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Effects of Lysine-to-Glycine Mutations in the ATP-Binding Consensus Sequences in the AddA and AddB Subunits on the *Bacillus subtilis* AddAB Enzyme Activities

BERT JAN HAIJEMA, ROB MEIMA, JAN KOOISTRA, AND GERARD VENEMA*

Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9751 NN Haren, The Netherlands

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The N-terminal regions of both subunits AddA and AddB of the *Bacillus subtilis* AddAB enzyme contain amino acid sequences, designated motif I, which are commonly found in ATP-binding enzymes. The functional significance of the motif I regions was studied by replacing the highly conserved lysine residues of the regions in both subunits by glycines and by examination of the resulting mutant enzymes with respect to their enzymatic properties. This study shows that the mutation in subunit AddB hardly affected the ATPase, helicase, and exonuclease activities of the AddAB enzyme. However, the mutation in subunit AddA drastically reduced these activities, as well as the k_{cat} for ATP hydrolysis. The apparent K_m for ATP in ATP hydrolysis did not significantly deviate from that of the wild-type enzyme. These results suggest that the lysine residue in motif I of subunit AddA of the AddAB enzyme is not essential for the binding of the nucleotide but has a role in ATP hydrolysis, which is required for the exonuclease and helicase activities of the enzyme.

The *Bacillus subtilis* AddAB enzyme plays an important role in DNA repair, homologous recombination, and cell viability (1, 2, 3, 17, 23, 24). The AddAB enzyme is the functional analog of the *Escherichia coli* RecBCD enzyme (22). The AddAB enzyme consists of subunits A (141 kDa) and B (135 kDa), encoded by the *addA* and *addB* genes (23), which are located in an operon structure (16). AddAB is a multifunctional enzyme. It possesses DNA-dependent ATP hydrolysis activity (31), ATP-dependent exonuclease activity on double-stranded (ds) (9) and single-stranded (ss) DNA (this paper), endonuclease activity on ssDNA (16), and helicase activity (36). Subunit AddA shares seven distinct regions of homology with several other helicases (23), e.g., DNA helicase pcrA of *Staphylococcus aureus* (20), MutB of *Haemophilus influenzae* (42), and UvrD (11), RecB (12), and Rep helicase of *E. coli* (14). One of these regions, motif I or mononucleotide binding site (18, 41), is also present in subunit AddB and is thought to be involved in ATP binding and hydrolysis (23). Structural analysis by nuclear magnetic resonance and X-ray diffraction of the ATPase adenylate kinase demonstrated that motif I is located near the MgATP binding site (13). Motif I consists of an α-helix, containing two hydrophobic residues that interact with the adenine-ribose moiety of ATP, and a lysine that may bind to the β and γ phosphates of ATP (13).

It was previously reported that the conserved lysine residues of motif I (Fig. 1) of both subunits were changed into glycines (17). The mutation AddBK14G (AddAB*) hardly affected the ATP-dependent helicase and exonuclease activities in crude cell extracts, whereas mutation AddAK36G (AddA*B) resulted in complete loss of both activities. A *B. subtilis* strain containing the *addA*B* genes showed reduced cell viability, recombination in transformation with chromosomal DNA, repair of DNA damaged by UV irradiation or mitomycin C treatment, and bacteriophage PBS1-mediated transduction, approximately to the level of those in a *B. subtilis addAB* deletion mutant. These results suggested that the helicase and/or exonuclease activities of AddAB are important in these processes.

To biochemically characterize the wild-type AddAB enzyme and both mutant enzymes AddA*B and AddAB*, the enzymes were overproduced and purified. The present paper describes the effects of the mutations in the motif I regions on the ATP dependency of the ATPase, helicase, and exonuclease activities and shows that the mutation in subunit AddA, in contrast to the mutation in AddB, drastically reduced the activities of the AddAB enzyme by reducing the maximal rate of ATP hydrolysis. The apparent affinity for ATP was not altered.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids are listed in Table 1.

Site-directed mutagenesis. The oligonucleotide-directed mutations in *addA* and *addB* were introduced by the gapped duplex method by using the pMa/c plasmid system developed by Stanssens et al. (38), modified as described by Baldrich et al. (4). The lysine codon AAA in the *addA* gene was changed into a glycine codon by using the mutagenic primer I with the sequence 5′-GGCTCT **GGT**ACCGCTGTGCTC, resulting in the *addAB*-A-K36G mutation (*addA*B*). The boldfaced GGT replaced the AAA lysine codon at position 36 with a glycine codon. The lysine codon AAA in the *addB* gene was changed into a glycine codon by using the mutagenic primer II with the sequence 5′-GGGAGTGG A**GGT**ACCAAGCTGATCATC, resulting in the *addAB*-B-K14G mutation (*addAB**). The boldfaced GGT replaced the lysine codon at position 14 with a glycine codon. The underlined nucleotides in primers I and II constitute an *Asp*718 restriction site. The fidelity of the mutations was checked by sequencing (35). Reconstruction of plasmids pIN881 and pIN882, containing the mutagenized *addAB** and *addA*B* genes, respectively, has been described previously (17).

Overproduction of AddA*B and AddAB*. Overproduction of the wild-type AddAB enzyme was achieved by gene amplification (30). The mutant AddAB enzymes were overproduced by the same procedure. Plasmids pSPT1989K and pSPT1990K were constructed as follows: from plasmid pSPT1988K (Fig. 2) (30), the 8.9-kb *Sal*I-*Bam*HI fragment, carrying the wild-type *addAB* genes, was removed and replaced by 8.9-kb *Sal*I-*Bam*HI fragments carrying the *addAB** and *addA*B* genes (obtained from plasmids pIN881 and pIN882, respectively [17], which are pWSK29 derivatives [43]), resulting in plasmids pSPT1989K and pSPT1990K, respectively. Plasmid pDE2 was constructed as follows: the Em^r gene was ligated into pD2 (17), a pMTL23 derivative (8), resulting in pDE2. *B. subtilis* strains 8GK03 and 8GK04 (17), carrying the *addA*B* and *addAB** genes in one copy, respectively, were transformed with *Sca*I-linearized pDE2. In this way, the Km^r gene of strains 8GK03 and 8GK04 was replaced by the Em^r gene

* Corresponding author. Mailing address: Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Phone: 31 50 3632093. Fax: 31 50 3632348. Electronic mail address: G.Venema @biol.rug.nl.

