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Escherichia coli single-stranded DNA binding protein stimulates the DNA deoxyribophosphodiesterase activity of exonuclease I

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ABSTRACT

The *E.coli* single-stranded binding protein (SSB) has been demonstrated *in vitro* to be involved in a number of replicative, DNA renaturation, and protective functions. It was shown previously that SSB can interact with exonuclease I to stimulate the hydrolysis of single-stranded DNA. We demonstrate here that *E.coli* SSB can also enhance the DNA deoxyribophosphodiesterase (dRpase) activity of exonuclease I by stimulating the release of 2-deoxyribose-5-phosphate from a DNA substrate containing AP endonuclease-incised AP sites, and the release of 4-hydroxy-2-pentenal-5-phosphate from a DNA substrate containing AP lyase-incised AP sites. *E.coli* SSB and exonuclease I form a protein complex as demonstrated by Superose 12 gel filtration chromatography. These results suggest that SSB may have an important role in the DNA base excision repair pathway.

INTRODUCTION

The *E.coli* single-stranded DNA binding protein (SSB) is a tetramer of 18.9 kDa subunits that binds single-stranded DNA and appears to be involved in several replicative, recombination, and repair functions (1,2). The function of the protein *in vivo* has been ascertained by characterization of *ssb* mutants. Mutants in SSB which were isolated as temperature-sensitive lethals exhibit increased UV sensitivity (3,4) and are defective in SOS repair (5,6) and in λ prophage induction (4). The mutants are also defective in the increased synthesis of RecA protein following DNA damage and demonstrate recombination abnormalities (7). Furthermore, the SSB protein has several *in vitro* functions such as directed priming of DNA synthesis at specific loci by the covering of single-stranded regions and enhanced fidelity and elongation of DNA polymerase II and DNA polymerase III holoenzyme (1,8).

SSB has been shown to inhibit the activities of some DNases. SSB converts the RecBCD nuclease into an ATP-dependent helicase (9). and it decreases the nucleolytic activity of DNA polymerases I and III (8). By contrast, SSB stimulates the activity of exonuclease I (10). Exonuclease I was originally identified as an 3′ → 5′ exonuclease acting on single-stranded DNA (11,12) and was later identified as the product of the sbcB locus (13). We have recently shown that a DNA deoxyribophosphodiesterase (dRpase) activity is associated with exonuclease I (14). This activity functions in DNA base-excision repair to remove deoxyribose-phosphate moieties following cleavage of DNA at an apurinic/apyrimidinic (AP) site by either an AP endonuclease or AP lyase (15–17).

In this report we demonstrate that SSB enhances the DNA dRpase activity of exonuclease I by stimulating the release of either 2-deoxyribose-5-phosphate from a polynucleotide substrate containing AP sites cleaved with endonuclease IV of *E.coli* or 4-hydroxy-2-pentenal-5-phosphate from a polynucleotide substrate containing AP sites cleaved with the AP lyase endonuclease III of *E.coli*. The role of SSB in DNA base excision repair will be considered.

MATERIALS AND METHODS

Enzymes and reagents

Exonuclease I and *E.coli* single-stranded DNA binding protein were purchased from U.S. Biochemicals. Poly(dA-dT), DNA polymerase I and the large-fragment (Klenow) of DNA polymerase I were purchased from Boehringer Mannheim. DNase I was purchased from Worthington, and lambda DNA was purchased from New England Biolabs. *E.coli* uracil-DNA glycosylase (18) and endonuclease IV (19) were prepared as described previously. Endonuclease III was a generous gift of Dr. Richard Cunningham, State University of New York, Albany. Molecular weight markers for gel filtration chromatography were purchased from Sigma. Molecular weight markers for SDS polyacrylamide gel electrophoresis were purchased from Bio-Rad.

Polynucleotide substrates

Poly(dA-dT) containing ^{32}P-labeled deoxyribose-phosphate moieties at incised AP sites was prepared as described previously (15,17). Poly(dA-dT) containing 5′ [^{32}P] dUMP residues

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following incorporation of [α-^{32}P] dUTP by the action of the Klenow fragment was subsequently treated with uracil-DNA glycosylase and then with either endonuclease IV to generate 5′ incised AP sites with 2-deoxyribose-5-phosphate ends or with endonuclease III to generate 3′ incised AP sites with 4-hydroxy-2-pentenal-5-phosphate ends. A lambda DNA substrate was prepared by nick-translation as described previously (14) and treated with uracil-DNA glycosylase and either endonuclease III or endonuclease IV.

