysis was detected with the Lovett and Roberts (4) buffer. This loss of NTP hydrolysis activity over a narrow range of B. subtilis RecA protein concentrations below 0.4 μM (the concentration used by Lovett and Roberts (4)) may have contributed to the inability of these investigators to detect the ATP hydrolysis activity of the B. subtilis RecA protein under their reaction conditions.

Three Strand Exchange Activity—The strand exchange activity of the B. subtilis RecA protein was evaluated using the same three strand exchange reaction that was used by Lovett and Roberts (4) in their characterization of the B. subtilis RecA protein. In this reaction, which was originally developed for the E. coli RecA protein, a circular φX ssDNA molecule and a linear φX dsDNA molecule are recombined to form a nicked circular DNA, 12 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 6.5% sucrose, 0.5 mM EDTA, 2.5 mM DTT, 20 mM KCl, 40 mM NaCl, and 1 mM potassium phosphate). In addition, the concentration of the B. subtilis RecA protein in our reactions (1 μM) was higher than that used by Lovett and Roberts (4) (0.4 μM). In an effort to determine the basis for the discrepancy between our results and those of Lovett and Roberts (4), we measured the dATP and ATP hydrolysis activities of the B. subtilis RecA protein over a range of protein concentrations, in both our standard reaction buffer and in the reaction buffer used by Lovett and Roberts (4).
dsDNA molecule and a linear ssDNA molecule. *E. coli* SSB protein, which strongly stimulates the strand exchange reaction promoted by the *E. coli* RecA protein, is included in the reaction solution (6, 10).

As shown in Fig. 4A, our preparation of *B. subtilis* RecA protein was able to promote strand exchange in the presence of either dATP or ATP. The detection of the ATP-dependent strand exchange activity, however, was dependent on the order in which the *B. subtilis* RecA protein and *E. coli* SSB were added to the reaction solution. The reactions shown in Fig. 4A were initiated by the addition of *E. coli* SSB after all other reaction components had been incubated at 37 °C. In contrast, if the reactions were initiated by the addition of *B. subtilis* RecA protein after all other reaction components had been incubated at 37 °C, a significant strand exchange reaction was detected with dATP but no activity was detected with ATP (Fig. 4B). This sensitivity of the ATP-dependent strand exchange reaction to the order of addition may account for the inability of Lovett and Roberts (4) to detect strand exchange by the *B. subtilis* RecA protein in the presence of ATP (the citation provided in their paper indicates that their reactions were initiated by adding *B. subtilis* RecA protein after the *E. coli* SSB). In contrast to the behavior of the *B. subtilis* RecA protein, the ATP- and dATP-dependent strand exchange reactions of the *E. coli* RecA protein proceeded with similar efficiencies regardless of whether the reactions were initiated by the addition of *E. coli* SSB (Fig. 4A) or by the addition of *E. coli* RecA protein (Fig. 4B).

The reason for the sensitivity of the strand exchange activity of the *B. subtilis* RecA protein to the order in which the *E. coli* SSB and *B. subtilis* RecA proteins are added to the reaction solution is not clear. However, it has been shown that the *E. coli* RecA protein is able to displace *E. coli* SSB protein from ssDNA in the presence of either ATP or dATP (11). In contrast, the results in Fig. 4B suggest that the *B. subtilis* RecA protein may be able to displace *E. coli* SSB from ssDNA in the presence of dATP but not in the presence of ATP. If this is the case, then when *B. subtilis* RecA protein and ssDNA are incubated in the presence of nucleotide cofactor before the reaction is initiated by the addition of SSB, the *B. subtilis* RecA protein may be able to form presynaptic complexes on the ssDNA and carry out strand exchange without interference from the SSB. Therefore, strand exchange is observed with both ATP and dATP when this order of addition is followed (Fig. 4A). In contrast, when the ssDNA is incubated with *E. coli* SSB before the reaction is initiated by the addition of *B. subtilis* RecA protein, the *B. subtilis* RecA protein may be able to displace the *E. coli* SSB from the ssDNA when dATP is present but not when ATP is supplied as the cofactor. Consequently, when this order of addition is followed, strand exchange is detected with dATP but not with ATP (Fig. 4B).