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           Subunit     Residue

           AddA        27- 37       LVAAAAGSGKT
  Motif I                            + +  ●●●●●
           AddB         5- 15       FLVGRSGSGKT
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FIG. 1. Sequence similarity between subunits AddA and AddB of motif I of the *B. subtilis addAB* gene products. Filled circles indicate identical amino acids, and plusses indicate conserved replacements. The boldfaced K in motif I represents the conserved lysine.

by a double crossover event, resulting in strains 8GE03 and 8GE04, respectively. Plasmids pSPT1989K and pSPT1990K were integrated into the chromosome of strains 8GE03 and 8GE04 by a Campbell-type crossover. These integrated plasmids were amplified by increasing the concentration of kanamycin to 50 μg/ml during growth in TY (trypton-yeast) medium. This resulted in strains 8GE03M and 8GE04M, carrying multiple copies of plasmids pSPT1989K and pSPT1990K in their chromosomes.

The antibiotic concentrations used for selection of transformants were as follows: kanamycin, 15 μg/ml; erythromycin, 2 μg/ml; or ampicillin, 100 μg/ml. *E. coli* strain MC1061 (44) was used as a cloning host for the construction of the plasmids described. General DNA technology methods were according to Sambrook et al. (34).

Purification of AddAB, AddA*B, and AddAB*. (i) Enzymatic assay. The purification of AddAB and AddAB* was monitored by assaying the ATP-dependent exonuclease activity. The enzyme unit is defined as follows: 1 U of ATP-dependent exonuclease equals the amount of protein (in milligrams) that converts 1 nmol of dsDNA, expressed as nucleotides, to acid-soluble nucleotides in 10 min at 37°C. The assays were performed as described in the section "Nuclease measurements." The reaction mixtures contained 1 μg of ^{3}H-labeled dsDNA, 75 μM ATP, and 5 μl of the fractions to be assayed, and they were incubated for 10 min. The purification of AddA*B was monitored by Western blotting (immunoblotting) by using antibodies directed against AddA (see below).

(ii) Enzyme purification. Cultures of *B. subtilis* 8GK003 (wild-type AddAB overproducer) (30), 8GE03M (AddA*B overproducer), and 8GE04M (AddAB* overproducer) were grown in 2 liters of minimal medium to competence. The cells were harvested by centrifugation and resuspended in 100 mM NaCl in buffer A, consisting of 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0), 1 mM β-mercaptoethanol, 5 mM $MgCl_2$, and 10% glycerol (vol/vol). Crude extracts were prepared by using a French press (Amicon, Beverly, Mass.) at 1,000 lb/in^2. Nonsolubilized material was removed by centrifugation (35,000 rpm, 90 min, 0°C) in a SW41Ti rotor by using a Beckman L8-70M ultracentrifuge. The yield was around 165 mg of protein. The protein solution was chromatographed on DEAE-Sepharose FF (Pharmacia, Uppsala, Sweden) with a 15-ml bed volume in an XK-16 column (Pharmacia) by using fast-performance liquid chromatography (Pharmacia). The enzyme was eluted with a gradient of 0.10 to 0.60 M NaCl in buffer A. Fractions containing the enzyme were pooled and dialyzed against 0.20 M NaCl in buffer A. The dialyzed fractions were chromatographed on a Mono Q 5/5 column (Pharmacia) by using fast-performance liquid chromatography. The enzyme was eluted with a gradient of 0.2 to 0.5 M NaCl in buffer A. The fractions containing the enzyme were dialyzed against 0.1 M NaCl in buffer A. The dialyzed solutions were chromatographed on an Econo-Pac heparin cartridge (5 ml) (Bio-Rad, Hercules, Calif.). The enzyme recovered from this column was concentrated by centrifugation through a Centricon-10 microconcentrator (Amicon). The glycerol concentration of the enzyme was increased to 50% (vol/vol). The enzymes were stored at −20°C. The yield was approximately 900 μg of enzyme, which was purified to apparent homogeneity as judged by silver-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels. The wild-type AddAB and the AddA*B and AddAB* mutant enzymes were all purified by the same procedure. The AddAB enzyme concentrations were determined with the D_C protein assay (Bio-Rad).

Chemicals and enzymes. The chemicals used were of analytical grade. ATP was obtained as disodium salt from Sigma (St. Louis, Mo.). Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturer (Boehringer GmbH, Mannheim, Germany).

Media. *B. subtilis* minimal medium consisted of Spizizen minimal salts (37) supplemented with glucose (0.5%) and casein hydrolysate (0.02%; Difco Laboratories, Detroit, Mich.). Amino acids, nucleotides (20 μg/ml), and vitamins (0.4 μg/ml) were added when required. TY medium and TY agar were prepared as described by Biswal et al. (5).

Competence and transformation. *B. subtilis* cells were made competent essentially as described by Bron and Venema (6). *E. coli* was made competent and transformed by the method of Mandel and Higa (28).

