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Defect in General Priming Conferred by Linker Region Mutants of *Escherichia coli dnaB*

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The *dnaB* gene of *Escherichia coli* encodes a bifunctional primase accessory protein/helicase necessary for chromosomal replication. Monomers of DnaB comprise two trypsin-resistant domains connected by a 45-amino-acid linker. To investigate the role of the linker in the structure and function of DnaB, we have purified and characterized three DnaB mutant proteins having single amino acid substitutions in the linker. We find that the mutant proteins retain the two-domain structure and assemble into hexamers that may be less stable than hexamers formed by wild-type DnaB. These mutant hexamers have hydrodynamic properties slightly different from those of the wild type, suggestive of a more open structure. The mutant proteins had reduced or absent ability to stimulate primase and also exhibited slight alterations in ATPase activity compared with the wild type. We conclude that the linker region promotes primase-DnaB interaction, but this effect may be indirect. We propose a model involving repositioning of N-terminal domains to explain the properties of the mutant proteins.

The *dnaB* genes of *Escherichia coli* and *Salmonella typhimurium* encode a helicase essential for chromosomal replication (12). This helicase activity provides template DNA strands that are used by the trailing DNA polymerase III for semiconservative and semidiscontinuous replication (6, 16). While DnaB translocates in a 5′ to 3′ direction on the lagging strand, it intermittently stimulates primase to synthesize RNA primers that will be extended into Okazaki fragments (1). Early experiments suggested that DnaB induces a template secondary structure that attracts primase (3). Recent reports, however, have demonstrated that primase can prime on unstructured oligonucleotides and also have provided indirect evidence for a primase-DnaB protein interaction (33–35).

The DnaB protein monomer is composed of 470 amino acids and has a molecular weight of 52,000 (22). The active native form is thought to be a hexamer (5, 25). Partial trypsin digestion produces two domains from native DnaB, an N-terminal domain (residues 15 to 125 or 15 to 127) and a C-terminal domain (residues 170 to 470) (23). The isolated C-terminal domain contains the site for ATP binding and hydrolysis and is the location of an amino acid substitution that eliminates detectable hydrolysis (7, 23, 29). Other activities of DnaB, including primase stimulation, are lost upon trypsin digestion. Therefore, their determinants within DnaB are unknown. In this report, we have studied mutations of DnaB that interfere with chromosomal replication when expressed in wild-type cells (21). Such genetic dominance suggested to us that the mutant proteins would be amenable to biochemical characterization because they would have an intrinsic defect and yet would have structures sufficiently similar to wild-type structures to interact with (and thus disrupt or sequester) some other components of replication. Such biochemical characterization is necessary because the genetic characterization of these mutants provided no clue as to the specific nature of their defect.

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MATERIALS AND METHODS

Materials. Materials were obtained as follows: chromatography medium from Pharmacia, DNA modification enzymes from New England Biolabs, ultrapure Na_2-nucleotides from Pharmacia, MgATP from Sigma, bacterial media from Difco, sequencing-grade trypsin from Sigma, polyvinylidene difluoride membranes from Bio-Rad, isopropyl-1-thio-β-D-galactopyranoside (IPTG) from United States Biochemical, and DE82 paper from Whatman. All other materials were from commercial suppliers and were the finest available.

Buffers. Lysis buffer was 50 mM Tris-HCl (pH 7.5), 2 mM Na_2EDTA, and 10% (wt/vol) sucrose. Buffer A contained 50 mM Tris-HCl (pH 7.5), 30 mM KCl, 5 mM $MgCl_2$, 25% (vol/vol) glycerol, and 1 mM Na_2ATP. Buffer B is buffer A with Na_2ATP omitted. Buffer C was composed of 50 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM Na_2ATP, 20% (vol/vol) glycerol, and 200 mM KCl. Primase buffer (26) contained 50 mM Tris-Cl (pH 7.5), 20% (vol/vol) glycerol, 1 mM Na_2EDTA, and 1 mM dithiothreitol.

Cloning and expression. The wild-type and mutant *dnaB* genes were expressed, respectively, in pET-3D and a pET-11D modified to contain an M13 origin of plus-strand synthesis (32, 40). The mutant plasmids were sequenced across *dnaB* in its entirety to ensure that no other changes were present. For expression, the plasmids were transferred to *E. coli* BL21/λDE3/pLysS (32) (for wild type) or the same strain without pLysS (for each mutant).

The expression strains were grown with shaking at 37°C in M9-TB broth (32) with ampicillin (100 μg/ml) to an optical density at 600 nm of approximately 0.7. IPTG was added to 0.4 mM to induce expression, and incubation was continued for 4 h. Next, the cells were collected by centrifugation at 4°C, washed in 50 mM Tris-Cl (pH 7.5), resuspended in lysis buffer (about 4 to 5 ml per g of cell paste), and quick-frozen in ethanol-dry ice.

