The AddAB Helicase/Nuclease Forms a Stable Complex with Its Cognate χ Sequence During Translocation*

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The Bacillus subtilis AddAB enzyme possesses ATP-dependent helicase and nuclease activities, which result in the unwinding and degradation of double-stranded DNA (dsDNA) upon translocation. Similar to its functional counterpart, the Escherichia coli RecBCD enzyme, it also recognizes and responds to a specific DNA sequence, referred to as Chi (χ). Recognition of χ triggers attenuation of the 3′- to 5′-nuclease, which permits the generation of recombinogenic 3′-overhanging, single-stranded DNA (ssDNA), terminating at χ. Although the RecBCD enzyme briefly pauses at χ, no specific binding of RecBCD to χ during translocation has been documented. Here, we show that the AddAB enzyme transiently binds to its cognate χ sequence (5′-AGCGG-3′) during translocation. The binding of AddAB enzyme to the 3′-end of the χ Bs-specific ssDNA results in protection from degradation by exonuclease I. This protection is gradually reduced with time and lost upon phenol extraction, showing that the binding is non-covalent. Addition of AddAB enzyme to processed, χ Bs-specific ssDNA that had been stripped of all protein does not restore nuclease protection, indicating that AddAB enzyme binds to χ Bs with high affinity only during translocation. Finally, protection of χ Bs-specific ssDNA is still observed when translocation occurs in the presence of competitor χ Bs-carrying ssDNA, showing that binding occurs in cis. We suggest that this transient binding of AddAB to χ Bs is an integral part of the AddAB-χ Bs interaction and propose that this molecular event underlies a general mechanism for regulating the biochemical activities and biological functions of RecBCD-like enzymes.

Double-stranded DNA breaks are a serious threat to all organisms. Even in the absence of any DNA-damaging agents, double-stranded DNA breaks occur frequently because of the collapse of stalled replication forks or the presence of daughter strand DNA gaps or nicks ahead of the fork (1–4). In bacteria, these lesions are usually dealt with by homologous DNA recombination, which is a powerful tool to faithfully repair double-stranded DNA breaks and to restore DNA replication forks (5). In Escherichia coli, the multifunctional heterotrimeric RecBCD enzyme is the main protein complex responsible for the processing of double-stranded DNA breaks. RecBCD enzyme can bind to double-stranded DNA (dsDNA) ends and translocate inward in a rapid and processive manner because of its bipolar helicase activity (6, 7). While translocating through the DNA lattice, unwinding is normally accompanied by nuclease degradation of both DNA strands until recognition of a specific eight-nucleotide sequence, called χ (5′-GCTGTTGG-3′). Upon χ recognition, the enzyme briefly pauses before resuming translocation at a slower pace and the vigorous 3′- to 5′-exonuclease activity is attenuated, whereas the 5′- to 3′-exonuclease is up-regulated (8, 9). This allows for the generation of long, 3′-overhanging, single-stranded DNA (ssDNA) that terminates at χ (referred to hereafter as χ-containing ssDNA). The χ-modified RecBCD enzyme also coordinates the preferential loading of the homologous pairing protein, RecA, onto the resulting χ-containing ssDNA (10). This facilitated loading of the RecA protein results in the preferential incorporation of χ-containing ssDNA into homologously paired recombination intermediates, and provides an efficient means of linking the processing and homologous pairing stages of homologous DNA recombination.