Western blot analysis. Equal amounts of protein (2.5 μg) were run on a homogeneous SDS–7.5% polyacrylamide PhastGel by using a PhastSystem (Pharmacia). Proteins were transferred to a nitrocellulose filter (BA 85; Schleicher & Schuell, Dassel, Germany) by PhastTransfer (Pharmacia). AddAB production was monitored with the specific antibodies directed against the AddA protein; this was followed by visualization of the antigen-antibody complexes by using alkaline phosphatase anti-rabbit immunoglobulin G conjugates (Protoblot Western Blot AP System; Promega, Madison, Mich.).

SDS-polyacrylamide gel electrophoresis. Protein samples were prepared and run on a homogeneous SDS–7.5% polyacrylamide PhastGel by using a PhastSystem (Pharmacia) as recommended by the supplier. After electrophoresis, the SDS-polyacrylamide gels were silver stained as recommended by Pharmacia.

Isolation of DNA. *B. subtilis* chromosomal DNA was purified as described by Venema et al. (40). Plasmid DNA was isolated by the method of Ish-Horowicz and Burke (21).

TABLE 1. Strains and plasmids

Strain or plasmid	Description and characteristics[a]	Source or reference
Strains		
B. subtilis		
2G8	*tyr-1 thy*	Laboratory collection
8GK01	*addAB* (Km^r) *amyE*::pIN5 (Tc^r)	17
8GK02	*addAB* (Km^r) *amyE*::pIN88 (Tc^r)	17
8GK03	*addAB* (Km^r) *amyE*::pIN881 (Tc^r)	17
8GK04	*addAB* (Km^r) *amyE*::pIN882 (Tc^r)	17
8GE03	*addAB* (Em^r) *amyE*::pIN881 (Tc^r)	This work
8GE04	*addAB* (Em^r) *amyE*::pIN882 (Tc^r)	This work
8GE03M	8GE03 derivative carrying multiple copies of pSPT1989K; amplification by using Km^r	This work
8GE04M	8GE04 derivative carrying multiple copies of pSPT1990K; amplification by using Km^r	This work
8GK003	8G5 derivative carrying multiple copies of pSPT1988K; amplification by using Km^r	30
E. coli		
MC1061	F^- *araD139* del(*ara-leu*)*7696 galE15 galK16* del(*lac*)*X74 rpsL* (Str^r) *hsdR2* (r_K^- m_K^+) *mcrA mcrB1*	44
Plasmids		
pIN881	Ap^r Tc^r, pIN5 derivative containing *addA*B* genes	17
pIN882	Ap^r Tc^r, pIN5 derivative containing *addAB** genes	17
pD2	Ap^r pMTL23 derivative, contains flanking regions of the *addAB* genes	17
pDE2	Ap^r Em^r, pD2 derivative	This work
pUC18	High-copy number *E. coli* vector, Ap^r	46
pSPT1988K	*E. coli* replicon carrying 8.9-kb *Sal*I-*Sma*I *addAB* fragment from *B. subtilis*, Ap^r Km^r	30
pSPT1989K	pSPT1988K derivative carrying 8.9-kb *Sal*I-*Sma*I *addA*B* fragment, Ap^r Km^r	This work
pSPT1990K	pSPT1988K derivative carrying 8.9-kb *Sal*I-*Sma*I *addAB** fragment, Ap^r Km^r	This work

[a] The colons represent disruptions by insertion of plasmid pIN5 or a pIN5 derivative. Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Em^r, erythromycin resistance.

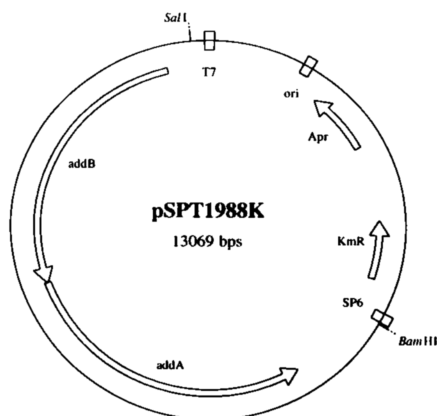

FIG. 2. Plasmid pSPT1988K. pSPT1988K consists of the entire plasmid pSPT18 (Pharmacia) into which the *addAB* genes and the kanamycin marker of *Streptococcus faecalis* were introduced (30).

Nuclease measurements. Nuclease measurements were essentially carried out as described by Kooistra et al. (24). Nuclease reactions contained 0.1 M glycine-NaOH buffer (pH 9.2), 2.5 mM β-mercaptoethanol, 0.05 M $MgCl_2$, 0.4 mg of bovine serum albumin per ml, ds chromosomal [^{3}H]DNA and AddAB enzyme. The ^{3}H-labeled DNA (1 μg/μl; specific activity, 10^5 cpm/μg) was isolated from *B. subtilis* 2G8. All components were mixed and placed in a 37°C water bath. Zero-time aliquots (50 μl) were removed and quenched with 30 μl of calf thymus DNA solution (2 mg/ml) and subsequent addition of 23 μl of trichloroacetic acid (50% vol/vol). ATP was added to initiate the reaction, and then aliquots (50 μl) were removed at various times and quenched with 30 μl of ice-cold calf thymus DNA solution (2 mg/ml). Subsequently, 23 μl of ice-cold trichloroacetic acid (50% wt/vol) was added. The quenched aliquots were left on ice for 30 min and then centrifuged for 15 min at 14,000 rpm in an Eppendorf centrifuge model 5417. The supernatant (100 μl) was mixed with 2 ml of Hydroluma scintillation fluid (Packard Instrument Company, Meriden, Conn.). Radioactivity was measured for 5 min. The concentration of acid-soluble DNA in the aliquots was calculated from the known specific radioactivity of the [^{3}H]DNA. Reactions with denatured *B. subtilis* [^{3}H]DNA (ssDNA) were done in exactly the same way as those with dsDNA. The ATP or [γ-S]ATP concentration was 75 μM, and the enzyme concentration was 20 nM. DNA was denatured by heating for at least 5 min at 100°C and chilling in ice water. The DNA was rendered completely sensitive to S1 nuclease digestion by this treatment.