Purification of DnaB, IN135, IT141, and LP156. DnaB and the linker mutants were purified identically. All steps were carried out at 4°C. The cell pellets were thawed, and then lysozyme was added to 0.2 mg/ml. After 45 min, the lysate was centrifuged at 86,000 × *g* for 1 h. Solid ammonium sulfate was gradually added to the supernatant, with stirring, to 20% saturation. After an additional 30 min of slow stirring, the suspension was centrifuged at 12,000 × *g* for 30 min. This supernatant was similarly adjusted to 40% saturation with ammonium sulfate and stirred slowly for an additional 30 min. The suspension was centrifuged at 12,000 × *g* for 30 min. The pellet was resuspended in a minimum volume of buffer A. The resuspended pellet was desalted over a Sephadex G-25 column previously equilibrated in buffer A. The protein peak was directly applied to a DEAE-Sephacel column (0.7 by 7 cm) equilibrated in buffer A. The column was loaded and run at 4 ml/h. After the sample was loaded, the column was washed to background (280 nm) with 190 mM KCl in buffer A and then with 240 mM KCl in buffer A. DnaB or the mutant protein was eluted in a 30-ml linear gradient from 240 to 500 mM KCl in buffer A. Aliquots from each column fraction were run on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (14). Fractions containing DnaB or mutant protein were pooled, precipitated with 60% ammonium sulfate, and pelleted for 30 min at 12,000 × *g*. To obtain highly pure protein free of nucleic acid, it was necessary to perform further purification in the absence of ATP. Therefore, the pellet was resuspended in a minimum volume of buffer B and desalted in buffer B as described above. The desalted

protein fraction was applied to a Mono-Q column equilibrated in buffer B. The Mono-Q column was washed with 10 volumes of 190 mM KCl in buffer B and then 240 mM KCl in buffer B. The protein was eluted with a linear gradient from 240 to 600 mM KCl in buffer B. Column fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and fractions containing DnaB or mutant were pooled and precipitated with 60% ammonium sulfate as described above. The pellet was resuspended in a minimum volume of buffer B and dialyzed against 4 liters of buffer B for at least 8 h. Following dialysis, the protein was divided into aliquots and stored at −80°C. The final protein yield was about 0.5 mg/liter of liquid culture for DnaB and approximately 0.1 mg/liter for IN135, IT141, and LP156.

Expression and purification of primase. *E. coli* BL21/λDE3 (32) containing plasmid pGNG1 (11) was grown in M9-TB broth containing ampicillin (100 μg/ml) to an optical density of 0.8 and then induced with 0.4 mM IPTG for 2 h. The cells were collected by centrifugation, resuspended in lysis buffer (about 4 to 5 ml per g of cell paste), and frozen in an ethanol-dry ice bath.

All purification steps were carried out at 4°C. The cell pellets were thawed, lysozyme was added to 1 mg/ml, and dithiothreitol was added to 1 mM. The lysate was slowly stirred for 45 min and then centrifuged for 30 min at 31,000 × *g*. Solid ammonium sulfate was added to the supernatant to 50% saturation with slow stirring. The suspension was stirred for an additional 30 min and then centrifuged for 30 min at 12,000 × *g*. The pellet was resuspended in a minimum volume of primase buffer and desalted over Sephadex G-25 equilibrated with primase buffer. The protein peak was applied to a DEAE column (0.7 by 6 cm) equilibrated in primase buffer. The column was washed to background at 2 ml/h. Protein was eluted with a linear gradient (0 to 150 mM NaCl in primase buffer). Fractions containing primase as judged by gel electrophoresis were pooled and precipitated with 60% ammonium sulfate. The pellet was desalted over Sephadex G-25 as described above and then applied to a Mono-Q column equilibrated in primase buffer. The column was washed until the absorbance was baseline, and then the protein was eluted with a 0 to 600 mM NaCl gradient in primase buffer. Fractions containing primase were pooled and precipitated with 60% ammonium sulfate as before. The pellet was resuspended and dialyzed for 10 h against 4 liters of primase buffer. The final yield of primase was approximately 6 mg/liter of broth culture.

Protein purity. DnaB was more than 95% pure with respect to protein contaminants, as judged by densitometry of SDS-polyacrylamide gels. Contamination by polynucleotides was checked by labeling 10 μg of protein preparation with 30 U of polynucleotide kinase and 10 μCi of [γ-^{32}P]ATP. The labeling reaction mixture was then applied to a denaturing 16% polyacrylamide gel. Some labeling reaction mixtures contained, in addition to protein, either 5 μg of an unlabeled 24-mer or 5 μg of unlabeled poly(dT) as a positive control. These molecules were readily labeled and detected on the gel. In some partially purified preparations of DnaB, discrete polynucleotide contaminants were detected; these were invariably removed by Mono-Q chromatography.

By the same techniques, primase was also determined to be greater than 95% pure and free of detectable polynucleotides.

Concentration determinations. The protein concentrations of DnaB, IN135, IT141, LP156, and primase reported throughout this work were determined by the Bradford method (8), using bovine serum albumin (BSA) as a standard. For DnaB, this value was 85% of the value determined by A_{280}, assuming a molar extinction coefficient of 1.85×10^5 cm^{-1} M^{-1} (hexamer) and a monomeric molecular weight of 52,000 (9). For primase, the concentration determined by the Bradford method was 97% of the UV absorbance value (E of 43,240 cm^{-1} M^{-1} and molecular weight of 65,500) (31).

The concentrations of double-stranded DNA and single-stranded DNA (ssDNA) were determined by A_{260}, assuming that 1 optical density unit equals 50 and 37 μg/ml, respectively (18).

Nucleotide concentrations were determined by A_{595}, using an extinction coefficient of 1.54×10^4 cm^{-1} M^{-1} (18).

Refractive index and gravimetric analysis were used to determine $MgCl_2$ concentration (37). The two methods gave identical values.

