Articles

Mechanism of DnaB Helicase of *Escherichia coli*: Structural Domains Involved in ATP Hydrolysis, DNA Binding, and Oligomerization

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Received January 7, 1999; Revised Manuscript Received May 10, 1999

ABSTRACT: We describe the delineation of three distinct structural domains of the DnaB helicase of *Escherichia coli*: domain α, amino acid residues (aa) 1−156; domain *β*, aa 157−302; and domain *γ*, aa 303−471. Using mutants with deletion in these domains, we have examined their role(s) in hexamer formation, DNA-dependent ATPase, and DNA helicase activities. The mutant DnaB*βγ* protein, in which domain α was deleted, formed a hexamer; whereas the mutant DnaBα*β*, in which domain *γ* was deleted, could form only dimers. The dimerization of DnaBα*β* was Mg^{2+} dependent. These data suggest that the oligomerization of DnaB helicase involves at least two distinct protein−protein interaction sites; one of these sites is located primarily within domain *β* (site 1), while the other interaction site is located within domain *γ* (site 2). The mutant DnaB*β*, a polypeptide of 147 aa, where both domains α and *γ* were deleted, displayed a completely functional ATPase activity. This domain, thus, constitutes the "central catalytic domain" for ATPase activity. The ATPase activity of DnaBα*β* was kinetically comparable to that of DnaB*β*, indicating that domain α had little or no influence on the ATPase activity. In both cases, the ATPase activities were DNA independent. DnaB*βγ* had a DNA-dependent ATPase activity that was kinetically comparable to the ATPase activity of wild-type DnaB protein (wtDnaB), indicating a specific role for C-terminal domain *γ* in enhancement of the ATPase activity of domain *β* as well as in DNA binding. Mutant DnaB*βγ*, which lacked domain α, was devoid of any helicase activity pointing to a significant role for domain α. The major findings of this study are (i) domain *β* contained a functional ATPase active site; (ii) domain *γ* appeared to be the DNA binding domain and a positive regulator of the ATPase activity of domain *β*; (iii) although domain α did not have any significant effect on the ATPase, DNA binding activities, or hexamer formation, it definitely plays a pivotal role in transducing the energy of ATP hydrolysis to DNA unwinding by the hexamer; and (iv) all three domains are required for helicase activity.

The DnaB helicase is the major replicative DNA helicase in *Escherichia coli* (*1*−*4*). It is a member of the hexameric DNA helicase family whose members are typically involved in DNA replication such as T4 DNA helicase, T7 DNA helicase, and SV40 T-antigen (*5*−*8*). The bacteriophage T7 encoded gene 4A-B helicase/primase protein complex is involved in the replication of T7 phage DNA while the bacteriophage T4 DNA helicase (gp41) is involved in the replication of the bacteriophage T4 genome (*6*, *9*, *10*). The T-antigen of the SV40 virus functions as a DNA helicase in the replication of the SV40 genome and can be utilized by eukaryotic DNA polymerases during in vitro replication of SV40 viral DNA (*8*, *11*, *12*). In addition to their hexameric structure (*5*−*8*), these helicases show a common polarity of movement, and all except T-antigen have a 5′ → 3′ polarity. All of these enzymes are thought to interact with the polymerase and/or primase and require a single-stranded DNA binding protein for efficient function. The DnaB helicase appears to be a prototypical replicative DNA helicase, and thus, understanding its mechanism of action would have broad implications on the general mechanism of action of replicative helicases.

DnaB helicase is a true multifunctional enzyme with a number of distinct enzymatic activities including ATP[1] hydrolysis, DNA binding, and association with other replication proteins, such as DnaG primase and DNA polymerase III holoenzyme (*13*). The interactions with replication proteins in the replication fork allow it to form the replisome

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ATPase, adenosine 5′-triphosphatase; AMPPNP, 5′-adenylyl imidodiphosphate; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl *β*-D-thiogalactoside; kDa, kilodalton; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; ssDNA, single-stranded DNA.

10.1021/bi990048t CCC: $18.00
Published on Web 08/06/1999

with primase and holoenzyme dimer in order to replicate the leading and lagging strands simultaneously. The energy of DNA-dependent ATP hydrolysis allows duplex DNA unwinding by the DnaB helicase and movement of the replisome synchronously with the progression of DNA unwinding and fork movement. DnaB helicase forms a hexamer which is partially Mg^{2+} dependent. DnaB helicase consists of 471 amino acid residues with a consensus ATP/GTP binding domain (*14*) and a "leucine zipper motif" (*15*, *16*) at the C-terminus. Although the primary structure of the DnaB helicase has been known for a number of years, the loci of its various activities and interaction sites remain unknown. Fluorescence studies of DnaB protein binding to oligonucleotides by Jezewska et al. (*17*, *18*) have yielded a number of unique information regarding stoichiometry and orientation of DNA binding. In addition, Yu et al. (*19*) demonstrated the binding of ssDNA in the inner channel of the T7 DNA helicase hexamer by direct electron microscopy of the protein−DNA complex. The crystal structure of DnaB protein has yet to be determined. However, in the absence of such information alternative approaches to understanding the structure and function can prove quite useful. For example, knowledge regarding the mechanism of action of bacteriophage Mu transposase has advanced tremendously in the past few years (*20*, *21*). Biochemical studies were carried out to define various structural domains that were assigned specific biological functions, and subsequently, subdomains of the individual domains were discovered that eventually led to a more detailed understanding of its mechanism (*21*−*23*). On the basis of previous trypsin cleavage studies and sequence analysis, we have defined specific structural domains in the DnaB protein. We have explored these structural domains in order to define the domains required for its enzymatic functions and assemble a basic picture of the interrelation of these loci in the eventual helicase action of the protein. Here we have defined the loci of various enzymatic activities and oligomerization and, in addition, elucidated the role of Mg^{2+} in the hexamer formation.