Enzyme assays

DNA dRpase activity was assayed in a standard reaction measuring the release of either 2-deoxyribose-5-phosphate (for 5′ incised AP sites) (14,15) or 4-hydroxy-2-pentenal-5-phosphate (for 3′ incised AP sites) (14,16). Reaction mixtures (100 μl) contained 50 mM Hepes-KOH, pH 6.5, 10 mM $MgCl_2$, 5 mM DTT, either poly(dA-dT) containing incised AP sites (18,000 cpm; 2−6 cpm/fmol) or lambda DNA containing incised AP sites (30,000 cpm; 6−7 cpm/fmol), 0.3 units (of exonuclease I with or without 0.9 μg of SSB. After incubation at 37°C for 30 min, release of sugar-phosphate was determined by precipitation with trichloroacetic acid in the presence of Norit charcoal or by HPLC anion exchange chromatography as described previously (14,15).

Binding of *E.coli* SSB to exonuclease I

To determine binding of exonuclease I to SSB, 60 units of exonuclease I plus 180 μg of SSB was loaded in a 200 μl volume of elution buffer onto a Superose 12 HR 10/30 FPLC column (Pharmacia) which was previously equilibrated with the elution buffer of 20 mM Tris−HCl, pH 7.8, 5 mM DTT, 1 mM NaEDTA, 100 mM NaCl, 5% glycerol. The column was eluted at a flow rate of 0.5 ml/min, and 0.2 ml fractions were collected and assayed for dRpase activity. The column was calibrated with alcohol dehydrogenase (150 kDa), bovine serum albumin (69 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa). In addition, two adjacent fractions from fraction numbers 7−20 were pooled, and an aliquot (150 μl) of each pooled fraction was added to 150 μl of 10% trichloroacetic acid, and the protein contained in each aliquot was precipitated by centrifugation at 4°C at 10,000 g. The precipitates were dissolved in 16 μl of gel loading buffer(62.5 mM Tris−HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.005% bromophenol blue), heated for 5 min at 100°C, and were loaded on a 12% polyacrylamide gel and separated according to the method of Laemmli (20). Following electrophoresis, proteins were visualized by silver staining (21).

RESULTS

E.coli SSB enhances the release of deoxyribose-phosphate at 5′ incised AP sites from a polynucleotide substrate

To determine if SSB interacts with exonuclease I to modulate the release of deoxyribose-phosphate moieties at 5′ incised AP sites, a poly(dA-dT) substrate was utilized containing [^{32}P]-labeled 2-deoxyribose-5-phosphate ends (Figure 1). This substrate has been used to assay DNA dRpase activity of *E.coli* and mammalian cells (14,15,17). A 100: molar ratio of SSB to exonuclease I was required for a two-fold increase in the amount of sugar-phosphate released from the substrate in 30 min, and this ratio was used in all subsequent reactions.

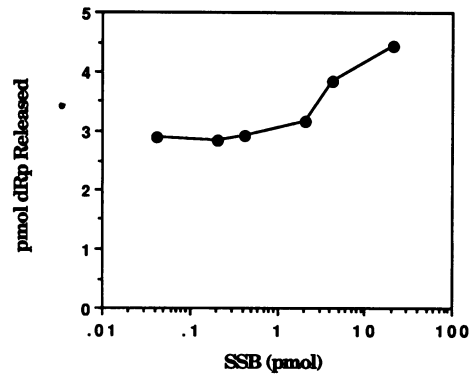

Figure 1. Release of 2-deoxyribose-5-phosphate from a poly(dA-dT) substrate containing 5′ incised AP sites. The poly(dA-dT) substrate was reacted with 0.13 pmol (0.3 units) of exonuclease I with increasing amounts of SSB protein. In the absence of SSB, 2.6 pmol of 2-deoxyribose-5-phosphate was released by exonuclease I in 30 min. Release of 2-deoxyribose-5-phosphate was determined by precipitation with trichloroacetic acid in the presence of Norit charcoal.