Helicase activity measurements. The reaction mixture for the determination of the helicase activity consisted of 20 mM Tris (pH 7.0), 0.2 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, SSB (5 μg/ml) (USB, Cleveland, Ohio), ds chromosomal ^{3}H-labeled DNA (1 μg/μl; specific activity, 10^5 cpm/μg), and AddAB enzyme. In these conditions the AddAB enzyme did not show ATP-dependent exonuclease activity (16). All components were mixed and placed in a 37°C water bath. Zero-time aliquots (50 μl) were removed and placed at 65°C for 10 min, at which temperature the enzyme was fully inactivated. The reaction was started by the addition of ATP, and aliquots (50 μl) were removed at various times and placed for 10 min at 65°C. After incubation at 65°C, the reaction mixtures were placed at 37°C, and 200 μl of an exonuclease 1 buffer, consisting of 67 mM glycin-NaOH buffer (pH 9.5), 10 mM 2-mercaptoethanol and 6.7 mM $MgCl_2$, and 3 U of exonuclease I (USB) was added. After incubation for 30 min at 37°C, the samples were chilled on ice, and then 200 μl of a calf thymus DNA solution (2 mg/ml) and 300 μl of trichloroacetic acid (15% wt/vol) were added. The samples were left on ice for 15 min and then centrifuged for 10 min at 12,000 cpm in an Eppendorf centrifuge model 5417. The supernatant (0.75 ml) was added to 5 ml of scintillation fluid, and the radioactivity was counted for 5 min. The concentration of acid-soluble DNA in the aliquots was calculated from the known specific radioactivity of the ^{3}H-labeled DNA.

ATPase activity measurements. Production of liberated P_i was determined by a colorimetric method with malachite green molybdate reagent (10). The reaction mixtures contained 20 mM Tris (pH 7.0), 0.2 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, DNA, and AddAB enzyme. The mixture was placed in a 37°C water bath. ATP was then added to initiate the reaction, and aliquots (50 μl) were removed at various times. The aliquots were mixed with 800 μl of malachite green molybdate reagent and incubated for 5 min at room temperature. After 5 min, 100 μl of 34% (wt/vol) citric acid was added; this was followed by a 40-min incubation at room temperature. The A_{660} was measured, and the amount of P_i liberated was calculated from a calibration curve (0 to 30 nmol of P_i per ml).

The reagent was prepared as follows: 340 mg of malachite green reagent (Sigma) was dissolved in 75 ml of doubly distilled water and mixed with a solution of ammonium molybdate (10.5 g) dissolved in 250 ml of 4 N HCl. The solution was made up to 1 liter in water and allowed to clarify on ice for at least 1 h, and it was then filtered through Whatman no. 3 filter paper.

The ssDNA used as a substrate in ATPase measurements was prepared as described in the section "Nuclease measurements."

RESULTS

AddAB overproduction. The highly conserved lysines of motif I of the subunits AddA and AddB of the AddAB enzyme were changed into glycines (see Materials and Methods), resulting in the mutant enzymes AddA*B and AddAB*, respectively. The wild-type enzyme and both mutant enzymes were overproduced by gene amplification. The overproduction of the wild-type AddAB and both mutant enzymes was monitored by Western blotting with specific antibodies directed against subunits AddA and AddB. Strong AddAB-specific signals were observed in the crude extracts of 8GK2003 (AddAB), 8GE03M (AddA*B), and 8GE04M (AddAB*), compared with the weak signals observed in the crude extract of 8GK02, which carries one copy of the *addAB* wild-type genes. These results indicate that the AddAB enzymes are overproduced.

To determine the level of overproduction, the ATP-dependent exonuclease activity on ^{3}H-labeled ds DNA was measured. As was expected, no activity was observed in the crude extracts of the *addAB* mutant *B. subtilis* 8G01. The strain overproducing the wild-type AddAB enzyme showed a considerably higher activity (approximately 30-fold) than that observed for strain 8GK02, which carries one copy of the *addAB* genes.

Purification of the AddAB, AddA*B, and AddAB* enzymes. The wild-type enzyme was purified by successive DEAE-Sepharose, Mono Q, and heparin-agarose affinity chromatography (Table 2 and Fig. 3). Cell extracts (Fig. 3, lane 1) and fractions containing the highest ATP-dependent nuclease activities after DEAE-Sepharose (lane 2), Mono Q (lane 3), and heparin-agarose chromatography (lane 4), respectively, are shown in Fig. 3. The AddAB enzyme was purified to apparent homogeneity after heparin-agarose chromatography as judged by silver-stained SDS-polyacrylamide gels (lane 4, wild-type enzyme). The yield of the wild-type enzyme was approximately 900 μg (19% recovery), and a 36-fold purification was achieved (Table 2). The mutant enzymes, AddAB* and AddA*B, were purified by the same procedure (Fig. 3, lane 6, mutant AddA*B, and lane 7, AddAB*). The wild type and the two mutant AddAB enzymes behaved similarly during all steps of the purification procedure, suggesting that the mutations in subunits AddA and AddB did not drastically affect the overall structure of the AddAB enzymes.