MATERIALS AND METHODS

Nucleic Acids, Enzymes, and Other Reagents. The plasmid pKA1 containing the wild-type DnaB gene was received as a kind gift from Drs. N. Nakayama and K. Arai of Kyoto University, Kyoto, Japan. Ultrapure deoxynucleotides were obtained from Amersham Pharmacia Biotech (Piscataway, NJ) and were used without further purification. [α-^{32}P]ATP and [γ-^{32}P]ATP were obtained from DuPont NEN (Boston, MA). T4 polynucleotide kinase was obtained from New England Biolabs Inc. (Beverly, MA). Poly(ethylenimine)−cellulose TLC strips were from J. T. Baker Chemical Co. (Pittsburgh, PA). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IO) and were of high purity ($\geq$95%), as determined by autoradiography of the phosphorylated products. Oligonucleotides used in polymerase chain reaction (PCR) were used without additional purification. The oligonucleotides used in helicase assays were further purified by electrophoresis in a 20% acrylamide−8 M urea gel.

Buffers. 1× TBE contained 89 mM Tris−borate (pH 8.3) and 2.5 mM EDTA. Buffer A contained 25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mg/mL BSA, and 5 mM DTT. Buffer B was 25 mM Hepes (pH 7.5), 10% glycerol, 5 mM $MgCl_2$, 0.1 M NaCl, and 1 mM DTT. Buffer C contained 25 mM Tris-HCl (pH 7.9), 10% sucrose, and 0.25 M NaCl. Buffer D contained 20 mM Tris-HCl, pH 8, 0.5 M NaCl, and imidazole at the indicated concentration.

WtDnaB and DnaB Mutant Proteins. The DnaB gene was amplified from the plasmid pKA1. The wild-type DnaB gene was isolated by polymerase chain reaction (PCR) under high-fidelity reaction conditions (*Pfu* DNA polymerase; Stratagene, La Jolla, CA) using pKA1 as the DNA template with oligonucleotide primers containing an *Nde*I site in the 5′-primer (5′-TCT CAT ATG GCA GGA AAT AAA CC-3′) and a *Bam*HI site in the 3′-primer (5′-CTC GGA TCC AGT TCA CGA AGA CG-3′). The PCR-amplified DNA was cut with *Nde*I/*Bam*HI enzymes and cloned into the expression vector pET29b (Novagen Corp., Milwaukee, WI) in the *Nde*I/*Bam*HI sites. The resultant plasmid was cut with *Pvu*I/*Bam*HI, and a *Pvu*I/*Bam*HI fragment of the wild-type DnaB gene from the plasmid pKA1 was inserted, which essentially replaces the wtDnaB coding region in the expression vector pET29bDnaB. A 90 bp region near the 5′-end was verified by sequencing. Unless otherwise indicated, this basic cloning strategy of genes using PCR was followed throughout our studies. The plasmid, pET29bDnaB, was used for expression of wtDnaB helicase in *E. coli* [BL21(DE3)] following a procedure described earlier (*24*). This plasmid was also the parent of all mutant and deletion clones of DnaB.

Expression of Wild-Type and Deletion Constructs of DnaB. Following verification of the desired inserts, the pET29b constructs were used to transform *E. coli* strain BL21(DE3), in which expression of the recombinant protein is under control of the lacUV5 promoter. The typical induction was as follows. Cultures of BL21(DE3) harboring the desired construct were grown to $OD_{600} = 0.4$ at 37 °C, at which time IPTG was added to 0.5 mM. The cultures were brought to room temperature and were shaken for 16 h. The cells were then harvested and resuspended in buffer C and frozen at −80 °C.

Purification of the Recombinant Proteins. Extracts were prepared from the induced *E. coli* cultures by treatment with lysozyme (0.1 mg/mL) at 4 °C for 30 min followed by sonication in buffer C containing an additional 0.25 M NaCl. The lysate was spun at 40000*g* for 30 min (fraction I). Following these steps the purification procedures varied with each protein and were as follows:

(*A*) *WtDnaB Protein*. WtDnaB protein was precipitated from fraction I using 0.18 g/mL ammonium sulfate. The precipitated protein (fraction II) was resuspended in buffer B and adjusted to the conductivity of buffer B containing 100 mM NaCl. It was then loaded onto a Q-Sepharose (HP) column equilibrated with buffer B containing 100 mM NaCl. The column was washed successively with buffer B containing 100 mM and then 300 mM NaCl. WtDnaB protein was then eluted with buffer B containing 400 mM NaCl. The column fractions were analyzed for protein concentration by the method of Bradford (*25*) and for purity by SDS−PAGE.

(*B*) *DnaBα Protein*. Purification of DnaBα protein was relatively simple as this protein was expressed as a $(His)_6$-tagged fusion protein. Following extraction of the induced cells, DnaBα was bound to a 1.0 mL His-bind resin column (Novagen, Milwaukee, WI), washed with buffer D containing 50 mM imidazole, and eluted isocratically with buffer D containing 100 mM imidazole. Following elution from the

column, the fractions were analyzed for protein concentration and for purity by SDS−PAGE. The peak fractions were pooled and dialyzed against buffer B containing 20% glycerol.

(*C*) *DnaBαβ Protein*. Following extraction of induced *E. coli* cells, the conductivity of fraction I was adjusted to that of buffer B containing 100 mM NaCl and loaded onto a 5.0 mL Q-Sepharose (HP) column. The protein was eluted isocratically from the column using buffer B containing 300 mM NaCl. The protein was further purified by size-exclusion HPLC. The peak fractions were then passed over a ssDNA cellulose column (1.0 mL), equilibrated with buffer B containing 100 mM NaCl to remove any residual wtDnaB. DnaBα*β* did not bind to ssDNA cellulose and was collected in the unbound fractions. The final product was analyzed for protein concentration and for purity by SDS−PAGE.

(*D*) *DnaBβ Protein*. Following extraction of the IPTG-induced *E. coli* cells harboring plasmid pET29bDnaB*β*, DnaB*β* was precipitated with 0.3 g/mL ammonium sulfate. The protein pellet obtained was resuspended in buffer B, and the conductivity was adjusted to that of buffer B containing 100 mM NaCl. The protein was then loaded onto a 5.0 mL Q-Sepharose (HP) column. DnaB*β* did not bind to the column at this ionic strength and was found in the unbound fractions. These fractions were pooled and injected in a S-Sepharose (HP) column equilibrated with buffer B containing 100 mM NaCl. This mutant did not bind at this salt concentration and was collected in the unbound fractions. The fractions containing protein were pooled and analyzed for protein concentration and for purity by SDS−PAGE.