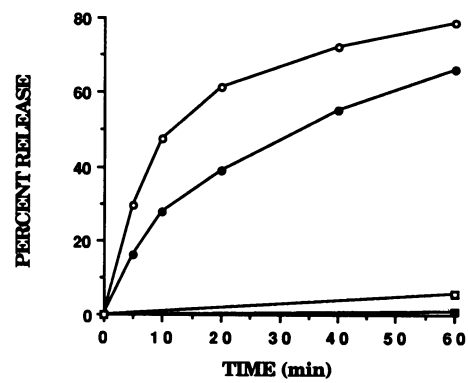

Figure 2. Time course for the release of 2-deoxyribose-5-phosphate from a poly(dA-dT) substrate containing 5′ incised AP sites. The release of 2-deoxyribose-5-phosphate was determined by precipitation with trichloroacetic acid in the presence of Norit charcoal. Reactions contained (●) exonuclease I; (○) exonuclease I plus SSB; (■) no enzyme; (□) SSB.

In figure 2, a time course for the release of deoxyribose-phosphate is shown. The addition of SSB to the reaction mixture resulted in a consistent increase in the amount of sugar-phosphate released from the poly(dA-dT) substrate. SSB was inefficient in the release of deoxyribose-phosphate in the absence of exonuclease I (<5% release in 60 min).

The addition of SSB to exonuclease I seemed to alter the rate of the reaction rather than the catalytic properties of the deoxyribophosphodiesterase activity. The apparent K_m for the release of 2-deoxyribose-5-phosphate was determined by Lineweaver−Burk analysis as shown in figure 3, and was found to have the value of 0.15 μM for 2-doexyribose-5-phosphate termini. This value is similar to the previously measured value of K_m (0.22 μM) for the reaction with exonuclease alone (14). The optimum pH for the dRpase reaction of exonuclease I/SSB was 6.5, as in the case of exonuclease I alone (14). Both the dRpase activity of exonuclease I and exonuclease I/SSB were inhibited by KCl (50% inhibition with 60 mM KCl, data not shown).

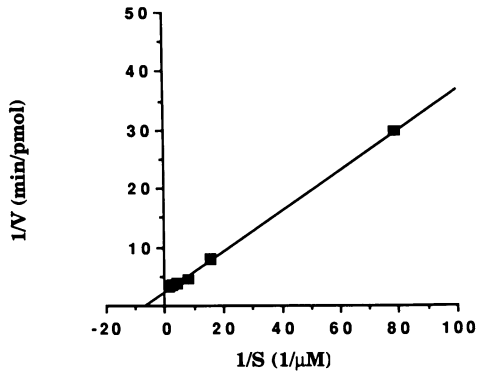

Figure 3. Lineweaver–Burk plot for the determination of K_m for the release of 2-deoxyribose-5-phosphate from a poly(dA-dT) substrate containing 5′ incised AP sites by exonuclease I and SSB. Substrate range, 0.01 μM to 0.90 μM; K_m = 0.15 μM.

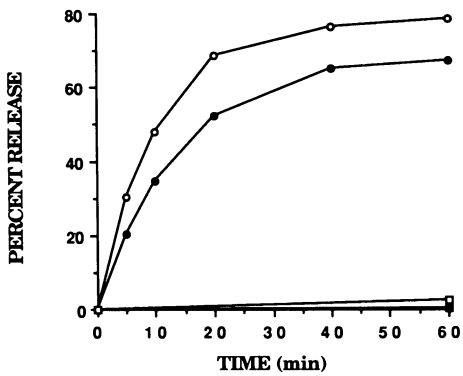

Figure 4. Time course for the release of 4-hydroxy-2-pentenal-5-phosphate from a poly(dA-dT) substrate containing 3′ incised AP sites. The release of 4-hydroxy-2-pentenal-5-phosphate was determined by precipitation with trichloroacetic acid in the presence of Norit charcoal. Reactions contained (●) exonuclease I; (○) exonuclease I plus SSB; (■) no enzyme; (□) SSB.