In the Gram-positive bacterium Bacillus subtilis, the AddAB enzyme is the functional homologue of RecBCD and is responsible for processing dsDNA ends. The AddAB helicase/nuclease is the founding member of a growing family of enzymes involved in the initiation of DNA recombination in Gram-positive bacteria (11). For two of these members, the B. subtilis AddAB enzyme (12) and the Lactococcus lactis RexAB enzyme (13, 14), it is already known that the biological and enzymatic activity of these heterodimeric enzymes is regulated by a short, specific DNA sequence. Thus, the paradigm established for the RecBCD-χ interaction appears to be broadly conserved in bacterial evolution. Comparative analysis of protein sequences reveals that the AddA subunit is equivalent to the RecB subunit, with the presence of typical helicase and ATPase motifs, together with a C-terminal nuclease motif (15–17). The AddB subunit shows weak resemblance to the RecC subunit and also to members of the UvrD/Rep family (Superfamily 1) of DNA helicases. It also carries a second conserved nuclease domain at its C terminus. This suggests that, unlike RecBCD, which possesses only one nuclease domain, degradation of each strand of the DNA duplex is carried out by individual nuclease sites in AddAB (18, 19). Despite the fact that AddAB enzyme only possesses two subunits, expression of AddAB enzyme or of the RexAB enzyme, fully complements an E. coli recBCD deletion strain for cell viability, UV survival, and conjugational recombination frequency (14, 20). In vivo, the AddAB enzyme effectively degrades linear dsDNA unless it carries a properly oriented 5′-nucleotide sequence (5′-AGCGG-3′), which is the analogue of the E. coli χ sequence in B. subtilis (referred to as χ Bs) (12). In vitro, the AddAB enzyme binds to dsDNA ends and uses energy derived from ATP hydrolysis to catalyze the unwinding and degradation of both DNA strands. Recognition of χ Bs by AddAB results in the attenuation of its 3′- to 5′-nuclease activity. The 5′- to 3′-nuclease activity remains unaffected, however, leading to the generation of long 3′-overhanging, ssDNA fragments terminating at χ Bs (18).
The manner by which the RecBCD or AddAB complexes recognize and interact with their cognate \( \chi \) sequence while translocating through DNA at rates of up to several hundred base-pairs (bp) per second is still unclear. In the case of RecBCD enzyme, early studies showed that under certain conditions, the RecBCD enzyme undergoes a \( \chi \)-dependent inactivation, characterized by the reduced ability of the enzyme to catalytically process \( \chi \)-containing DNA molecules (21, 22). Under these conditions, the enzyme dissociates into subunits after DNA processing (23). However, recent single molecule microscopy experiments revealed that although \( \chi \) recognition results in a brief pause of the enzyme at \( \chi \) that is followed by a reduced rate of translocation (9), the heterotrimeric form of the enzyme persists and continues to translocate after \( \chi \) recognition (24). Thus, subunit dissociation does not explain how \( \chi \) recognition results in a persistent change in enzyme activity that can be maintained for distances of up to 30 kilobases but yet is fully reversible upon dissociation.

Interestingly, single molecule studies showed that the newly generated \( \chi \)-containing ssDNA remains associated with the translocating RecBCD enzyme (9), although the mechanistic significance of this observation is yet to be fully explored. Here, we report that AddAB also undergoes a \( \chi \)-independent inactivation process, and we provide the first direct evidence that this behavior occurs by the non-covalent binding of the enzyme to the \( 3' \)-end of the newly generated \( \chi \) ssDNA that persists during the course of translocation. We suggest that this previously undisclosed interaction represents the underlying regulatory event that is responsible for all of the enzymatic changes that are elicited by interaction with \( \chi \) and that this molecular event reversibly switches the RecBCD/AddAB family of enzymes from DNA-destroying to DNA-repairing enzymes.

**EXPERIMENTAL PROCEDURES**

Reagents and Proteins—Chemical reagents and ATP were purchased from Sigma. Shrimp alkaline phosphatase and [\( \gamma-\text{32P} \)]ATP were purchased from United States Biochemical Corp and PerkinElmer, respectively. Proteinase K was from Roche Molecular Biochemicals. Restriction endonucleases and T4 polynucleotide kinase were from New England Biolabs (NEB). SSBind was prepared as described previously (25). AddAB protein was purified as described below.

**Cloning of the addA and addB Genes of B. subtilis for Overexpression Purposes**—Cloning of the addA and addB genes from B. subtilis was achieved by PCR from genomic DNA. Primers were designed based on the published sequence of the B. subtilis genome (26), which allowed the amplification of the entire addA and addB genes immediately flanked by suitable restriction endonuclease recognition sequences (Ndel and Xhol for addA and Ncol and BamHI for addB). For addA, the forward and reverse primer sequences were 5’-GGC-GATGCTATAGAATACATCTAAATCCGGCGAC-3’ and 5’-GCTT-ATGGCTCGAGCTATAATGTCGAGATGTCGCCC-3’, respectively. For addB, the sequences were 5’-GGC-GATGCTATAGAATACATCTAAATCCGGCGAC-3’ and 5’-GCTTCTCCTGCGATGGA-GACGAGTTTTTATGAGG-3’ and 5’-GCTGCTGCGGATCTTTGAGGATAATG_GTC-3’. The template DNA was purified from B. subtilis strain 168 supplied by Dr. Sabine Autrett (Errington Laboratory, University of Oxford, UK). The addA and addB sequences were then inserted into the pET22b and pET28a vectors (Novagen), respectively, at the restriction sites listed above using standard cloning techniques to create plasmids pAddA-22b and pAddB-28a. DNA sequencing (MWG-Biotech) was used to confirm that the final sequences of the entire genes, as well as the promoter regions of the vectors, were identical to those published previously.