Nuclease activities of the purified enzymes. The exonuclease activities of the purified wild-type AddAB enzyme are shown in Fig. 4. The AddAB enzyme showed a high exonuclease activity

TABLE 2. Purification of the *B. subtilis* AddAB enzyme

Fraction	Total protein (mg)	Total act [U (%)][a]	Sp act (U/mg of protein)	Purification (fold)
Crude extract	165	75 (100)	0.45	1.0
DEAE-Sepharose	52	40 (53)	0.77	1.7
Mono Q	19	25 (33)	1.32	3.0
Heparin-agarose	0.9	14 (19)	15.55	36.0

[a] The ATP-dependent exonuclease activity was determined and expressed as units, in which 1 U equals the amount of protein (in milligrams) that converts 1 nmol of DNA, expressed as nucleotides, to acid-soluble products in 10 min at 37°C. The reaction mixtures contained 3 nmol of ^{3}H-labeled chromosomal *B. subtilis* DNA expressed as nucleotide residues and 75 μM ATP. Other conditions and unit definitions are as described in Materials and Methods.

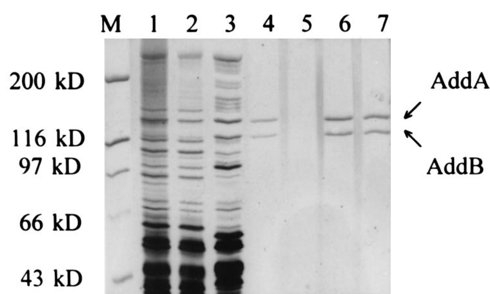

FIG. 3. SDS-polyacrylamide gel electrophoresis of protein fractions containing the highest ATP-dependent exonuclease activities. Samples (1 μl) were denatured by boiling for 5 min in SDS-gel loading dye and run on a 7.5% polyacrylamide gel containing SDS. After the run the gel was silver stained. Lane M, molecular mass markers; lane 1, crude extract of strain 8GK2003 (AddAB); lane 2, fraction (AddAB) after DEAE-Sepharose chromatography; lane 3, fraction (AddAB) after Mono Q chromatography; lane 4, fraction (AddAB) after heparin-agarose chromatography; lane 6, fraction (AddA*B) after heparin-agarose chromatography; and lane 7, fraction (AddAB*) after heparin-agarose chromatography.

on dsDNA as the substrate in the presence of ATP, but no activity at all in the absence of ATP and in the presence of the ATP analog [γ-S]ATP. These results indicate that the AddAB enzyme requires ATP hydrolysis to degrade DNA. Different results were obtained with respect to the exonuclease activity on ssDNA. High activity was again observed in the presence of ATP, but in contrast to the exonuclease activity on dsDNA, the enzyme also showed a low exonuclease activity in the absence of ATP and in the presence of [γ-S]ATP (Fig. 5B), indicating that the enzyme also has a low ATP-independent exonuclease activity on ssDNA.

The ATP-dependent exonuclease activities of the wild-type and the mutant enzymes, AddA*B and AddAB*, are shown in Fig. 4. The AddA*B mutant showed little ATP-dependent exonuclease activity on both dsDNA (Fig. 4A) and ssDNA (Fig. 4B), in contrast to the AddAB* mutant, which showed only slightly reduced exonuclease activities on both dsDNA and ssDNA.

The exonuclease activities of the enzymes on dsDNA showed similar dependencies on the ATP concentration (Fig. 5A). The rate of DNA degradation increased up to 20 μM ATP after which a small decrease was observed. At all ATP concentrations, the respective rates for the AddA*B and AddAB* mutants were approximately 30- and 2-fold smaller than that of the wild-type enzyme. The observation that the mutant enzymes had an ATP dependency similar to that of the wild-type enzyme suggests that the reduction in the exonuclease reaction rates of the mutants is caused not by a reduction in the affinity of the mutant enzymes for ATP, but by a reduced ability to hydrolyze ATP.

DNA unwinding activities. The ATP-dependent DNA unwinding activities catalyzed by the wild-type and mutant enzymes are shown in Fig. 6. Similar to what was observed for its exonuclease activity, the DNA unwinding activity of the mutant AddA*B enzyme was strongly reduced, whereas the activity of the mutant AddAB* enzyme was only slightly less compared with that of the wild-type enzyme.

The ATP dependencies of the helicase activities of both the wild-type and mutant enzymes were similar (Fig. 5B): maximum rates for the wild-type and mutant enzymes were reached at 50 μM ATP. These results suggest, analogous to what was observed for the dsDNA exonuclease activity, that the mutations did not alter the apparent affinity for ATP. However, at all ATP concentrations tested, the respective rates for the AddA*B and AddAB* mutant enzymes were approximately 30- and 2-fold less than that of the wild-type enzyme. Therefore, the decrease of the maximum rate of DNA unwinding is probably caused by a reduced ability to hydrolyze ATP.

ATP hydrolysis catalyzed by AddAB, AddA*B, and AddAB*. The wild-type enzyme hydrolyzes ATP in the presence of linear dsDNA and ssDNA as substrates. The ATPase activity on dsDNA and ssDNA proceeds proportionally with time for at least 4 min (Fig. 7); this is in contrast to the helicase activity (Fig. 6), which proceeds proportionally with time for only sev-