(*E*) *DnaBβγ Protein*. DnaB*βγ* was precipitated from fraction I using 0.2 g/mL ammonium sulfate. The pellet was resuspended in buffer B, and the conductivity was adjusted to that of buffer B containing 100 mM NaCl. The extract was loaded onto a 5.0 mL high-performance Q-Sepharose open column. The protein was eluted isocratically with buffer B containing 400 mM. The fractions were analyzed for protein concentration and for purity by SDS−PAGE.

SE-HPLC Fractionation. The molecular weight of the wild-type and various truncated polypeptides was determined using size-exclusion HPLC (SE-HPLC). Homogeneous preparations of the respective proteins were fractionated on a TSK-QC (1.0 × 15 cm) gel filtration column equilibrated with buffer B containing 150 mM NaCl. In addition, fragment α was also fractionated on a TSK-125 (0.8 × 30 cm) column, using the same running buffer as described above. The TSK-125 column displays greater resolution for lower molecular weight proteins. The column fractions were analyzed for protein concentration and for purity by SDS−PAGE. The corresponding molecular weights were determined by comparison of elution volumes with a least-squares analysis of the elution volumes of molecular weight standards.

Assay for ATPase Activity. The ATPase activity assays were carried out as previously described (*26*). The amount of DnaB protein (wild type and mutants) used in the assays was selected such that the rate of hydrolysis would be linear in the time range examined. A standard 10 μL reaction mixture contained 10 mM $MgCl_2$, 200 pmol of M13mp18 ssDNA (unless otherwise indicated), 100 μM [α-^{32}P]ATP, and DnaB proteins as indicated, in buffer A. The reactions were incubated at 37 °C for 30 min (unless stated otherwise) and terminated by the addition of 2 μL of 200 mM EDTA followed by chilling on ice. Two microliter aliquots were applied to poly(ethylenimine)−cellulose strips which were prespotted with an ADP−ATP marker. The strips were developed with 1 M formic acid and 0.5 M LiCl and dried. The ADP−ATP spots were located by UV fluorescence. The portions containing ATP and ADP were excised and counted in a liquid scintillation counter using a toluene-based scintillator.

Assay for DNA Helicase Activity. The helicase assays were based on the methods as described (*27*). The substrate was prepared by hybridizing a synthetic 60-mer oligonucleotide to M13mp19 ssDNA as previously described (*27*). The oligomer was complementary to a 50 bp sequence between nucleotides 6268 and 6317 of M13mp19 ssDNA and contained five nucleotide tails (nonhomologous regions) on both 5′ and 3′ termini. The purified substrate was diluted to a final concentration of 17 fmol/μL ($1-2 \times 10^4$ cpm/μL) with 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

Assay Conditions. Reaction mixtures were set up on ice as follows. A standard 20 μL reaction volume contained buffer A, 10 mM $MgCl_2$, 3.4 mM ATP, 17 fmol of substrate, and the indicated amount of DnaB protein. The mixtures were incubated at 37 °C for the times indicated, and the reactions were terminated by the addition of 4 μL of 2.5% SDS, 60 mM EDTA, and 1% bromophenol blue. A fraction (25%) of each reaction mixture was analyzed on 8% polyacrylamide gels in 1× TBE and 0.1% SDS. The electrophoresis was carried out in 1× TBE and 0.1% SDS for 1 h at 160 V. Following electrophoresis the gels were dried and exposed to Fuji RX film at −80 °C for 12 h. If needed, helicase activity was quantitated by scintillation counting of excised substrate/product bands from the dried gels as described by LeBowitz and McMacken (*28*) and/or by scanning densitometry of the corresponding autoradiogram.

Other Methods. Protein concentrations were estimated according to the method of Bradford (*25*), using DnaB protein as a standard. The concentration of DnaB protein standard was determined by the method of Gill and von Hippel (*29*).

RESULTS

Design of Constructs Dividing the DnaB Gene into Three Domains: *α*, *β*, *and γ*. DnaB helicase has many exceptional structural and functional features including hexamer formation, DNA binding, DNA-dependent ATPase, DNA helicase, and interaction with other replication proteins such as DNA primase and DNA polymerase III (*1*). To precisely define the domains responsible for hexamer formation and the enzymatic activities of DnaB protein, we have designed several distinct N- and C-terminal deletion constructs. The predicted secondary structure of DnaB polypeptide chain indicated at least two hinge regions (*30*) which we have used to define the boundaries of these domains.

Previous studies with proteolytic fragments of DnaB protein generated by limited trypsin digestion (*16*, *31*), as well as the protein sequence, indicated that domain *β* contains a type I nucleotide binding motif (*14*) located between aa 230−240 and, thus, is important for the ATPase function.

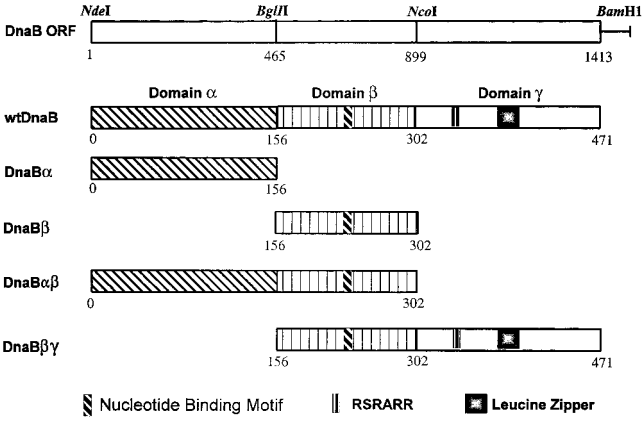

FIGURE 1: Schematic diagram of the DnaB open reading frame and the various mutant proteins utilized in this study. The domain designations corresponding to a given region of the 471 amino acid open reading frame are indicated. The Walker type I ATP/GTP binding motif is shown as a nucleotide binding motif, and the RSRARR indicates the basic amino acid rich sequence, which corresponds to the putative DNA binding domain. The leucine zipper refers to the leucine-rich region that has homology with a leucine zipper consensus sequence (*15*, *16*).