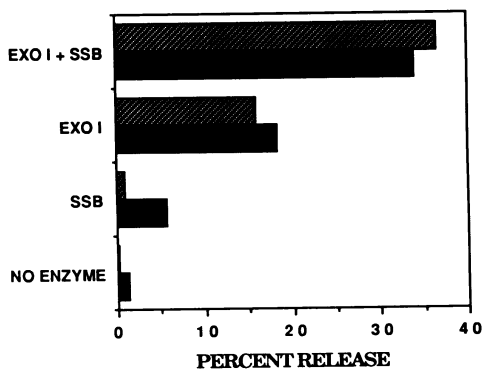

Figure 5. Release of 2-deoxyribose-5-phosphate from a lambda DNA substrate containing 5′ incised AP sites (■)and 4-hydroxy-2-pentenal-5-phosphate from a lambda DNA substrate containing 3′ incised AP sites (▨). The lambda DNA substrate was reacted with exonuclease I in the presence and absence of SSB protein for 30 min. Release of sugar-phosphate was determined by precipitation with trichloroacetic acid in the presence of Norit charcoal.

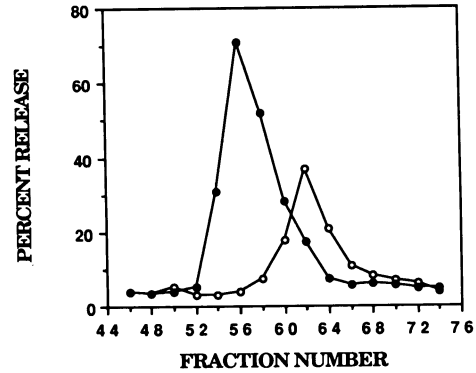

Figure 6. Gel filtration chromatography of exonuclease I and exonuclease I/SSB on Superose 12. Fractions (0.2 ml) were assayed for dRpase activity using the poly(dA-dT) substrate containing 5′ incised AP sites. The release of 2-deoxyribose-5-phosphate was determined by precipitation with trichloroacetic acid in the presence of Norit charcoal. (○) exonuclease I (60 units); (●) exonuclease I (60 units)+ SSB (180 μg).

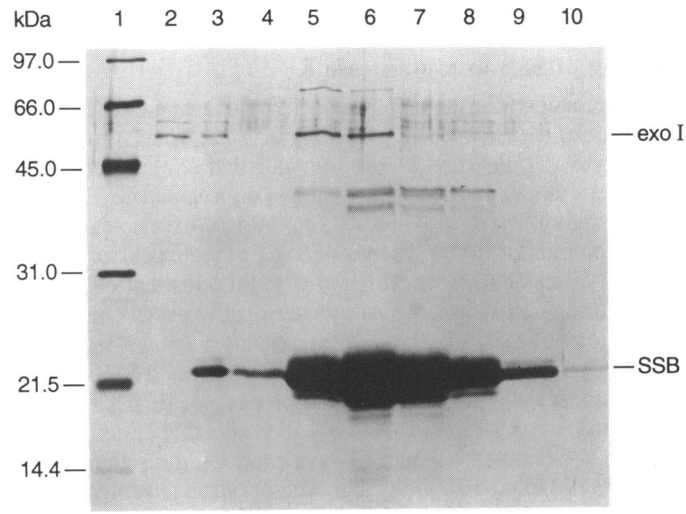

Figure 7. Separation of proteins in active fractions following gel filtration chromatography on Superose 12. Proteins in active fractions were resolved on a 12% polyacrylamide gel, and were visualized by silver staining. Lane 1, molecular weight markers (hen egg white lysozyme, soybean trypsin inhibitor, bovine carbonic anhydrase, hen egg white ovalbumin, bovine serum albumin, rabbit muscle phosphorylase b); lane 2, exonuclease I (3 units); lane 3, exonuclease I (3 units) plus SSB (1 μg); lane 4, fractions 52–53; lane 5, fractions 54–55; lane 6, fractions 56–57; lane 7, fractions 58–59; lane 8, fractions 60–61; lane 9, fractions 62–63; lane 10, fractions 64–65. Fraction numbers correspond to those shown in figure 6.

Release of deoxyribose-phosphate at 3′ incised AP sites from a polynucleotide substrate

AP lyases such as endonuclease III of *E.coli* cleave DNA at AP sites leaving the 3′ sugar-phosphate group 4-hydroxy-2-pentenal-5-phosphate (22). This unsaturated sugar-phosphate group is released by the dRpase activity of exonuclease I (14). When a poly(dA-dT) substrate containing ^{32}P-labeled 4-hydroxy-2-pentenal-5-phosphate termini was reacted with exonuclease I

in the presence of SSB (Figure 4), a consistent i50% stimulation in the amount of sugar-phosphate product released was seen. SSB in the absence of exonuclease I was ineffective in the release of 4-hydroxy-2-pentenal-5-phosphate (<2.5 % release in 60 min).