**Expression and Purification of AddAB Protein**—For expression of AddAB protein, the pAddA-22b and pAddB-28a plasmids were freshly transformed into E. coli B834 (DE3) cells (Novagen). An overnight starter culture of these cells was used to inoculate 12 liters of phosphate-buffered LB medium supplemented with 50 \( \mu \)g/ml ampicillin and 30 \( \mu \)g/ml kanamycin. The cells were grown at 37 °C until they reached mid-log phase. At this point 1 mM IPTG and a further 25 \( \mu \)M ampicillin were added to the medium. The growth temperature was simultaneously dropped to 25 °C, which increases the yield of soluble proteins. Growth was continued for a further 3–4 h before the cells were harvested, resuspended in 150 ml of 50 mM Tris-Cl pH 7.5, and 10% sucrose, and stored at −80 °C. All subsequent purification steps were performed at 4 °C. Cells were lysed in the presence of 0.1 mM phenylmethylsulfonyl fluoride using Brij lysis. Briefly, lysosome was added to 0.2 mg/ml, followed by a 30-min incubation with stirring. Next, EDTA was added to 1.5 mM followed by stirring for 30 min. Finally Brij-58 was added slowly to a final concentration of 0.3% over 30 min with stirring. The soluble cell extract was recovered by centrifugation and ammonium sulfate was added to a final concentration of 30%. The precipitate was recovered by centrifugation and ammonium sulfate was added to a final concentration of 50%. The precipitate was recovered by centrifugation, resuspended in buffer B (20 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 0.1 mM dithiothreitol) + 100 mM NaCl, and dialyzed against the same buffer overnight. The protein sample was loaded onto a Fast-Flow Q-Sepharose column (Amersham Biosciences-GE Healthcare) in Buffer B + 100 mM NaCl, washed with buffer B + 300 mM NaCl, and eluted with a gradient to 600 mM NaCl. This eluate was dialyzed against 50 mM potassium phosphate buffer, pH 7.5, + 1 mM dithiothreitol. It was then loaded in this buffer onto a Bio-Gel hydroxyapatite column (Bio-Rad) and eluted with a gradient to 300 mM potassium phosphate buffer. After dialysis against buffer B + 100 mM NaCl, the sample was loaded in the same buffer onto a Hi-Trap heparin column (Amersham Biosciences-GE Healthcare). At this stage, the sample contains a molar excess of AddA protein but, unlike AddAB protein, AddA does not bind to the heparin. The AddAB protein was eluted with a gradient to 500 mM NaCl, and peak fractions were collected. The sample was diluted with B buffer to a conductivity equivalent to buffer B + 100 mM NaCl and loaded onto a 10-ml mono-Q column (Amersham Biosciences-GE Healthcare). After washing with buffer B + 200 mM NaCl, AddAB protein was eluted with a gradient to 600 mM NaCl. Most of the protein was present in a major peak at ~40% (equivalent to ~350 mM NaCl) through the gradient. This protein was collected, dialyzed against buffer B + 100 mM NaCl + 50% glycerol, and stored at −80 °C. Concentration was determined using a theoretical extinction coefficient (\( e = 251,900 \text{ M}^{-1} \text{cm}^{-1} \)), and the protein was judged to be greater than 95% pure by SDS-PAGE analysis. Functionality was assessed using a dye-displacement assay and protein titrations, as described earlier (27). The protein preparations used here were between 25 and 50% active.

**DNA Substrates**—Plasmids, pADGF0 (\( \chi_0 \)), pADGF6406-1 (carrying one \( \chi_0 \) site, \( \chi' \)) and pADGF1 (carrying three \( \chi_0 \) sites in both orientations, \( \chi' + \chi' \)) were described previously (18). Tailed DNA substrates were prepared as described previously (18). Labeling at the 5’-end was carried out using T4 polynucleotide kinase with [\( \gamma-\text{32P} \)]ATP after shrimp alkaline phosphatase treatment of the linearized DNA. The oligonucleotides used here were SKHN55, 5’-TCACAAACAGAATGATGTCGATCGTTCCTAAGTAAAT-3’ (\( \chi_0 \)) and SKHN56, 5’-TCACAAA-ACAGAATGATGTCGATCGTTCCTAAGTAAAT-3’ (\( \chi_0 \)).

**Helicase Inactivation Assay**—Standard reactions contained 25 nM Tris acetate (pH 7.5), 1 mM dithiothreitol, 0.5 mM magnesium acetate, 6.25 \( \mu \)M nucleotides linear DNA (corresponding to 1.03 nM molecule), 2 \( \mu \)M E. coli single-stranded DNA binding protein (SSB),...
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1 mM ATP, and 0.26 mM functional AddAB (AddAB:dsDNA ends ratio = 1:8). The DNA substrates were linearized by BamH1 digestion. Reactions were initiated by addition of AddAB enzyme and incubated at 37 °C. Samples were taken at the indicated time points, deproteinized by digestion with Proteinase K, and processed by electrophoresis through 1% agarose gels at 4 V/cm for 4 h in 1× TAE buffer. After separation of the reaction products, the gels were dried and exposed to PhosphorImager screens and quantified using the ImageQuant software (Molecular Dynamics).