Trypsin digestion. Trypsin digestions were carried out in buffer B at 20°C in a total volume of 40 μl. The DnaB or mutant concentration was 1 mg/ml. The ratio of DnaB or mutant to trypsin was 50:1 (wt/wt). Nucleotides, when present, were at 5 mM. The reaction mixtures were assembled on ice in the absence of trypsin. They were then incubated at 20°C for 1 min to allow temperature equilibration and nucleotide binding. After 1 min, a 4-μl pseudo-zero aliquot was removed. The digestion was initiated by addition of trypsin. At 0.5, 5, and 60 min, aliquots of the reaction mixtures were removed and immediately quenched by heating for 2 min at 95°C in sample buffer.

N-terminal sequencing of peptide fragments. Fragments derived from trypsin digestion of DnaB and the linker mutants were N-terminally sequenced at the Case Western Reserve University Core Facility (17, 20).

Gel filtration chromatography. Gel filtration over a 25-ml Superose-6 column was carried out with a Pharmacia FPLC (fast protein liquid chromatography) system (5, 24). The flow rate in all determinations was 0.1 ml/min. The column was first equilibrated at 4°C in buffer C. The void volume was determined with blue dextran. The column was then calibrated by the method of Monty and Siegel (30) with the standards contained in a Pharmacia high-molecular-weight calibration kit. DnaB or linker mutants (100 μg in 100 μl) were applied to the column together with 30 μg of cytochrome *c*. The Stokes radius was calculated from the sample elution volume. The elution volume of the cytochrome C internal marker did not detectably vary from run to run.

Sedimentation analysis. DnaB or linker mutant (100 μg) was mixed with BSA (44 μg), cytochrome *c* (105 μg), β-galactosidase (44 μg), and catalase (44 μg) in a total volume of 100 μl. This mixture was applied to the top of a 5-ml linear 20 to 45% glycerol gradient composed in buffer C. The gradients were centrifuged for 16 h at 50,000 rpm in a Beckman SW50.1 rotor at 4°C. Fractions of 100 μl were collected from the bottom of the tube. Half of each fraction was analyzed by gel electrophoresis. The stained gels were scanned with a laser densitometer to determine the sedimentation positions of the sample and reference standards. The sedimentation coefficient of DnaB or linker mutants was calculated by interpolation (5, 24, 36).

ATPase assays. ATPase assays were conducted at 30°C in 50 mM Tris-Cl (pH 7.5)–8 mM $MgCl_2$–3 mM MgATP–[α-^{32}P]ATP (approximately 80 dpm/pmol) with or without DNA effector. Partially assembled reaction mixtures were prewarmed to 30°C for 1 min, and then reactions were initiated by addition of DnaB or mutant. Aliquots of 1.5 μl were removed at the indicated time points and spotted onto polyethyleneimine-cellulose thin-layer chromatography (TLC) plates. The TLC plates were developed in 1 M formic acid–0.5 M LiCl as previously described (2). The relative proportions of ATP and ADP at each time point were determined by radioanalytic scanning (Ambis, Inc.). Initial rates of hydrolysis of ATP to ADP plus P_i were determined by linear regression of plots of ADP formed versus time. ATP utilization was limited to 20% in all determinations in which specific activities are reported unless noted otherwise in a figure legend.

General priming. General priming assays were conducted as previously described (4). The assays were performed at 30°C in a total volume of 460 μl with these final concentrations: 28 μg of DnaB or mutant per ml, 12 μg of primase per ml, 20 mM Tris-Cl (pH 7.5), 4% (wt/vol) sucrose, 8 mM dithiothreitol, 40 μg of BSA per ml, 0.8 mM Na_2ATP, 8 mM $MgCl_2$, 0.16 mM poly(dT) as nucleotide, 4 μg of rifampin per ml, and about 40 μCi of [α-^{32}P]ATP (final specific activity, ca. 240 cpm/pmol). Partially assembled reaction mixtures were prewarmed to 30°C, and then reactions were initiated by addition of primase and then DnaB in rapid succession. Aliquots of 10 μl were withdrawn from the complete reaction mixture at the indicated time intervals, and the reaction was terminated by spotting onto DE81 paper. Additionally, 5-μl aliquots were withdrawn at the indicated time intervals, mixed with denaturing gel loading buffer, and placed on ice until they could be analyzed by gel electrophoresis to determine product lengths. The DE81 filter papers were washed four times with 0.3 M ammonium formate (pH 7.8)–10 mM sodium pyrophosphate (26). The DE81 papers were then washed once in 95% ethanol, allowed to dry, and counted in Ecoscint liquid scintillation fluid.

A second set of general priming assays was conducted as described above, with the following modifications: DnaB and mutant protein concentrations (150 μg/ml), primase (64 μg/ml), MgATP (3 mM) in place of Na_2ATP, poly(dT) (0.8 mM as nucleotide), and [α-^{32}P]ATP (50 cpm/pmol). The total volume of these assays was 1,400 μl. At the indicated time points, a 10-μl aliquot was removed from the reaction mixture and spotted onto DE81 paper, and a 1-μl aliquot from each reaction was spotted onto a polyethyleneimine-cellulose TLC plate. The DE81 filters were treated and counted as above to determine poly(A) synthesis. The TLC plates were treated as previously described for the ATPase assays.

RESULTS

The three mutant proteins that we studied have substitutions of asparagine in place of isoleucine at position 135 (IN135), threonine for isoleucine at position 141 (IT141), and proline for leucine at position 156 (LP156). These were the only linker region mutations identified in a study of dominant *dnaB* mutations (21). All three of these proteins were purified from overexpression strains to >95% purity (Fig. 1A, 0 min). The fractions used in experiments reported here were free of ATP and detectable polynucleotides.