In addition, our laboratory identified a leucine zipper like motif located within domain γ, indicating that this domain may play a role in DNA binding and oligomerization (*15*). We have investigated the contributions of DnaB protein domains in its structure and function by carrying out selective deletions of specific regions within the DnaB gene. As shown in Figure 1, the 1413 base pair (bp) open reading frame of the DnaB gene contains two unique restriction sites: *Bgl*II at 465 bp and *Nco*I at 899 bp. We have introduced an *Nde*I site at the ATG start codon and a *Bam*HI site after the stop codon. Together, these sites provided a simple strategy for subcloning DnaB gene and expressing their truncated gene products in terms of three separate domains. The 465 bp *Nde*I−*Bgl*II fragment coded for the N-terminal region designated as domain α; the 435 bp *Bgl*II−*Nco*I fragment coded for the middle one-third of the polypeptide chain that contained the ATP binding motif and was designated domain β; and the *Nco*I−*Bam*HI fragment encoded the C-terminal domain γ which also contained the leucine zipper motif. Deletion clones were generated using these restriction sites and subcloning the resulting fragments in pET29b as described in Materials and Methods. Proteins were expressed from these constructs to determine their effects on *in vitro* ATPase and DNA helicase activities, as well as the ability to form oligomers.

Proteins from various deletion constructs were overexpressed in *E. coli* and were purified chromatographically to homogeneity. An SDS−PAGE profile of the purified proteins is presented in Figure 2. The wtDnaB protein migrated on SDS−PAGE with an expected mass of ~52 kDa (lane 2, Figure 2). The relative masses of the mutant proteins from SDS−PAGE were as follows: DnaBα, 18.6 kDa; DnaBαβ, 37.3 kDa; DnaBβ, 15 kDa; and DnaB$\beta\gamma$, 34.8 kDa (lanes 3−6, Figure 2). Protein preparations were essentially devoid of contaminating endogenous DnaB protein as determined by SDS−PAGE analysis. The purified mutant proteins were then used for *in vitro* biochemical studies.

Analysis of the Contributions of Various Domains to the Oligomeric Structure. The wild-type DnaB protein has a

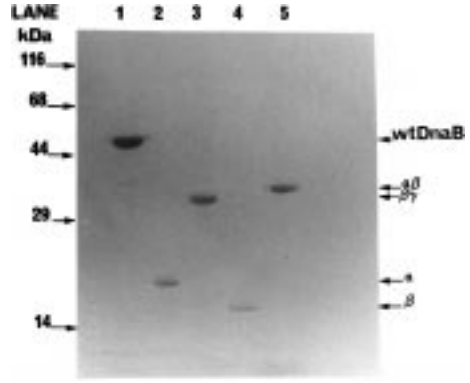

FIGURE 2: SDS−PAGE analysis of the purified deletion mutant proteins used in this study. Following overexpression of the various constructs, the proteins were purified to homogeneity by ion-exchange chromatography as described in Materials and Methods. Aliquots of the indicated protein samples were analyzed by 10−18% SDS−PAGE. Following electrophoresis the gel was stained with Coomassie brilliant blue R-250. Lanes: 1, wtDnaB; 2, DnaBα; 3, DnaB$\beta\gamma$; 4, DnaBβ; and 5, DnaBαβ.

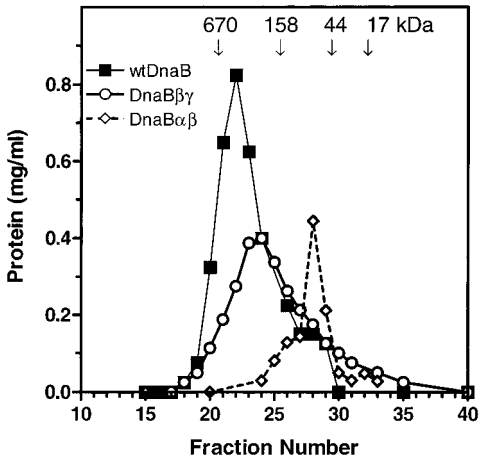

FIGURE 3: Size-exclusion HPLC of wtDnaB, DnaBαβ, and DnaB$\beta\gamma$. Procedures for fractionation of the polypeptides are described in Materials and Methods. Samples of the collected fractions were analyzed for protein. Gel filtration standards (Bio-Rad Laboratories, Richmond, CA) were as follows: thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), egg ovalbumin (44 kDa), and equine myoglobin (17 kDa). Native molecular masses were estimated relative to a linear regression curve generated from the elution profile of the markers. Proteins were as follows: wtDnaB (solid line, ■), DnaB$\beta\gamma$ (bold line, ○), and DnaBαβ (broken line, ◇).

hexameric quaternary structure (*1*, *32*). It was anticipated that, in order to form a hexameric structure, DnaB protein would require more than one protein−protein interaction site. Indeed, the T7 helicase has been shown to have two interaction sites by Notarnicola et al. (*7*). Thus, analysis of the oligomeric structures of the deletion mutants might likely yield useful information regarding the makeup and assembly of the hexamer. The different mutant proteins were analyzed by size-exclusion HPLC (SE-HPLC) to determine their state of oligomerization. As shown in Figure 3, DnaB protein eluted at a volume corresponding to a mass of 310 kDa, which is consistent with a hexameric structure. DnaBαβ, in which the C-terminal domain γ was deleted, eluted at a volume corresponding to a mass of ~80 kDa (Figure 3), which given a monomeric molecular mass of 39 kDa is suggestive of a dimeric native structure. Analysis of DnaBβ, in which

both N-terminal and C-terminal domains were deleted, also indicated a possible dimeric structure with a strong tendency toward aggregation (data not shown). The aggregated DnaBβ eluted near the void volume of the gel filtration columns used in this study. DnaB$\beta\gamma$ protein retained the ability to form hexamers, as inferred from its native molecular mass of 240 kDa (Figure 3). Taken together, the facts that polypeptides containing either domains β or $\alpha\beta$ can form only dimers and that the presence of domain γ (site 2) allows for the formation of hexamers suggest that a protein−protein interface required for hexamer formation is likely located within domain γ. The other protein−protein interface is likely located within the N-terminus of domain β and domain α (site 1).