- **Protection Assay**—Typical reactions contained 25 mM Tris acetate, pH 7.5, 1 mM magnesium acetate, 1 mM ATP, 1 mM dithiothreitol, 10 μM nucleotide linear pADG6406-1 (corresponding to 1.6 nM molecules), 2 μM SSB protein, and 3.2 nM (unless otherwise specified) functional AddAB enzyme. Assays were performed at 37 °C. Tailed DNA substrates were prepared as described (18) so that AddAB can only access the linear dsDNA from one entry site. Two minutes after initiation of the reaction by the addition of the enzyme, Exonuclease I (NEB), a 3’- to 5’- ssDNA exonuclease, was added to a final concentration of 0.8 units/μL. Samples were taken at the indicated time points and processed as described above. When indicated, a phenol extraction was carried out 30 s after the addition of Exonuclease I, followed by an ethanol precipitation. The pellets were then resuspended in water and further desalted by passage through a G-25 spin column (Amersham Biosciences-GE Healthcare) equilibrated with water. Standard buffer components were then added back to a final volume of about half of the initial volume to compensate for the loss of DNA during the procedure. The samples were then treated with various combinations of enzymes, as indicated. Reaction mixtures were incubated for 1 min after each new addition, and the products were separated by agarose gel electrophoresis as described above.

- **Competition Experiment**—The reaction conditions were similar to the ones described for the χ protection assay, except that a 100-fold molar excess of unlabeled oligonucleotides was added after the addition of AddAB enzyme. The oligonucleotides used here either contained or did not contain a χ06 sequence at their 3’-ends (SKNH55 or SKNH56, respectively). The concentration of the SSB protein was 4.5 μM. The reaction mixture was incubated at 37 °C for 2 min before the addition of each component. The reaction was initiated by addition of ATP. Exonuclease I was added to the reactions after 2 min. Samples were taken at the indicated time points and processed as described above.

**RESULTS**

The AddAB Enzyme Shows χ06-Dependent Inactivation—We first investigated the consequences of χ06 recognition on the ability of the AddAB enzyme to process a linear DNA substrate. For this, we used three different DNA substrates carrying zero (χ0), one (χ1), or three (χ+++ ) χ06 sites (Fig. 1A). These DNA substrates were linearized, radiolabeled at their 5’-ends, and incubated with a limiting amount of enzyme (1 functional AddAB enzyme/8 DNA ends; 12.5% saturation). Substrate usage was then followed as a function of time after separation of the reaction products by agarose gel electrophoresis. In the case of the RecBCD enzyme, it is known that, at least under certain conditions (21), interaction with χ can result in a χ-dependent, reversible inactivation of the enzyme. As shown in Fig. 1B, processing of a χ0DNA substrate by AddAB enzyme is catalytic, with substrate usage reaching near completion after 5 min, in agreement with results obtained using a more sensitive real time dye-displacement assay. However, in the presence of three χ06 sites, the efficiency of the reaction was markedly lower (Fig. 1B). Quan-

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*F. Chédin and S. C. Kowalczykowski, unpublished observations.*
RecBCD enzyme loads E. coli RecA protein onto the 3'-end of the ssDNA produced during dsDNA processing (10). Similarly, Exo I activity can be inhibited by the formation of secondary structure in ssDNA (28).

Here, we used a tailed DNA substrate either carrying or lacking a single XBs site (Fig. 2A), and we initiated the reaction with a saturating amount of AddAB enzyme. Tailed DNA ensures that the AddAB enzyme enters and translocates through the DNA from only one end. As described earlier (18), processing of the XBs DNA by the AddAB enzyme resulted in the unwinding and degradation of much of the dsDNA substrate, but some full-length ssDNA is also produced (Fig. 2B, lanes 1–3). Addition of Exo I after completion of the reaction (2 min) led to the rapid and complete disappearance of this full-length ssDNA, indicating that this DNA species was fully available for degradation by Exo I. Unexpectedly, a novel prominent band appeared after treatment by Exo I (Fig. 2B, lanes 4–6). Using alkaline gel electrophoresis, we determined that this DNA species migrates at a position consistent with a 5'-end-labeled DNA fragment ending at the primosome assembly site (PAS) present on these substrates (data not shown). This PAS site corresponds to the B. subtilis pAMβ1 PAS site, which was described as capable of adopting a strong secondary structure (29). We therefore conclude that this stable DNA fragment occurs because of inhibition of degradation by Exo I by the strong DNA secondary structure formed at PAS.