Physical characterization of mutant proteins. We anticipated on the basis of genetic properties that the mutant proteins could have physical characteristics resembling those of the wild type, and this expectation was reinforced by the similar purification behaviors of the mutant proteins. We have characterized the proteins by partial trypsin digestion, gel filtration chromatography, and velocity sedimentation. The results of trypsin digestion (Fig. 1) indicate that the mutant proteins assume a two-domain structure similar to that of wild-type DnaB. This is shown by the production of a 33-kDa carboxyl-terminal fragment, fragment II, and a 12- to 14-kDa amino-terminal fragment, fragment III (Fig. 1A). The amino-terminal sequences of fragments II and III derived from IN135, IT141,

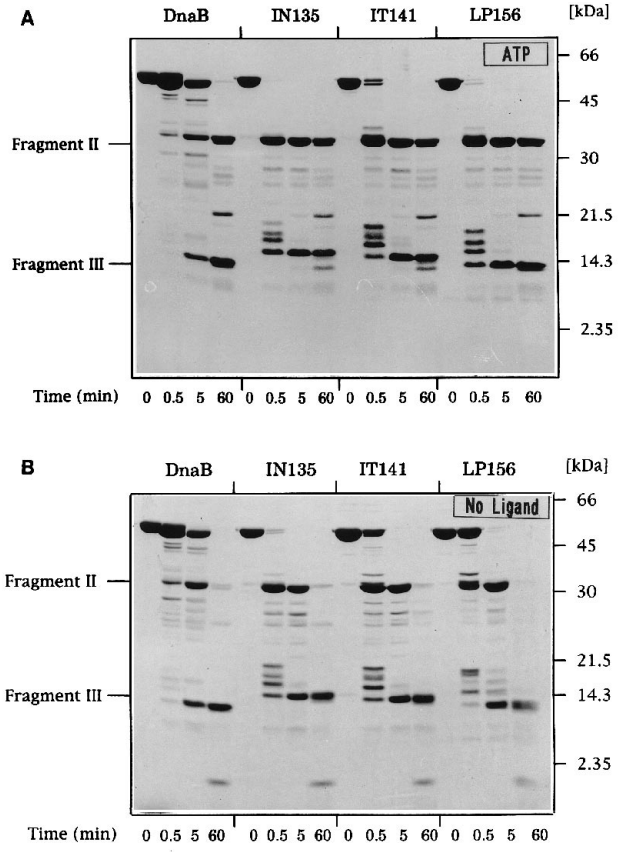

FIG. 1. Trypsin digestion of DnaB and the three linker mutants. ATP, when present, was at a concentration of 5 mM. Samples were analyzed by electrophoresis through a 10 to 18% polyacrylamide gradient gel containing SDS.

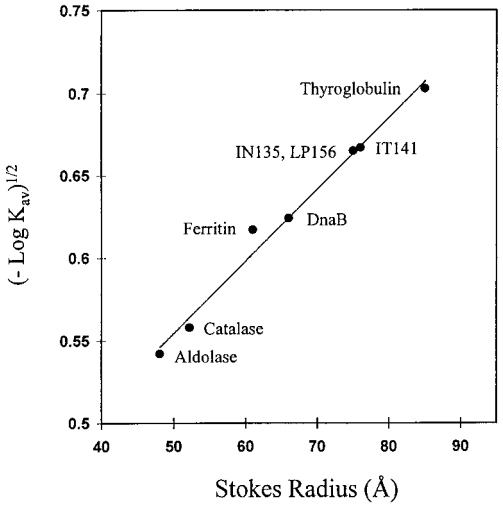

FIG. 2. Stokes radius determination for DnaB and the linker mutants. Protein elution volumes were determined by A_{260}. $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is protein elution volume, V_0 is the void volume as determined by elution of blue dextran, and V_t is total column bed volume (25 ml); 1 Å = 0.1 nm. The results of all determinations are summarized in Table 1.

and LP156 were determined and correspond exactly to the sequences of these fragments derived from wild-type DnaB. We noticed two differences between the mutant and wild-type digestion patterns. The first difference is that the mobility of fragment III varied slightly among the different proteins. Since all of the fragments III have identical N-terminal sequences, the mobility differences probably reflect different C-terminal sequences of the fragments III. The second difference between the mutants and wild type is that the production of fragment II from all three mutant proteins occurred much faster than that from the wild type (e.g., 30 s of digestion for the mutants degraded much more of the full-length protein than did 5 min for the wild type). Both of these differences between mutant and wild type suggest that the linker region of the mutants assumes an altered conformation that affects the trypsin accessibility to its cleavage targets.

Digestion in the presence of ADP or ATPγS produced results very similar to those with ATP (data not shown).

In the absence of nucleotide, the initial stages of digestion proceeded much as in the presence of nucleotide (Fig. 1B). With additional time, however, fragment II was nearly completely digested while fragment III remained trypsin resistant. These results indicate that as with wild-type DnaB, the carboxyl-terminal fragment of each mutant is protected against trypsin digestion by bound nucleotide. This result shows that the mutant proteins bind nucleotide, a conclusion that is confirmed by ATP hydrolysis (below).