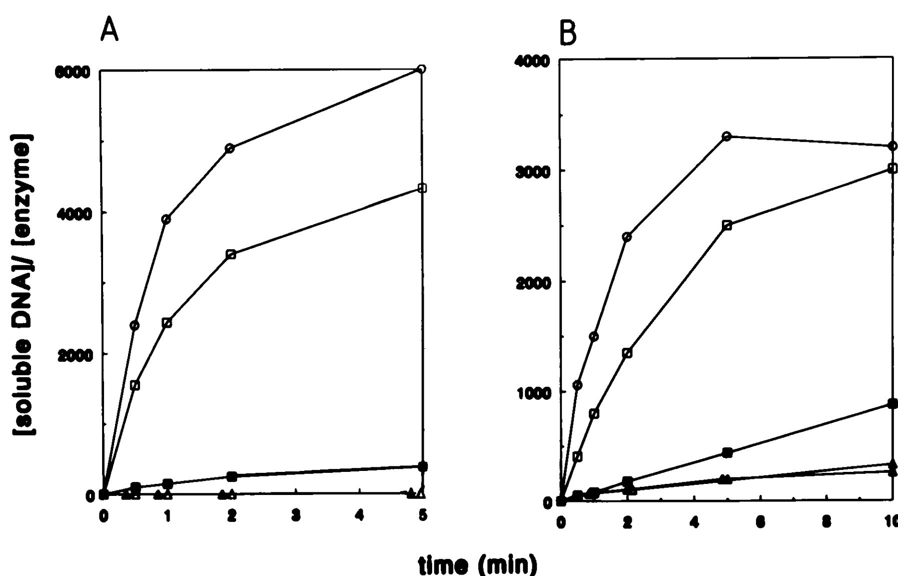

FIG. 4. ATP-dependent exonuclease activities of the AddAB, AddA*B, and AddAB* enzymes on dsDNA (A) and on ssDNA (B). Reaction mixtures contained 20 nM enzyme, ^{3}H-labeled DNA (273 μM nucleotides), and either AddAB and 75 μM ATP (○), AddAB and 0 mM ATP (△), AddAB and 75 μM [γ-S]ATP (▲), AddA*B and 75 μM ATP (■), or AddAB* and 75 μM ATP (□). Other conditions were as described in Materials and Methods.

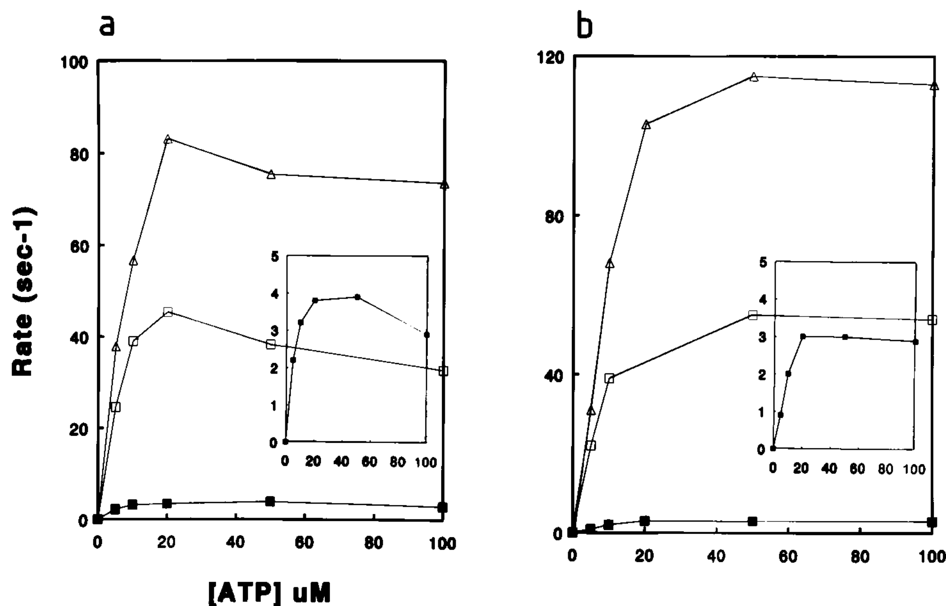

FIG. 5. ATP-dependent exonuclease reaction rates (A) and DNA unwinding rates (B) at various concentrations of ATP catalyzed by the AddAB (△), AddA*B (■), and AddAB* (□) enzymes. The reaction mixtures contained ^{3}H-labeled DNA (273 μM nucleotides) and 20 nM enzyme. The initial rates were determined as follows: after 0, 5, 10, and 20 s of incubation, the exonuclease and unwinding activities were determined. From the time curves, the initial rates were estimated. Other conditions were as described in Materials and Methods. Inset, ATP-dependent exonuclease rates (A) and DNA unwinding rates (B) at various concentrations of ATP catalyzed by the AddA*B enzyme.

eral seconds, indicating that the enzyme hydrolyzed more ATP than was required for the helicase activity. No ATPase activity was observed when covaletly closed circular DNA was used as the substrate (data not shown) or in the absence of DNA (Fig. 7).

The ATP hydrolysis kinetic constants $K_{m(\mathrm{ATP})}$ and k_{cat} were determined for the wild-type and the mutant enzymes with dsDNA (pUC18 DNA, linearized with *Eco*RI restriction endonuclease, 24 nM DNA ends) as the substrate. At this DNA concentration, a maximal rate of ATP hydrolysis was reached, both for the wild-type and for the mutant AddAB enzymes (data not shown). These results indicate that the lysine-to-glycine mutations did not affect the apparent affinity for DNA [$K_{m(\mathrm{DNA})}$]. The concentration of 24 nM DNA ends corresponded to approximately one molecule of AddAB bound at each DNA end, indicating that the affinity of AddAB for DNA is high, although still 10- and 50-fold lower, respectively, as found for the *E. coli* (39) and *H. influenzae* (45) RecBCD enzymes.

The respective k_{cat} values of AddA*B and AddAB* were approximately 5 and 60% of that of the wild-type enzyme (Fig. 8A and Table 3). The mutant enzymes showed similarly reduced rates for the exonuclease and DNA unwinding activities. No significant change in the apparent affinity for ATP [$K_{m(\mathrm{ATP})}$] was detected for either mutant enzyme compared with the wild-type AddAB enzyme (Fig. 8A and Table 3). These results indicate that Lys-36 of subunit AddA is involved in the ATP hydrolysis reaction catalyzed by AddAB and is apparently not essential for the binding of ATP. Remarkably, the apparent affinity for ATP [$K_{m(\mathrm{ATP})} \approx 1$ mM ATP] (Fig. 8A and Table 3) observed for ATP hydrolysis is considerably less than the apparent affinity for ATP in DNA unwinding (<50 μM ATP) as deduced from Figure 5B.