When a XBs-containing substrate was used, a 5'-end-labeled top strand XBs-specific DNA fragment was observed in addition to full-length ssDNA (Fig. 2B, lanes 7–9), as described earlier (18). Addition of Exo I after 2 min led to the appearance of the PAS fragment generated by degradation of the full-length ssDNA species up to the PAS site, showing that Exo I was functional. In sharp contrast, however, the XBs-specific ssDNA was largely resistant to degradation (Fig. 2B, lanes 10–12). Such protection of the XBs-specific fragment from Exo I degradation was unexpected. Unlike the RecBCD system, where the RecA protein is strictly required to observe protection of XBs-specific fragments from degradation, RecA protein was absent from our assays. Quantification of the protection over time revealed that although Exo I degraded almost all of the full-length ssDNA and about one-half of the XBs-specific ssDNA within 30 s, the remaining half of the XBs-specific ssDNA persisted with a half-life of ~10–15 min (Fig. 2C). Protection of the XBs-specific fragment was not limited to this particular XBs site but was found for all three XBs sites tested (data not shown). Finally, protection of the XBs-specific fragment

**FIGURE 2.** XBs-specific ssDNA is protected from exonuclease I degradation by binding of the AddAB enzyme. A, plasmids pADGF0 (XBs0) or pADG6406-1 (XBs-1) were tailed so that AddAB can only access the DNA from one end (18). The distance (in nucleotides) from the XBs site, or the PAS site, to the labeled 5'-end is indicated. B, the indicated substrates were reacted with AddAB enzyme (at an AddAB to dsDNA ends ratio of 1 to 2) for 2 min, after which 0.8 units/μl exonuclease I enzyme were added. Aliquots were withdrawn at the indicated times, and the distribution of reaction products was analyzed by agarose gel electrophoresis. A schematic depiction of the main product species, as had been established previously by Chédin et al. (18), is shown on the right side. C, the relative percentages of XBs-specific and of full-length ssDNA remaining after addition of exonuclease I are plotted as a function of time. The results are the average of two independent experiments, and each point is presented with the observed variation. D, processing by AddAB enzyme was as described in B except that SSB was omitted from the time course shown in right panel.

RecBCD enzyme loads E. coli RecA protein onto the 3'-end of the χ-containing ssDNA produced during dsDNA processing (10). Similarly, Exo I activity can be inhibited by the formation of secondary structure in ssDNA (28).

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from Exo I degradation was observed under all conditions tested (low and high magnesium ion concentrations; data not shown). Protection was also not because of the formation of a secondary DNA structure, because Exo I could clearly degrade the full-length ssDNA from its 3'-end down to the PAS site, which is located a few hundred base pairs downstream of the $\chi_{Bs}$ studied in Fig. 2B. We therefore surmised that protection from Exo I degradation might be because of the binding of a protein to the 3'-$\chi_{Bs}$-specific end generated upon attenuation of the AddAB enzyme nuclease activity at $\chi_{Bs}$.

Only two proteins are present in our reactions, AddAB enzyme and SSB protein. To test whether protection could be due to binding of the AddAB enzyme to the end of the $\chi_{Bs}$-specific fragment, we performed reactions in the absence of SSB protein. As with the RecBCD enzyme, the catalytic activity of the AddAB enzyme is reduced in the absence of SSB protein (30), particularly at higher magnesium ion concentrations and at higher ratios of DNA ends to enzyme. Under the saturating conditions used here (1 enzyme/end), most of the linear dsDNA was processed in 2 min despite the absence of SSB (Fig. 2D, lanes 5 and 6). As had been described for the RecBCD enzyme (30), DNA degradation by AddAB enzyme is greater in the absence of SSB, as judged by the absence of full-length ssDNA (compare lane 2 with 6). Because of this more extensive degradation, the yield of $\chi_{Bs}$-specific ssDNA was reduced. Nevertheless, protection of the remaining $\chi_{Bs}$-specific ssDNA from Exo I degradation was observed (60 ± 10% protection, compared with 50 ± 4% by AddAB in the presence of SSB as judged 60 s after addition of Exo I; Fig. 2D, lanes 7 and 8). This, therefore, rules out the possibility that SSB was responsible for the protection of the $\chi_{Bs}$-specific ssDNA, a conclusion that is consistent with its documented property of stimulating DNA degradation by Exo I. We therefore conclude that $\chi_{Bs}$-specific ssDNA is being protected from Exo I degradation by the binding of the AddAB enzyme to the 3'-end of the $\chi_{Bs}$-containing ssDNA. Because the protection gradually decreases with time (Fig. 2C), we speculated that the binding of AddAB to the 3'-end was non-cova lent and subject to slow dissociation.