Molecular weight determination. We used the method of Monty and Siegel (30) to estimate native molecular weights for wild-type DnaB and each mutant protein. In this method, the molecular weight is determined from independent measurements of Stokes radius (Fig. 2) and sedimentation coefficient (Fig. 3). The results of these measurements and molecular weight calculations, summarized in Table 1, suggest that the wild type and each mutant assume a hexameric form under the conditions of the sedimentation and gel filtration analysis.

ATPase activity. Wild-type DnaB exhibits a DNA-stimulated ATPase activity. The extent of stimulation ranges from 8- to 50-fold in various reports (2, 25, 36). We measured the ATPase activities of the mutant proteins in the presence or absence of ssDNA [M13 or poly(dT)] and compared the results with those for the wild type measured under the same conditions. In almost all experiments, ATP consumption was less than 20% of the initial ATP at time points used, and free magnesium was maintained at a level above that sufficient to stabilize wild-type DnaB hexamers (9, 36).

All of the mutant proteins, as well as the wild type, had readily detectable ATPase activity, yielding ADP and inorganic PO_4 (data not shown). We attribute this activity to DnaB, not to a contaminating protein, because the peak of activity coincided with the peak of DnaB protein (of wild type or mutant) during the gel filtration experiments used to determine the Stokes radius.

In an early study, ATP hydrolysis by wild-type DnaB exhibited a protein concentration-dependent lag phase below 9 μg of DnaB per ml (25). This lag was thought to reflect protein oligomer assembly. We analyzed the dependence of ATP hydrolysis on protein concentration for the three mutants as well as the wild type. In our experiments, hydrolysis was initiated by adding protein from a concentrated stock into the reaction cocktail. Not surprisingly, we obtained linear initial rates at all protein concentrations tested (10 μg/ml and above) for the mutants and wild type.

We did note some differences in the ATPase activity in the mutants compared with the wild type. Specifically, whereas the wild-type protein had a DNA-independent specific activity of ~530 pmol of ATP hydrolyzed per min per μg when measured at protein concentrations of 30 to 250 μg/ml, the mutant pro-

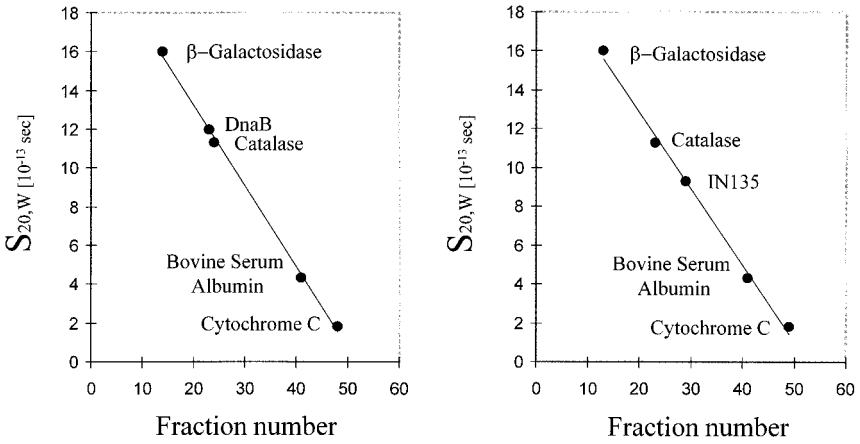

FIG. 3. Sedimentation analysis. The sedimentation positions for DnaB and the marker proteins were determined as described in Materials and Methods. The results of one determination for DnaB and IN135 are shown to highlight the change in sedimentation of the mutant relative to DnaB. The results of all determinations are presented in Table 1.

tein specific activity increased with increasing protein concentration over the same protein concentration range (Fig. 4).

The mutant proteins also differed with respect to stimulation by ssDNA. Whereas the wild-type ATPase was stimulated fourfold by M13 ssDNA under the conditions used, the mutants were not detectably stimulated by M13 ssDNA (data not shown). Both the mutants and the wild type were stimulated by poly(dT), but the stimulation of the mutants was less (Fig. 5).

Assay of primase stimulation. As reported previously, under specific conditions, primase is unable to synthesize RNA primers on a poly(dT) template except in the presence of DnaB (1, 4). Our preparations of wild-type DnaB and primase reproduce these observations. Control experiments containing only one protein demonstrate that neither our DnaB nor our primase is contaminated with other proteins (such as RNA polymerase) capable of poly(A) synthesis.

Compared with wild-type DnaB, the IT141 mutant exhibits impaired primase stimulation, and the other two mutants give undetectable primase stimulation (Fig. 6). These differences could reflect altered or absent binding of the mutant DnaB proteins to poly(dT), but the ability of poly(dT) to stimulate the ATPase activity of these mutant proteins challenges this explanation. Nonetheless, the standard reaction conditions for the ATPase and priming assays are rather different, leaving open the possibility that conclusions drawn from one assay are not applicable to the other.

To evaluate the role of DNA binding in the priming assays, we designed a modified set of conditions allowing simultaneous determination of ATPase and priming activity (coupled assay). The results of this assay show that even as poly(dT) stimulates ATP hydrolysis by IN135 and LP156, there is no detectable poly(A) synthesis on these templates. Therefore, it appears that IN135 and LP156 bind to poly(dT) but are then unable to interact, or interact productively, with primase (Fig. 7).