The Entire ATPase Domain Is Contained within Domain β. The availability of highly purified truncated constructs allowed us to examine the role of each of these domains in the DNA-dependent ATPase activity of DnaB protein. As described above, DnaBβ contains the type I ATP binding motif. We wanted to determine whether domain β contained the entire ATPase catalytic site or whether other functional domains (α and/or β) are also required for ATPase activity.

The kinetic data of ATP hydrolysis by the wild-type DnaB helicase are shown in Figure 4, and those of DnaB$\alpha\beta$, DnaBβ, and DnaB$\beta\gamma$ proteins are shown in Figures 5, 6, and 7, respectively. There was no significant difference between DnaB$\alpha\beta$ and DnaBβ in their kinetics of ATP hydrolysis, and in both cases the rates were DNA independent: $V_{max} = 3.2 \times 10^4$ and 3.9×10^4 pmol min^{-1} mg^{-1} for DnaB$\alpha\beta$ and DnaBβ, respectively. Comparison of DnaB$\alpha\beta$ (Figure 5) and Dnaβ (Figure 6) demonstrated that domain β contained the central catalytic site for ATPase activity and appeared to contain all of the essential structural features required for ATP hydrolysis despite its small size. The mutant DnaB$\beta\gamma$ hydrolyzed ATP at a rate comparable to that of wtDnaB protein in the presence and absence of DNA: V_{max} for DnaB$\beta\gamma = 7.4 \times 10^6$ and 4.8×10^5 pmol min^{-1} mg^{-1}, respectively (Figure 7). These data suggest that although the ATPase activity of DnaBβ itself was DNA independent, the addition of domain γ restored the DNA-dependent ATPase activity to wild-type level (Figure 7). Analysis of the ATPase activity in the presence of DNA supported the putative localization of the DNA binding domain of DnaB helicase to be within the γ domain as demonstrated by the relative DNA dependence of ATPase activity of DnaB$\beta\gamma$ as compared to those of DnaB$\alpha\beta$ or DnaBβ, which were both DNA independent (Figures 5−7).

The affinity of ATP binding (as determined by K_m) of wtDnaB, DnaB$\alpha\beta$, DnaBβ, and DnaB$\beta\gamma$ proteins did not seem significantly different and ranged from 20 to 150 μM (Table 1). The data also indicated that DNA binding did influence ATP binding affinity somewhat as assessed from K_m for ATP by wtDnaB protein and its mutants. The major effect of DNA binding was the significant stimulation of the overall rate or V_{max} of ATP hydrolysis by wtDnaB and DnaB$\beta\gamma$ (Figures 4 and 7, Table 1). A comparison of the kinetics of ATP hydrolysis between DnaB$\alpha\beta$ and DnaBβ (Figures 5 and 6) suggests that the presence or absence of domain α did not result in a measurable difference in the rate of ATP hydrolysis although a small but significant effect (~2-fold) was observed in the binding of ATP. The most marked stimulation of the rate of ATP hydrolysis was

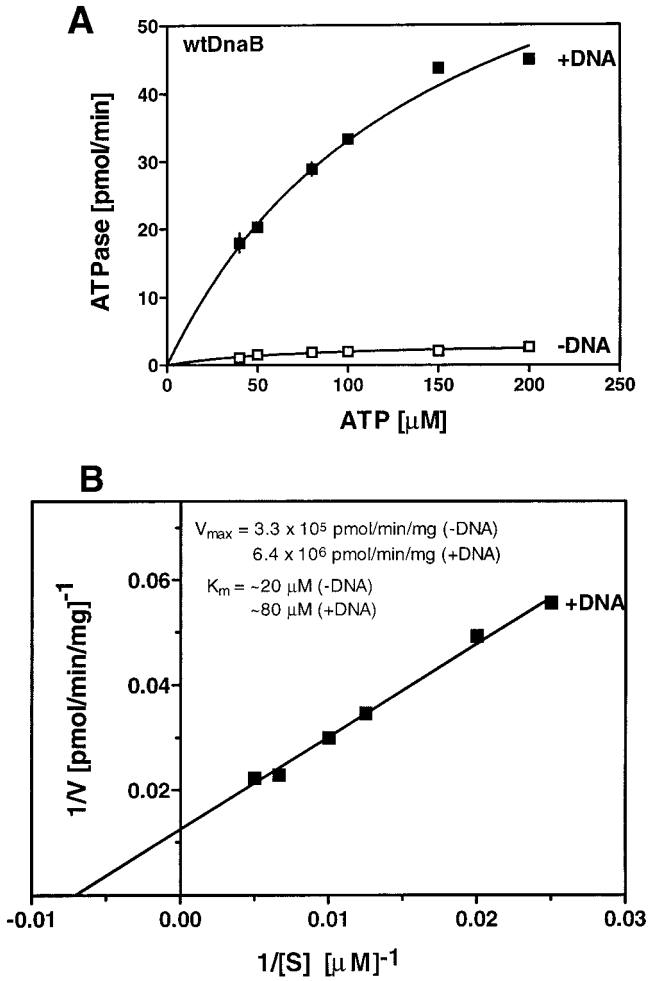

FIGURE 4: Kinetic analysis of the ATPase activity of wild-type DnaB protein. (A) ATPase activity (V) vs ATP concentration ([S]) in the presence and absence of 200 pmol of M13mp19 ssDNA. The curve was generated using a nonlinear regression analysis of the data. (B) Double reciprocal plot ($1/V$ vs $1/[S]$) of the ATPase activity in the presence and absence of DNA. The ATPase assay was carried out as described in Materials and Methods.

observed when domain γ was present. These results indicate that domain γ stimulated the basal ATPase activity of DnaBβ about 34-fold in the absence of DNA, and by the virtue of its ability to bind ssDNA, it also stimulated the basal ATPase activity again about 5.5-fold in a synergistic manner (Table 1). However, the effects of domain γ on the ATPase activity were observed only with the rate of the ATP hydrolysis and did not appear to have any effect on the affinity (or K_m) of the protein for ATP binding.