To test this idea, we examined the ssDNA-dependent ATP and dATP hydrolysis activities of the *B. subtilis* and *E. coli* RecA proteins in the presence and absence of *E. coli* SSB. The reaction solutions contained 5.7 μM dX ssDNA, 2.0 μM *B. subtilis* or *E. coli* RecA protein, and either 0 or 0.3 μM *E. coli* SSB. In all cases, the NTP hydrolysis reactions were initiated by the addition of RecA protein. Under these conditions, which simulate those used for the strand exchange reaction shown in Fig. 4B, there is a sufficient amount of both RecA protein and *E. coli* SSB to completely cover all of the ssDNA present; maximal rates of ATP and dATP hydrolysis will occur when the ssDNA is completely covered by RecA protein. The time courses of ATP and dATP hydrolysis that were determined for the *B. subtilis* and *E. coli* RecA proteins in the presence and absence of *E. coli* SSB are shown in Fig. 5. Note that in this section, the observed rates of dATP or ATP hydrolysis are reported (rather than turnover numbers), because the exact number of *B. subtilis* or *E. coli* RecA monomers that can bind to a dX ssDNA molecule and be activated for ATP or dATP hydrolysis is not clear.

In the absence of *E. coli* SSB, the *E. coli* RecA protein catalyzed ATP and dATP hydrolysis at rates of 14 and 32 μM min⁻¹, respectively (Fig. 5A). Because ATP and dATP are hydrolyzed with similar turnover numbers when ssDNA is in excess relative to RecA protein (Table I), the variation in hydrolysis rates that is observed under strand exchange conditions (Fig. 5A, where ssDNA is limiting) likely reflects differences in the degree to which the *E. coli* 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. Under the same conditions, the *B. subtilis* RecA protein hydrolyzed ATP and dATP at rates of only 4 and 8 μM min⁻¹, respectively (Fig. 5B). Because the turnover numbers that were measured for the *B. subtilis* RecA protein-catalyzed ATP and dATP hydrolysis in the presence of excess ssDNA are only 2-fold lower than those measured for *E. coli* RecA protein (Table I), the 4-fold lower rates of dATP and ATP hydrolysis by the *B. subtilis* RecA protein under strand exchange conditions (Fig. 5B) indicate that the *B. subtilis* RecA protein may be less effective than the *E. coli* RecA protein in binding to regions of secondary structure in ssDNA, at least under these reaction conditions.
The apparent differences in the ATP-dependent strand exchange activities of the *E. coli* and *B. subtilis* RecA proteins may be attributable to the fact that *E. coli* SSB protein was used as an accessory factor for both proteins. It is conceivable that the *B. subtilis* RecA protein-promoted strand exchange reaction would proceed more efficiently in the presence of ATP if the SSB protein from *B. subtilis* was used instead of the *E. coli* SSB protein. Although the gene for the *B. subtilis* SSB protein has been identified (14), a purification and characterization of the *B. subtilis* SSB protein has not yet been reported. It may be desirable to isolate the *B. subtilis* SSB protein for use as a strand exchange accessory factor before a more extensive investigation of the *B. subtilis* RecA protein is undertaken.

REFERENCES


Our results demonstrate that the RecA protein from *B. subtilis* can catalyze the ssDNA-dependent hydrolysis of both dATP and ATP. The turnover numbers for ATP and dATP hydrolysis are similar and nearly independent of protein concentration when measured under our standard reaction conditions. Under the reaction conditions used by Lovett and Roberts (4), however, there is a loss of NTP hydrolysis activity at protein concentrations below approximately 0.4 μM. This phenomenon may account for the inability of these investigators to detect ATP hydrolysis activity of the *B. subtilis* RecA protein (4).

Although the *B. subtilis* and *E. coli* RecA proteins can both hydrolyze either ATP or dATP, the *B. subtilis* protein differs from the *E. coli* protein in that its ATP hydrolysis reaction is inhibited, rather than stimulated, by *E. coli* SSB. This inhibition, which may be due to the inability of the *B. subtilis* RecA protein to displace *E. coli* SSB from ssDNA in the presence of ATP, is apparently responsible for the sensitivity of the *B. subtilis* RecA-promoted strand exchange reaction to the order in which *B. subtilis* RecA protein and *E. coli* SSB are added to the reaction solutions. The inhibitory effect is such that when strand exchange is initiated by the addition of the *B. subtilis* RecA protein to an otherwise complete reaction, no strand exchange is detected with ATP. This effect may account for the inability of Lovett and Roberts (4) to detect strand exchange by *B. subtilis* RecA protein in the presence of ATP. We show here, however, that if the *B. subtilis* RecA protein is incubated with the ssDNA and ATP before *E. coli* SSB is added, a vigorous strand exchange reaction does occur. This result demonstrates that the *B. subtilis* RecA protein can use ATP as a cofactor for the strand exchange reaction.4

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4 The properties of the *B. subtilis* RecA protein are reminiscent of the previously described mutant *E. coli* RecA340 protein. The *E. coli* RecA340 protein competes more effectively with SSB for ssDNA binding in the presence of dATP than with ATP and as a result, shows a strong preference for dATP over ATP as a cofactor in the three strand exchange reaction (13).