TABLE 1. Hydrodynamic properties of DnaB and the linker mutants[a]

Protein	Stokes radius (Å[b])	Sedimentation coefficient $s_{20,w}$	Calculated molecular weight[c] (10^3)	Inferred oligomeric form
DnaB	66 ± 1	11.9 ± 0.5	330	Hexamer
IN135	77 ± 2	9.1 ± 0.3	290	Hexamer
IT141	76 ± 2	9.8 ± 0.1	310	Hexamer
LP156	77 ± 1	9.5 ± 0.4	290	Hexamer
DnaB[d]	59–60	11.3–11.5	250–290	

[a] Determined from three separate protein preparations.
[b] 1 Å = 0.1 nm.
[c] Determined by using the formula $M = 6\pi\eta Nas/(1 - vq)$ (30), where η is solvent viscosity, N is Avogadro's number, a is Stokes radius, s is sedimentation coefficient, q is solvent density, and v is partial specific volume (0.73 ml/g) calculated from amino acid composition (10).
[d] Previously reported values determined with similar methods (5, 24, 36).

DISCUSSION

In this work, we sought to describe the biochemical defect(s) in three mutant DnaB proteins having alterations in the trypsin-sensitive linker region of DnaB. All three proteins were impaired in the ability to stimulate primase in general priming assays, while at the same time retaining their ability to carry out poly(dT)-stimulated ATPase activity. These are the first mutants described with this particular biochemical phenotype (see references 15, 19, 27, and 29 for other DnaB mutant characterizations). The results presented here, together with the characterization of a mutant profoundly impaired in ATP hydrolysis (29), make it clear that primase stimulation and ATP hydrolysis are independent activities of DnaB.

General priming is distributive; that is, each association of primase with a template complex leads to synthesis of a single primer, followed by dissociation (4). This dissociative mechanism is also thought to occur at the replication fork (38). Therefore, a key question is understanding how primase is attracted to the template. Primase alone at a low concentration is inactive on naked ssDNA molecules such as M13 or G4 viral DNAs. Priming by primase on such DNA molecules requires either single-strand-binding protein (SSB) in the case of G4 templates or DnaB in the case of M13 templates. It has long been uncertain whether such priming accessory proteins work by inducing a particular secondary structure in the template or by interacting directly with primase. Some of this uncertainty stemmed from the stabilization of specific hairpin structures in the replication origin of G4 DNA in the presence of SSB. The requirement for SSB at the G4 origin is now understood to reflect the necessity of preserving the primase recognition se-

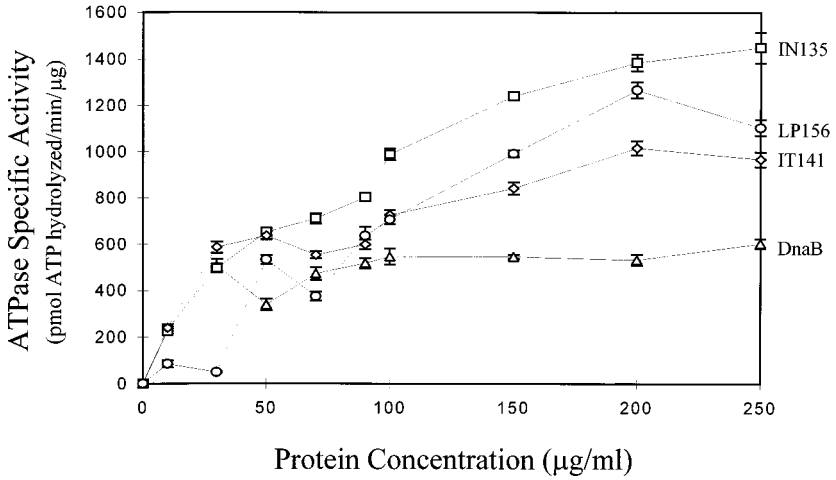

FIG. 4. The linker mutants display a protein concentration dependence in their DNA-independent ATPase activities. Protein at the indicated concentration was assayed for ATPase activity in the presence of 3 mM MgATP and absence of DNA effector. The velocity of ATP hydrolysis was determined from the slope of plots of ATP hydrolyzed versus time. These velocities were then converted to specific activity by taking into account protein concentration. The error was calculated from triplicate samples.

quence in an unstructured form by assimilating nearby sequences into hairpins (33). In support of this view, primase was found to be capable of synthesizing primers on unstructured oligonucleotides in the absence of additional proteins (34). In contrast, DnaB appears to stimulate primase through a protein-protein interaction. This has been studied most successfully with proteolytic domains of primase. In assays carried out with high levels of protein, a large N-terminal fragment of primase contained intrinsic (i.e., accessory-independent) priming activity that was about twice as active as intact primase. In contrast to intact primase, this N-terminal fragment was unable to participate in any DnaB-dependent priming reactions. The missing C-terminal portion, studied as a separate 16-kDa fragment, inhibited DnaB-dependent priming activities of intact primase. This inhibition was relieved by increasing the amount of primase (35). From this study, it was inferred that primase interacts with DnaB through the inhibitory C-terminal domain of primase. Thus, in the current view, an interaction between DnaB and primase attracts primase to DNA and activates it by sequestering the inhibitory domain.

This view of DnaB-assisted priming leads to a simple explanation for the impaired primase stimulation of the DnaB mutants studied here. Since the mutants can bind to poly(dT) (as indicated by ATPase assays), they appear to be impaired in their interaction with primase. This impairment could take the form of reduced (or absent) binding to primase or binding that is unproductive. Our results do not distinguish these possibilities; however, we prefer the former because it avoids the assumption of an unproductive mode of binding.