The kinetic constants $K_{m(\mathrm{ATP})}$ and k_{cat} for ATP hydrolysis of the wild-type and the mutant enzymes with ssDNA (170 μM nucleotide residues of ss chromosomal DNA) as a substrate were also determined. At this DNA concentration, a maximal rate for ATP hydrolysis was reached, both for the wild-type and the mutant enzymes (data not shown). The $K_{m(\mathrm{ATP})}$ values for the ATPase activity with ssDNA as the substrate were similar ($\approx$ 1 mM ATP) for all three proteins (Fig. 8B and Table 3), indicating that the binding of ATP by the mutant enzymes had not changed. The $K_{m(\mathrm{ATP})}$ values are similar to those for ATP hydrolysis with dsDNA as the substrate. The respective k_{cat} values of AddA*B and AddAB* were approximately 4 and 31% of that of the wild-type enzyme (Fig. 8B and Table 3). Similarly reduced values for the mutant enzymes

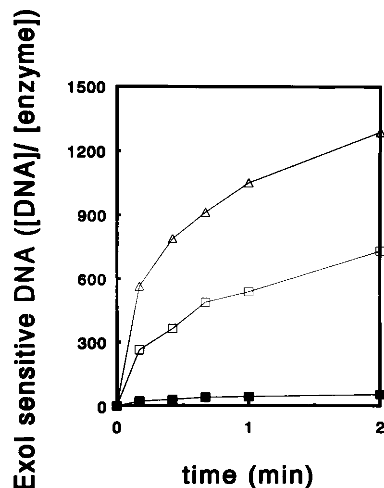

FIG. 6. DNA unwinding catalyzed by the AddAB (△), AddA*B (■), and AddAB* (□) enzymes. The reaction mixtures contained ^{3}H-labeled DNA (273 μM nucleotides), 20 nM enzyme, and 1 mM ATP. Other conditions were as described in Materials and Methods.

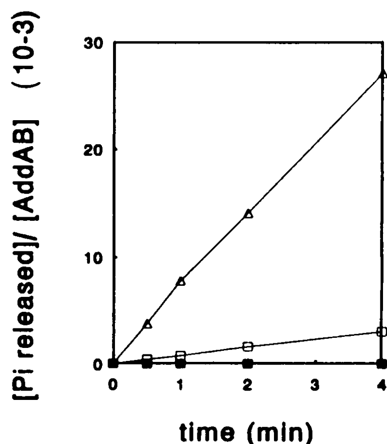

FIG. 7. ATP hydrolysis catalyzed by the AddAB enzyme (10 nM). The reaction mixtures contained 1 mM ATP and dsDNA (linear pUC18 DNA, 24 nM DNA ends) (△), ssDNA (heat-denatured chromosomal DNA, 170 μM nucleotides) (□), or no DNA (■). Other conditions were as described in Materials and Methods.

were observed with dsDNA as a substrate. These results again indicate that the lysine-to-glycine substitutions in motif I of AddA and AddB affected ATP hydrolysis but not ATP binding.

DISCUSSION

The *B. subtilis* AddAB enzyme, involved in DNA repair and homologous recombination (23, 24), consists of two subunits, AddA and AddB, and each subunit contains an N-terminal region, designated motif I (23), which is thought to be involved in ATP binding and/or hydrolysis. To better understand the functional significance of the motif I regions in subunits AddA and AddB with respect to the ATPase, helicase, and exonuclease activities of the enzyme, the highly conserved lysines of motif I of both subunits were replaced with glycines.

The wild-type AddAB enzyme is a potent ATP-dependent exonuclease on dsDNA (9) and ssDNA, but only at a high pH (9.2). At conditions of physiological pH (7.5) and ATP concentration (1 mM), no activity was observed (data not shown). This is in contrast to the *E. coli* RecBCD enzyme, which is highly active in these conditions (32, 33). Possibly, the exonuclease activity of the AddAB enzyme is absent in these conditions. Alternatively, the exonuclease activity under physiological conditions may be overlooked because of the method used, since the activity was measured as the amount of DNA degraded to acid-soluble products. DNA fragments released by the exonuclease activity under these conditions that exceed 15 to 25 bp would not be detected by this method of measurement.

In addition to a high ATP-dependent activity, a low ATP-independent exonuclease activity was also observed with ssDNA as the substrate, although a contaminating activity in the enzyme preparation cannot be excluded. This ATP-independent activity has not been observed for the *E. coli* RecBCD enzyme (15).

The lysine-to-glycine substitution in subunit AddB resulted in an enzyme which had relatively high levels of ATPase, helicase, and exonuclease activities. Therefore, the role of the lysine in the motif I region of AddB in these activities appears to be limited. Also, in vivo, this mutation had little effect on DNA repair and homologous recombination (17). Therefore, the functional significance of the lysine in motif I of subunit AddB remains unclear. AddB might bind ATP as a regulatory or allosteric effector of the activity of the holoenzyme by making the enzyme more efficient or more processive. Such an auxiliary function has been observed for some ATP-binding subunits such as those of the *E. coli* DNA polymerase III (29), the bacteriophage T4 DNA polymerase complex (7), and the *E. coli* RecBCD enzyme (27). A lysine-to-glutamine substitu-