Domain α Is Required for DNA Helicase Function. To act as a helicase, the DnaB protein must have ATPase activity, DNA binding ability, and the ability to form a hexamer. On the basis of the results described above, it was not anticipated that DnaB$\alpha\beta$ or DnaBβ would be capable of unwinding DNA. However, DnaB$\beta\gamma$ possessed a hexameric structure, in addition to other functional requirements such as DNA binding, for DNA helicase activity. The purified truncated proteins were examined in standard DNA helicase assays in order to assess their ability to unwind DNA, and the results are presented in Figure 8. We have directly compared DNA helicase activities of wtDnaB and all of the deletion mutants described here. WtDnaB unwound a 50 bp partial duplex as anticipated; however, none of the mutants

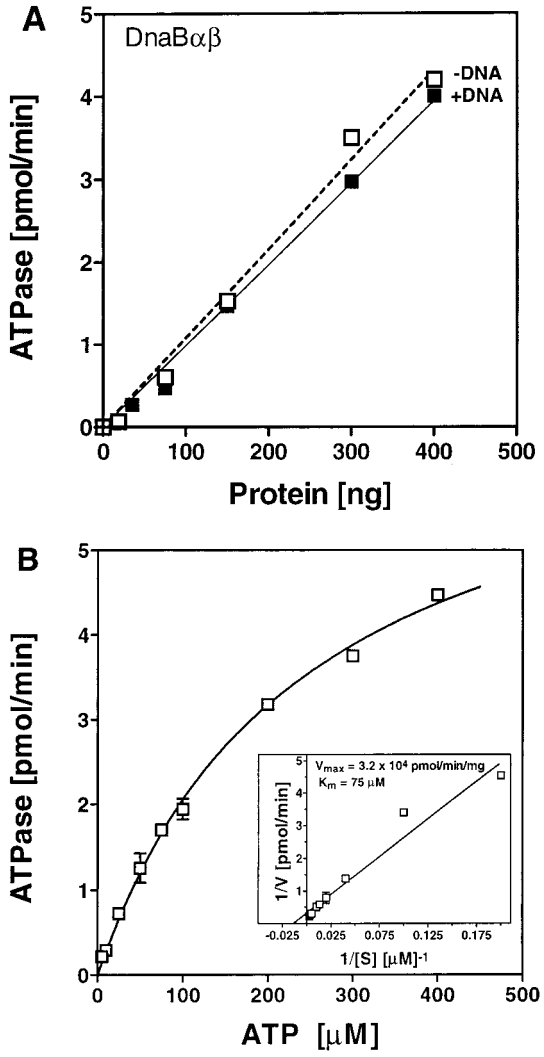

FIGURE 5: Characterization of the ATPase activity of DnaBαβ protein. (A) Analysis of the DNA dependence of ATP hydrolysis. Protein titration of ATPase activity in the presence and absence of 200 pmol of M13mp19 ssDNA. (B) Kinetic analysis of the ATPase activity of DnaBαβ. ATPase assays were carried out with varying substrate concentrations, as indicated, in the absence of 200 pmol of M13mp19 ssDNA. Inset: The Lineweaver−Burk plot (inset) was generated using linear regression analysis of the data.

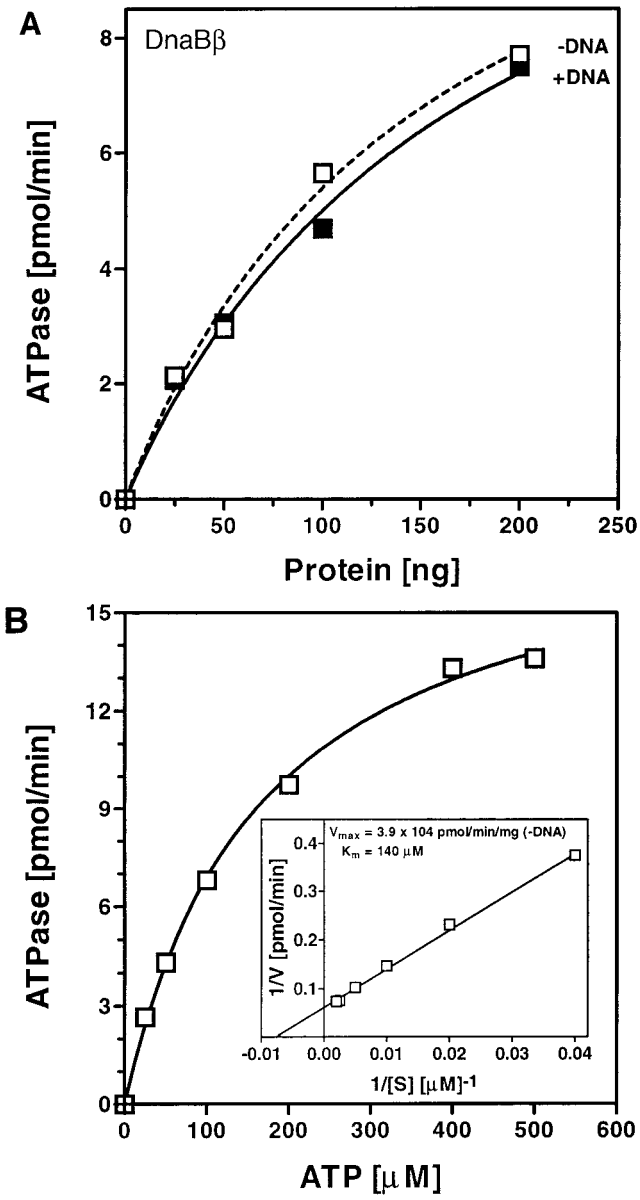

FIGURE 6: Characterization of the ATPase activity of DnaBβ. (A) Analysis of the DNA dependence of ATP hydrolysis. Protein titration of ATPase activity in the presence and absence of 200 pmol of M13mp19 ssDNA. (B) Kinetic analysis of the ATPase activity of DnaBβ. ATPase assays were carried out at various substrate concentrations in the absence of 200 pmol of M13mp19 ssDNA. The Lineweaver−Burk plot ($1/V$ vs $1/[S]$) shown in the insert was obtained by linear regression analysis of the data.