No reports published to date have identified a primase interaction site on DnaB. Neither of the major proteolytic fragments of DnaB can stimulate primase (23). It is tempting to take the data that we have presented as evidence that primase interacts with the linker region of DnaB. This may be the case, but it is not the only possible interpretation. The hydrodynamic measurements on the mutant DnaB proteins indicate that the overall shape of the hexamer is somewhat altered in the mutants compared with the wild type. Visualization of wild-type

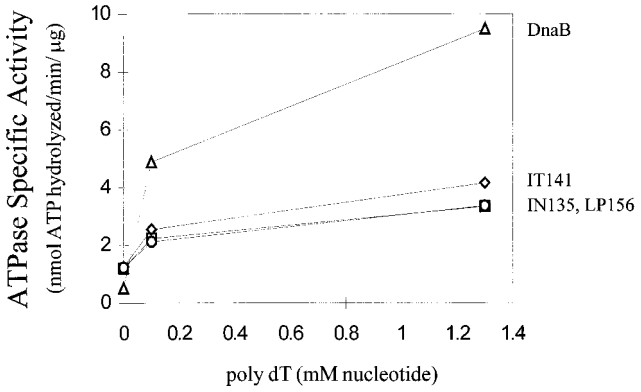

FIG. 5. The ATPase activities of the linker mutants and DnaB are stimulated by poly(dT). ATPase assays were done in the presence of 3 mM MgATP and the indicated concentrations of poly(dT). The specific activities were determined from the slope of plots of ATP hydrolyzed versus time that were corrected for protein concentration (150 μg/ml). The standard error was calculated from triplicate samples and was less than 10% for all points. The substrate utilization in the case of DnaB was limited to 40%.

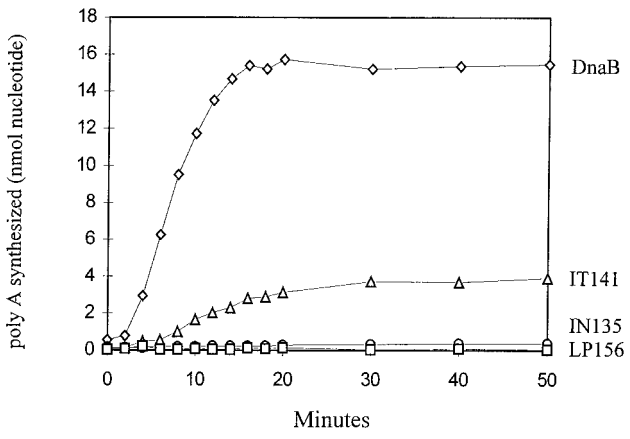

FIG. 6. General priming. Each point is the average of duplicate determinations. All values are normalized to the original volume of 460 μl.