Protection of $\chi_{Bs}$-Specific ssDNA Occurs by Binding of AddAB Enzyme during Translocation —Two models can explain the protection of the $\chi_{Bs}$-containing ssDNA from degradation by Exo I. In the first model, binding to the 3'-end of the $\chi_{Bs}$-specific ssDNA by AddAB enzyme is an integral part of the recognition process and occurs in cis during translocation. After binding, the enzyme would continue to unwind and degrade DNA while bound to the $\chi_{Bs}$-containing 3'-end until it reaches the end of the duplex DNA or dissociates. In the second model, processing of the duplex DNA into full-length ssDNA and $\chi_{Bs}$-specific ssDNA occurs without any binding, but after dissociating from the DNA, AddAB enzyme would rebind in trans to the 3'-end of the $\chi_{Bs}$-specific ssDNA.

To distinguish between these two possibilities, we tested whether adding AddAB back to reactions from which all proteins had been removed after processing would result in protection of the $\chi_{Bs}$-containing ssDNA. For this, tail $\chi_{Bs}$-containing DNA was first reacted for 2 min with a saturating amount of AddAB enzyme as described above, followed by addition of Exo I for 30 s to degrade any susceptible ssDNA. The reaction was then stopped by phenol extraction. The DNA, stripped of all proteins, was recovered by ethanol precipitation. We consistently noticed that only a portion (59 ± 11% (n = 8)) of the $\chi_{Bs}$-specific ssDNA was recovered after that treatment, compared with the PAS fragment (Fig. 3, lanes 3 and 4). This specific loss of $\chi_{Bs}$-specific fragments can be explained if some of the ssDNA that was bound by AddAB was directed to the phenol phase during the extraction, as has been observed for other DNA-binding proteins. Addition of the AddAB enzyme back to the recovered reaction products for 1 min did not lead to any detectable change in the product distribution (Fig. 3, lane 5). However, the addition of Exo I to the recovered reaction products resulted in the complete loss of the $\chi_{Bs}$-specific ssDNA, indicating that the factor that was protecting the 3'-end had been removed by the phenol extraction (a similar loss of protection was observed after treatment of the reaction products with Proteinase K; data not shown). Note that the PAS fragment was also partially degraded upon addition of Exo I (Fig. 3, lane 6). This was consistently observed in independent experiments, although the extent of the degradation was variable. We speculate that the secondary structure that confers protection to the PAS fragment can become at least partially unfolded as a result of phenol extraction and ethanol precipitation, thus allowing Exo I to degrade a subset of molecules. Importantly, incubation with AddAB for 1 min followed by addition of Exo I failed to restore protection to the $\chi_{Bs}$-specific ssDNA (Fig. 3, lane 7). This indicates that binding of the AddAB enzyme to the 3'-end of the $\chi_{Bs}$-specific fragment in trans after dissociation of the complex does not account for the protection that is observed simultaneously with processing by AddAB enzyme.

Our results suggest that the 3'-end of the $\chi_{Bs}$-specific ssDNA is being bound directly by the AddAB enzyme and that this binding occurs in cis, during translocation of the enzyme through DNA. Note that this binding does not result in the inhibition of the enzyme in cis because the reaction products are fully unwind. This observation, in turn, implies that the AddAB enzyme, although bound to the 3'-end at the $\chi_{Bs}$-specific ssDNA, can continue to travel through the DNA to which it is bound (i.e. in cis); however, these very same AddAB-$\chi_{Bs}$-specific ssDNA complexes cannot initiate unwinding on subsequent DNA molecules until they have dissociated, thus accounting for the inactivation observed with $\chi_{Bs}$-containing substrates.

To confirm that binding of AddAB to the $\chi_{Bs}$-specific ssDNA occurred during translocation, we also performed competition experiments with ssDNA in trans. In these experiments, a prebound AddAB-dsDNA complex was challenged with a 100-fold molar excess of unlabeled, 35-nucleotide-long, single-stranded oligomers either carrying or lacking the $\chi_{Bs}$ sequence at their 3'-end. These oligonucleotides were
AddAB binds specifically to the 3'-end of the 100-fold molar excess of single-stranded 35-mers carrying (Yb⁻), or not (Yb⁺), at their 3'-end were added to preformed complexes of AddAB and dsDNA. The reaction mixture was incubated for 2 min at 37 °C after each addition. The reaction was initiated after preincubation of all components by addition of ATP, allowed to proceed for 2 min after which exonuclease I was added to all reactions. The reactions were kept at 37 °C for another minute before the separation of the products by agarose gel electrophoresis.