Release of deoxyribose-phosphate from a lambda DNA substrate

To determine whether the enhanced dRpase activity of exonuclease I was specific for poly(dA-dT) containing incised AP sites, a lambda DNA substrate was prepared containing either [^{32}P]-labeled 2-deoxyribose-5-phosphate groups at 5′ termini or [^{32}P]-labeled 4-hydroxy-2-pentenal-5-phosphate groups at 3′ termini. These DNA substrates were reacted with both exonuclease I and SSB. The addition of SSB to the reaction mixture containing exonuclease I resulted in a 1.5 fold increase in the amount of 2-deoxyribose-5-phosphate released from lambda DNA containing 5′ incised AP sites (Figure 5). When a lambda DNA substrate containing 3′ incised AP sites was treated with exonuclease I and SSB, a 2.3 fold increase in the release of 4-hydroxy-2-pentenal-5-phosphate was seen. These results suggest that the dRpase activity of exonuclease I/SSB behaves in a similar fashion with both poly(dA-dT) and lambda DNA containing incised AP sites.

Binding of SSB to exonuclease I

One mechanism for the enhanced activity of exonuclease I in the presence of the *E.coli* SSB protein is that the proteins may form a complex. To test this possibility, purified exonuclease I and SSB were mixed and chromatographed on a Superose 12 FPLC gel filtration column (Figure 6). When exonuclease I is chromatographed in the absence of SSB, a single peak of dRpase activity elutes at 12.4 ml (fraction 62). Proteins that elute from the column at this volume would have an expected molecular weight of 50−55 kDa. Exonuclease I has a known molecular weight of 53,174 Da (23). When exonuclease I and SSB are premixed and chromatographed, a major peak of dRpase activity elutes at 11.4 ml (fractions 56−58). Proteins that elute from the column at this volume would have an expected molecular weight of 120−130 kDa. The molecular weight of the tetramer of SSB is 75.6 kDa. The elution position of the SSB/exonuclease I protein mixture suggests that an SSB tetramer and exonuclease I form a protein−protein complex.

To confirm that the 120−130 kDa dRpase activity contained SSB and exonuclease I, the protein was analyzed by SDS polyacrylamide gel electrophoresis (Figure 7). Fractions 54−57 (lanes 5 and 6), which had the maximal dRpase activity, contained both exonuclease I and SSB. As seen in lanes 8 and 9, a high level of SSB is still present in fractions that elute from the column between 12−12.6 ml, which corresponds to a molecular weight range of 70−80 kDa, as expected for the elution of SSB in the tretrameric form.

DISCUSSION

We have demonstrated that the *E.coli* SSB protein can stimulate the dRpase activity of exonuclease I. The maximal increase in the reaction rate required an excess of SSB protein to exonuclease I (molar ratio ~100:1) . A large excess of SSB to exonuclease I is also required to stimulate the 3′ → 5′ exonuclease activity of the enzyme (8). Whether SSB can function *in vivo* to stimulate the dRpase or exonuclease activity of exonuclease I at a lower molecular ratio is unknown.

The results presented in this work suggest that SSB may also function as part of the DNA base excision repair pathway. SSB may be part of a repair protein complex involving exonuclease I, and possibly a DNA polymerase.

In addition to the dRpase activity of exonuclease I , we have recently demonstrated that exonuclease I is also capable of removing 3′ phosphoglycolate end-groups from DNA (24). However, SSB did not stimulate this reaction (data not shown). The fpg protein (fapy-DNA glycosylase), the product of the *mutM* gene, also contains a dRpase-like activity that appears to remove 5′ terminal deoxyribose-phosphates via a β-elimination mechanism (25). Whether this enzyme interacts in any way with *E.coli* SSB remains to be determined.

DNA dRpase activities have also been partially purified from mammalian cells, including human cells (17). With cell-free extracts of human cells, the human SSB is required for nucleotide excision repair (26). If the human SSB was found to enhance the human dRpase activity, this would be strong evidence that this protein may also play a role in base excision repair in human cells

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