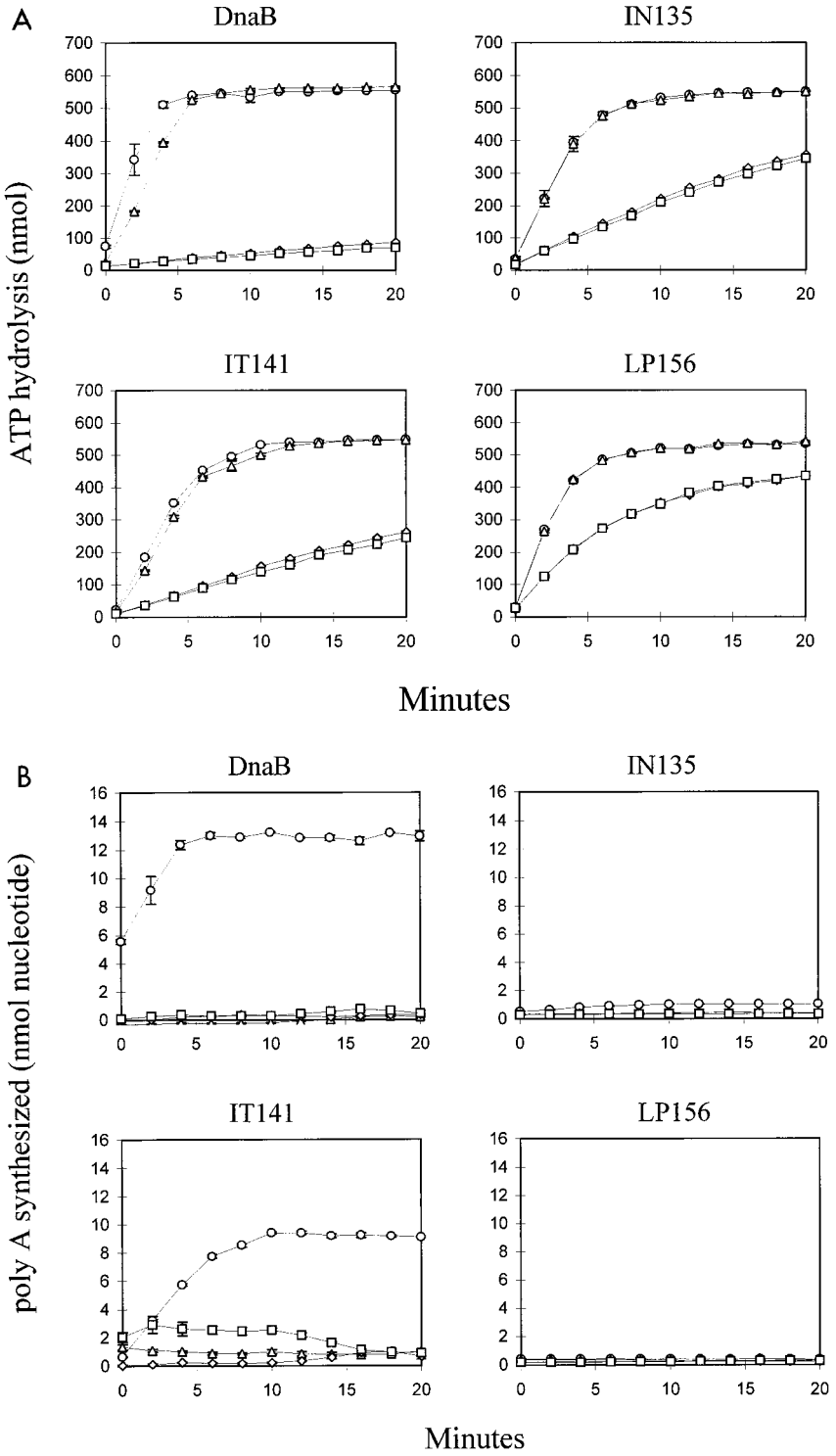

FIG. 7. ATPase and poly(A) synthesis (coupled ATPase and general priming assay). The complete reaction mixture contained DnaB (or mutant protein), primase, poly(dT), and reaction mix components (Materials and Methods). Circles, complete reaction; triangles, primase omitted; squares, poly(dT) omitted; diamonds, primase and poly(dT) omitted. The error was calculated from triplicate samples.

DnaB hexamers by electron microscopy has suggested a flattened triangular shape, constructed of a hexagon of C-terminal domains (each touching two neighbors) with three pairs of N-terminal domains forming the vertices of the triangle (28). Trypsin digestion studies indicate that the C-terminal domain of each mutant is structurally indistinguishable from that of the wild type. Thus, it is reasonable to propose that in the mutant hexamers, the hexagon formed by the C-terminal domains is intact and any abnormal folding of the linker region would be reflected in repositioning of the N-terminal domains, i.e., the

triangular vertices. Our hydrodynamic measurements are consistent with the suggestion that the relative positioning of the N-terminal domains is altered in the mutants. Such domain movements have the potential to block or reveal a primase interaction site located, in principle, anywhere in DnaB.

The experiments of Biswas et al. have implicated the N-terminal domain of DnaB in oligomerization, even though the isolated N-terminal domain behaves as a monomer (7). The oligomerization role of the N terminus is nicely accounted for in the structural proposal of San Martin et al., whereby N termini are seen to interact in pairs at the triangular vertices (28). One implication of the latter model is that domain repositioning, as we have proposed for the linker mutants, might be expected to disrupt the normal interaction of N-terminal domain pairs, thereby leading to increased instability of DnaB mutant hexamers compared with the wild type. We noted that for both wild-type and mutant proteins, the specific activity in the DNA-independent ATPase assay increased with increasing protein concentration. In the case of the wild type, the specific activity plateaued by 30 μg/ml, whereas for the mutants, we did not reach a clear plateau even though we tested concentrations as high as 250 μg/ml. Since an earlier study (25) suggested that hexamers are the most active form of DnaB (for ATPase), our results suggest that mutant DnaB hexamers are less stable than wild-type hexamers, consistent with the domain-repositioning model. This instability of hexamers may also contribute to the accelerated trypsin digestion of the mutant proteins if the preferential target is a lower oligomer of DnaB.

A specific version of the repositioning model may be suggested by the recent work of Yu et al. (39). Electron microscopy revealed not only DnaB hexamers displaying C3 symmetry like those reported by San Martin et al. (28) but also a large fraction (about 50%) of hexamers showing C6 symmetry. These C6 structures were related to the C3 structures by the movement of three N-terminal domains. The relationship of this observation to our results reported here remains uncertain.

An appealing aspect of the domain repositioning model is that almost any mutation that disrupts the proper folding of the linker would be expected to have a similar biochemical phenotype. The defects caused by the mutations that we have studied are remarkably similar, differing only in the degree of severity. Only one other linker region mutation has been studied thus far, a mutation from Ala to Val at position 130 (AV130 change) (27). In contrast to the mutations studied here, the AV130 change yields a temperature-sensitive phenotype in vivo and a thermolabile protein in vitro. At the permissive temperature, all tested functions of the AV130 mutant appear to be intact, whereas they are all severely reduced at the nonpermissive temperature. Because it is uncertain what happens to the overall structure of the AV130 mutant at the nonpermissive temperature, this mutant provides little insight into linker region function.

In addition to its role in stimulating primase action, DnaB is also a helicase driven by ATP hydrolysis. It is clear from our current and previous (29) results that ATP hydrolysis is not coupled to primase stimulation. The mutant proteins studied here exhibited changes in two characteristics of the ATPase activity: a higher DNA-independent activity compared with the wild type and a reduced ability to be stimulated by poly(dT). These features could be related. They could both be explained if the mutant protein assumes a conformation like that of the DNA-bound wild type. Alternatively, the DNA-independent hyperactivity could have some other explanation, and the poor stimulation could reflect weak DNA binding. The detailed effects of the three mutations reported here on ATPase activity, if significant, are most likely to be evidenced in the helicase activity. This remains a subject for future study.

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