readily bound by the AddAB enzyme in electrophoretic mobility shift assays (data not shown). The reactions were initiated by addition of ATP. If binding to the Yb⁺-specific ssDNA only occurs by reassociation after dissociation from the unwound DNA, then the excess of cold ssDNA competitors should block rebinding to the 3'-end of the Yb⁺-specific ssDNA. When present at a saturating concentration (Fig. 4), the processing of dsDNA by AddAB enzyme in the presence of excess competitor ssDNA resulted in the complete production of the expected full-length ssDNA and Yb⁺-specific ssDNA. This is consistent with the behavior of the RecBCD enzyme, for which addition of ssDNA oligonucleotides after formation of the enzyme-dsDNA complex has little effect on enzyme activity (31). More importantly, however, the Yb⁺-specific ssDNA was still protected from Exo I, whereas the full-length ssDNA was not, irrespective of whether the competitor oligonucleotides carried a Yb⁺ sequence at their 3'-extremity (Fig. 4, lanes 5–8) or not (lanes 9–12). Thus, this result suggests that AddAB binds specifically to the 3'-end of Yb⁺-specific ssDNA during the course of translocation and processing, and not subsequently after dissociating from the DNA processing products.

DISCUSSION

In this study, we showed that the AddAB helicase/nuclease binds to its cognate regulatory sequence, Yb⁺, in cis during unwinding, after which it continues to process the downstream dsDNA while still bound to the 3'-end of the Yb⁺ sequence. This conclusion is based on two observations. First, we showed that the ability of AddAB enzyme to initiate new cycles of dsDNA processing was reduced in response to Yb⁺ suggesting that the enzyme is inactivated by a prior encounter with its regulatory sequence. Second, we observed that the Yb⁺-specific ssDNA, generated by processing of Yb⁺-containing dsDNA, was protected from degradation by Exo I because of the binding of AddAB enzyme to the 3'-end of the Yb⁺-specific ssDNA. Protection was specific to these fragments and was observed in reactions where AddAB was the only protein present. The full-length ssDNA that was generated during DNA unwinding was not protected by the AddAB enzyme against Exo I degradation, and the removal of proteins by phenol extraction or Proteinase K treatment rendered the Yb⁺-specific ssDNA sensitive to Exo I. In the absence of any treatment, protection from Exo I was slowly lost over time, indicating that AddAB enzyme dissociated from the Yb⁺-specific ssDNA with a half-life of 10–15 min.

Our observation that AddAB remains bound to the end of the Yb⁺-specific fragments provides a simple explanation for the Yb⁺-dependent inactivation of the helicase activity of the enzyme (Fig. 1). Assuming that Yb⁺-bound AddAB molecules are not competent to initiate a new round of processing, the number of functional AddAB molecules in the reaction is expected to gradually decrease as a consequence of Yb⁺ recognition in the previous cycle. The fact that recognition of Yb⁺ is not 100% efficient (it is estimated to be ~25% per individual site) and that dissociation of the AddAB-Yb⁺-ssDNA complexes does occur, albeit slowly, explains why multiple rounds of DNA unwinding can, and do, occur. Interestingly, reversible χ-dependent inactivation was also described for RecBCD enzyme, although it is observed only at low free magnesium ion concentrations (21), and the RecBCD enzyme can be reactivated by the addition of magnesium salt. In contrast, Yb⁺-dependent inactivation of AddAB was observed under all conditions tested, and attempts to reactivate the AddAB enzyme resulted in only very partial, if any, reactivation (data not shown). One reason behind this difference might be related to the strength of the interaction between AddAB and its Yb⁺ sequence. Indeed, in the case of the RecBCD enzyme, our attempts to detect protection of χ-specific DNA fragments from Exo I under inactivating conditions were unsuccessful (data not shown), indicating that even under such conditions, the RecBCD enzyme does not bind to χ with a sufficient stability to afford protection.