including DnaB$\beta\gamma$ had any helicase activity. DnaB$\beta\gamma$ could not unwind DNA despite the fact that it possessed a hexameric structure and the DNA-dependent ATPase activity comparable to the wild type. These findings were quite significant because these data clearly indicate that domain α is indispensable for DNA unwinding despite the fact that it did not have any enzymatic activity nor does it play a significant role in ATPase and DNA binding activities and oligomerization.

Effect of Mg^{2+} on the Enzymatic Activities of DnaB Protein. The Mg^{2+} chelated form of ATP undergoes hydrolysis by DnaB helicase and therefore is required for the enzyme's ATPase and DNA helicase activities; however, in the case of DnaB helicase Mg^{2+} is also known to be useful for maintaining the integrity of the hexameric structure especially during purification of the helicase (*2*). The effect(s) of this structural requirement of Mg^{2+} on the enzymatic activities as well as locus of Mg^{2+} action on DnaB hexamer formation/stabilization remain(s) unknown. We attempted to correlate the effect of Mg^{2+} on DnaB subunit interaction with that of its enzymatic activities. WtDnaB protein was purified in the presence and absence of Mg^{2+}, and an analysis of its *in vitro* ATPase and helicase activities was carried out. As shown in Figure 9A, no noticeable difference was observed in the DNA unwinding ability of DnaB purified in the absence of Mg^{2+} provided the protein was maintained at higher concentrations (>100 μg/mL). The helicase activity was decreased about 50% (as determined by scanning densitometry) only when the protein was kept overnight (16 h at 4 °C) at 100 mg/mL in the absence of Mg^{2+} (Figure 9B). A small decrease (≤5%) in the ATPase activity was also observed in dilute protein preparations purified in the absence of Mg^{2+} (Figure 9C).

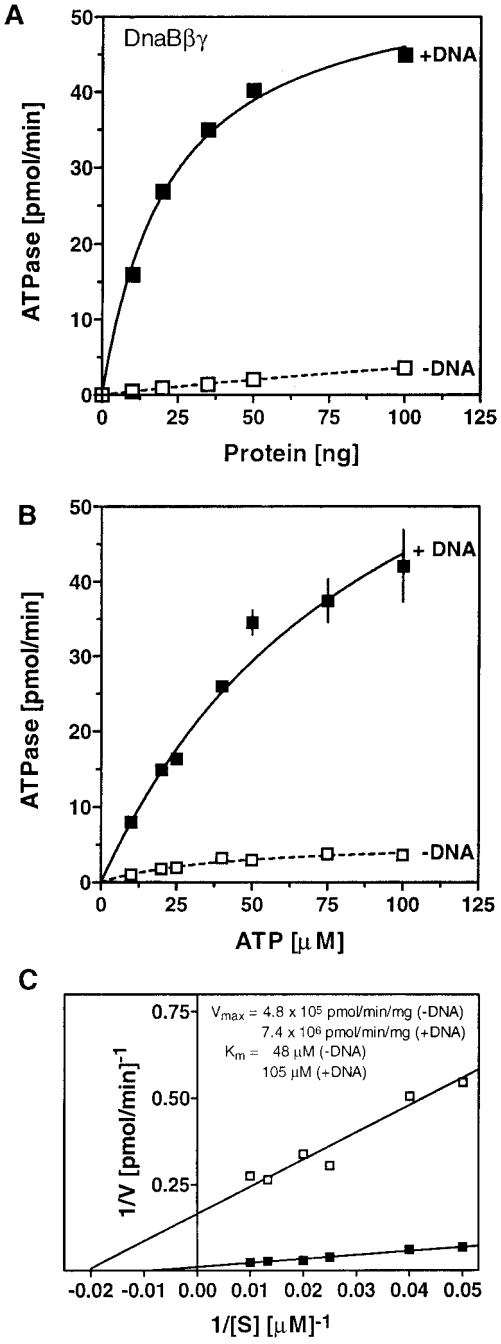

FIGURE 7: Characterization of the ATPase activity of DnaB$\beta\gamma$. (A) Analysis of the DNA dependence of ATP hydrolysis. Protein titration of ATPase activity in the presence and absence of 200 pmol of M13mp19 ssDNA. (B and C) Kinetic analysis of the ATPase activity of DnaB$\beta\gamma$. (B) ATPase activity (V) as a function of [S] in the presence and absence of ssDNA. (C) The Lineweaver−Burk plot obtained by linear regression analysis of double reciprocal plots of the two sets of data shown in (B).

Locus of Mg^{2+} Interaction in the DnaB Helicase. The results presented above regarding the oligomeric structures of the truncated DnaB proteins appear to suggest that hexamer formation involves two distinct sites of protein−protein interactions: one appeared to be located primarily at the N-terminus of domain β and the other is located within domain γ. It is important to know whether Mg^{2+} interacts with one or both of these sites. Therefore, we have examined the effects of Mg^{2+} interaction with DnaB helicase and its deletion mutants with respect to their oligomeric structures. SE-HPLC was carried out with wtDnaB and mutant proteins purified in the presence and absence of Mg^{2+}. The chromatography buffers were also prepared accordingly, and the oligomeric structures formed for these proteins under the two different conditions were compared (Figure 10). This analysis was initially carried out using the full-length wtDnaB protein, as shown in Figure 10A. Under the conditions used, it is interesting to note that no noticeable difference was observed in the native molecular weight of DnaB protein in the presence or absence of Mg^{2+} (Figure 10A). Similarly, the elution profile of DnaB$\beta\gamma$ protein, which is consistent with a hexameric structure, did not appear to be dependent upon the presence of Mg^{2+} either (Figure 10B). In contrast, a comparison of the elution profiles for DnaB$\alpha\beta$, in the presence and absence of Mg^{2+}, was distinctly different from that of wtDnaB. DnaB$\alpha\beta$ had an elution profile consistent with that of a monomer in the absence of Mg^{2+} but was able to form dimers when Mg^{2+} was present (Figure 10C). Taken together, these data suggest that Mg^{2+} plays a role in the protein−protein interactions in the N-terminal dimerization (site 1).