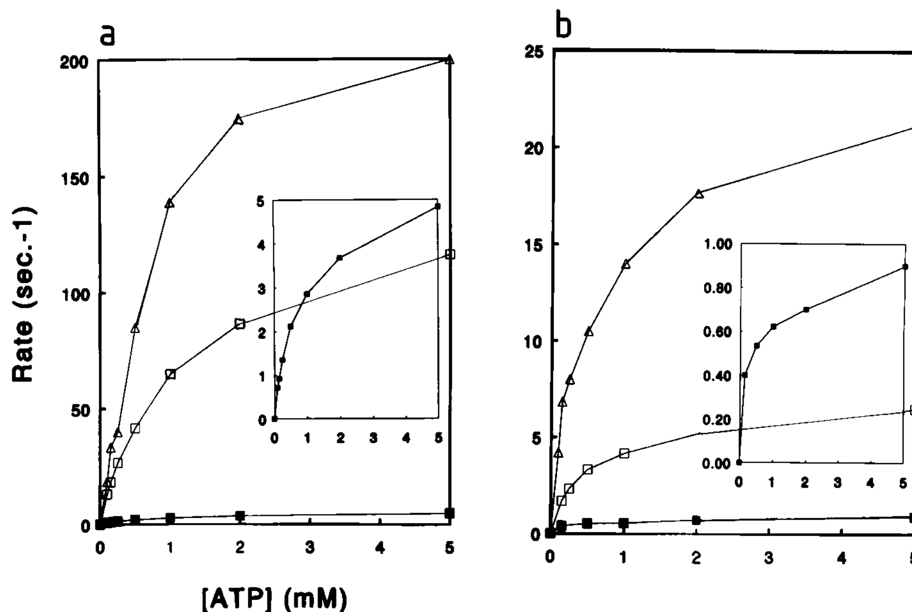

FIG. 8. ATPase reaction rates with dsDNA (A) or ssDNA (B) as a substrate at various concentrations of ATP catalyzed by the AddAB (△), AddA*B (■), and AddAB* (□) enzymes. The reaction mixtures contained 10 nM enzyme and, as a dsDNA substrate, *Eco*RI-linearized pUC18 DNA, 24 nM DNA ends, or, as a ssDNA substrate, heat-denatured chromosomal *B. subtilis* DNA (170 μM nucleotides). The results are the averages of at least three independent determinations. Other conditions were as described in Materials and Methods. Inset, ATPase reaction rates with dsDNA (A) or ssDNA (B) as a substrate at various concentrations of ATP catalyzed by the AddA*B enzyme.

TABLE 3. The kinetic constants k_{cat} and K_m for DNA-dependent ATP hydrolysis catalyzed by AddAB, AddA*B, and AddAB*[a]

Enzyme	k_{cat} (s^{-1})		K_m (mM)	
	dsDNA substrate	ssDNA substrate	dsDNA substrate	ssDNA substrate
AddAB	247 ± 13	26 ± 0.7	0.93 ± 0.13	1.1 ± 0.16
AddA*B	14 ± 1	1 ± 0.1	0.84 ± 0.08	0.8 ± 0.05
AddAB*	143 ± 4	8 ± 0.5	1.19 ± 0.09	1.1 ± 0.16

[a] The k_{cat} and K_m values were derived from the double reciprocal plots presented in Fig. 8. *Eco*RI-linearized pUC18 DNA and heat-denatured *B. subtilis* chromosomal ssDNA were used as dsDNA and ssDNA substrates, respectively (see legend to Fig. 8 for details).

tion in the motif I region of subunit D (25) reduced the processivity of the RecBCD enzyme (27), whereas the enzymatic activities were only slightly reduced (26).

The lysine-to-glycine substitution in subunit AddA reduced the exonuclease activities on dsDNA and ssDNA and the helicase and ATPase activities to comparable degrees, suggesting that the motif I region of subunit AddA, in contrast to the motif I region of subunit AddB, is essential for the ATP-binding and/or hydrolysis to promote the helicase and the exonuclease activities of the AddAB enzyme. The reduced exonuclease, helicase, and ATPase rates of the AddA*B mutant appear to be due to a reduced ability to hydrolyze ATP and not to a reduced ability to bind ATP, because the ATP dependencies of the wild-type and mutant AddA*B enzyme were not altered, whereas the maximal rates of the mutant enzyme for the activities tested were strongly reduced. A similar mutation was introduced into subunit B of the *E. coli* RecBCD enzyme (19), the functional analog of AddAB. The effects of this mutation corresponded to those of the mutation in the AddA subunit of the AddAB enzyme. The lysine-to-glutamine mutation in subunit B of RecBCD also resulted in highly reduced enzymatic activities due to a lower rate of ATP hydrolysis, whereas the apparent affinity for ATP appeared to be unchanged. However, one important difference was observed: the RecBCD mutant still showed approximately wild-type levels of ATP-dependent exonuclease activity on ssDNA (19), whereas the mutation in the AddA subunit resulted in almost complete loss of this activity, indicating that ATP hydrolysis by subunit AddA is also required for the ATP-dependent exonuclease activity on ssDNA.

The helicase and exonuclease activities of AddAB were maximal at ATP concentrations of approximately 50 μM and higher, as estimated from Fig. 5, although the reaction conditions of both activities were different. The apparent affinity for ATP in ATP hydrolysis with dsDNA or ssDNA as the substrate was considerably lower ($K_m \approx 1$ mM ATP) than that observed for the helicase activity. This, together with the observation that ATPase activity, with dsDNA or ssDNA as a substrate, is proportional with time for at least 4 min, whereas the helicase activity is proportional with time for only several seconds, suggests that, after a certain time, the ATP hydrolysis is not involved in DNA unwinding. The additional hydrolysis of ATP may be required for an unknown enzymatic property of the AddAB enzyme. The similar apparent affinity for ATP in ATP hydrolysis with dsDNA or ssDNA as a cosubstrate suggests that this unknown property involves a reaction with ssDNA. Conceivably, after DNA unwinding, the AddAB enzyme remains attached to the ssDNA to keep it in a conformation suitable for further processing by the recombination machinery.

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