The binding of AddAB to Yb⁺ occurs only in cis during translocation. This is evidenced by two different results. First, treatment of reaction
Stable Binding of AddAB Enzyme to χ

products with phenol, which extracts all non-covalently bound proteins from DNA, renders the χinh-specific fragments sensitive to treatment by Exo I even when AddAB was added back prior to addition of Exo I (Fig. 3). Second, the presence of competitor ssDNA did not affect the protection of χinh-specific ssDNA, confirming that binding to χinh probably occurs without prior dissociation of the enzyme from DNA (Fig. 4). Together, these results suggest that binding of AddAB to χinh probably occurs as part of the normal recognition mechanism. It is also clear that binding to χinh does not lead to any detectable impairment in the ability of AddAB enzyme to continue processing the dsDNA to which it is bound. This, in turn, suggests the existence of a growing loop of ssDNA of AddAB enzyme to continue processing the dsDNA to which it is depicted in Fig. 5, the 3' enzymatic conformational changes, are correlated with a brief pause in the direct evidence that such a transient complex with detection of fluorescent spot after χ recognition that both grew in intensity and remained associated with the enzyme (9). This spot was presumed to correspond to the expected χ-specific ssDNA, but the fact that it translocated with the enzyme suggested that it was anchored to it, rather than being spooled out freely. Our observation here that the AddAB enzyme forms a stable complex with the 3'-end of the χinh-specific ssDNA provide the first direct evidence that such a transient complex with χ indeed exists.

These data for AddAB enzyme, together with the larger collection of genetic, biochemical, and structural (32) findings for the RecBCD enzyme that have been cited earlier, suggest a general model by which χ sequences regulate the biological function of their respective enzymes. Specifically, as depicted in Fig. 5, the 3'-terminated strand of a broken dsDNA molecule is scanned for the presence of a χ sequence during translocation and is fed toward the domain responsible for nucleolytic degradation. Upon recognition of χ, the χ sequence itself, which is now at the 3'-end of the processed ssDNA, binds tightly to the enzyme, preventing movement of this DNA strand into the nucleolytic site and thereby limiting degradation of the χ-containing ssDNA. This molecular event underlies the observed attenuation of the 3' to 5'-nuclease activity by χ. Binding, and the expected enzymatic conformational changes, are correlated with a brief pause in the translocation of the enzyme at χ. Because the χ-containing ssDNA is bound to the enzyme via the χ sequence, continued translocation beyond χ causes a loop of ssDNA to form that travels with the enzyme and that serves as a substrate for the next step of homologous recombination. This next step requires the assembly of a RecA protein filament on the χ-containing ssDNA, to the exclusion of the dsDNA-binding protein; this "loading" of RecA protein is mediated by direct protein-protein interactions with RecBCD enzyme (it is presently unknown whether AddAB can load its cognate RecA protein onto ssDNA). Persistence of a loop of ssDNA during the course of translocation also means that RecA protein needs to assemble onto this ssDNA loop. The assembly of RecA protein filaments occurs in the 5'→3' direction; however, the RecBCD/AddAB enzyme is traveling in the opposite direction (as defined by translocation along the χ-containing DNA strand 3'→5'). Thus, as we had postulated previously, assembly of the RecA nucleoprotein filament must be discontinuous, with repeated loading events by RecBCD/AddAB enzyme serving as nucleation points that facilitate subsequent polymerization in the presumed standard 5'→3' direction. As long as the 3'-end of the χ-containing ssDNA is bound to the translocating enzyme, a homologous pairing event will produce a parame- nomic joint molecule, which can be converted into a more stable plec tonic joint by the action of a topoisomerase. Alternatively, the parame nic joint molecule can be converted to a plec tonic joint when the 3'-end is released by dissociation of the helicase complex, and then the 3'-end can be used to initiate DNA replication. This dissociation frees the AddAB enzyme to reinitiate another processing cycle, and, finally, the subsequent steps of recombinational repair ensue.

This simple model accounts for many observed features of the RecBCD family of enzymes. Namely, both χ recognition and binding are dependent on a properly oriented recognition site and the changes elicited by this interaction are only observed in cis. Furthermore, once a χ sequence has been recognized and bound, the enzyme becomes insensitive to the presence of additional χ sequences (33, 34). Both genetic and biochemical findings show that both χ-binding and χ-elicited effects can persist for distances of ~30 kbp downstream of χ (9). However, although χ binding persists during translocation, it is fully reversible upon dissociation of the enzyme from DNA. In the case of the RecBCD enzyme, dissociation is typically rapid, allowing the enzyme to catalytically process additional dsDNA molecules. In contrast, in the case of the AddAB enzyme described here, dissociation is slower, causing inactivation of the enzyme and permitting detection of the AddAB-χinh complex directly. This model does not require that any subunit of the complex be modified or ejected in any way. In agreement, recent single molecule experiments conclusively show that the RecD subunit is not ejected from the RecBCD enzyme upon χ recognition (24). Thus, we concluded that the regulation of recombination activity by χ sequences is a direct consequence of binding by the regulatory χ sequence to the RecBCD/AddAB enzyme; this binding, in turn, elicits an allosteric change in the enzyme that persists for the duration of the translocation event needed to initiate recombination.

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