Table 1: Comparison of ATPase Activities of DnaB Protein Fragments in the Presence and Absence of DNA

	−DNA		+DNA		
DnaB	V_{max}, units/mg	K_m, μM	V_{max}, units/mg	K_m, μM	K_d, μM
wtDnaB	1.7×10^6	50	6.4×10^6	70	7.5
DnaB$\alpha\beta$	3.2×10^4	75	3.2×10^4	75	NA[a]
DnaBβ	4.1×10^4	140	4.1×10^4	140	NA
DnaB$\beta\gamma$	1.4×10^6	140	7.7×10^6	150	5
DnaBα	NA	NA	NA	NA	NA

[a] NA: not applicable.

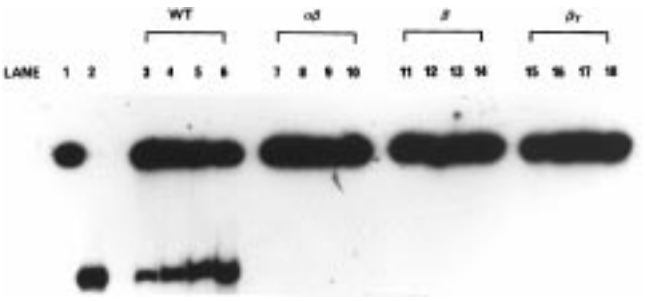

FIGURE 8: DNA helicase activity of the various truncated polypeptides. Protein titration of the DNA unwinding activity on a 50 bp partial duplex substrate. Details of the assay procedure are given in Materials and Methods. Lanes: 1, native substrate, no enzyme added; 2, heat-denatured substrate, no enzyme added; 3−6, wild-type DnaB protein, 50, 100, 200, and 400 ng, respectively; 7−10, DnaB$\alpha\beta$, 50, 100, 200, and 400 ng, respectively; 11−14, DnaBβ, 50, 100, 200, and 400 ng, respectively; 15−18, DnaB$\beta\gamma$, 50, 100, 200, and 400 ng, respectively.

DISCUSSION

The DnaB helicase is multifunctional protein with complex quaternary structure (*1*). Arai et al. (*33*) demonstrated using gel filtration and cross-linking studies of recombinant DnaB protein that the DnaB protein is a hexamer of six identical subunits. Electron microscopic data of DnaB helicase in solution have suggested that the protein forms a circular array, similar to that of a doughnut, with a central opening of approximately 40 Å (*32*). It is not known how various regions of the 471 amino acid polypeptide chain contribute to hexamer formation as well as toward its ATPase and

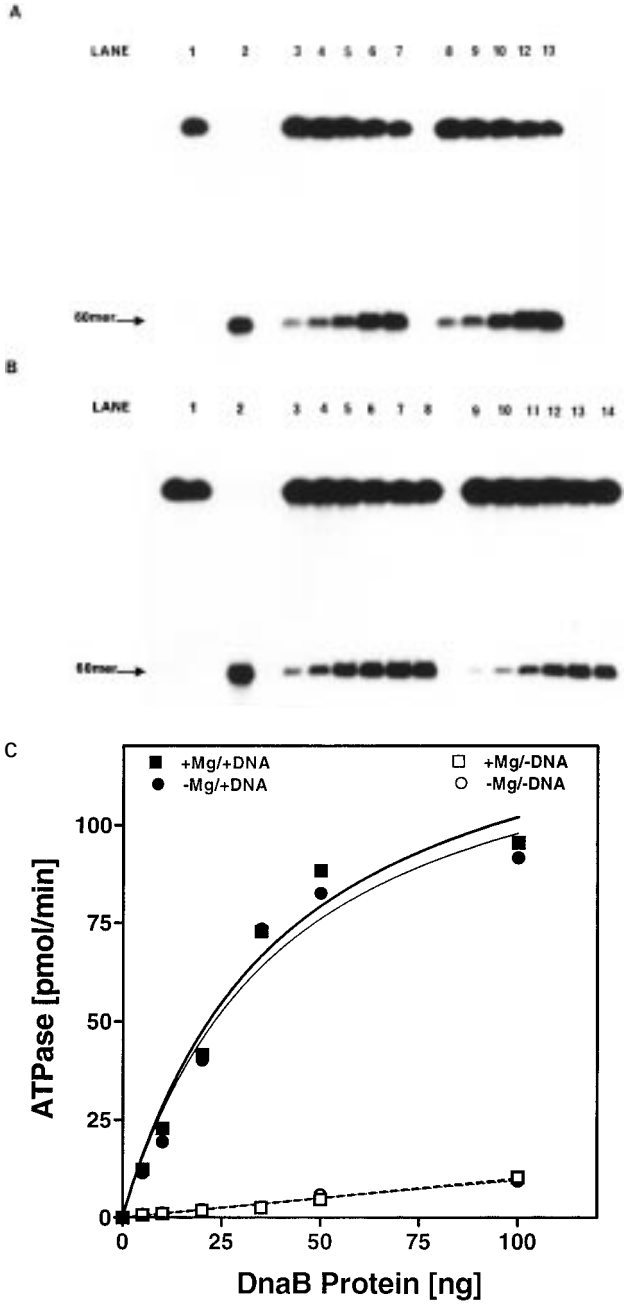

FIGURE 9: Effect of Mg^{2+} on the helicase and ATPase activities of DnaB Protein. (A) Titration of the helicase activity using DnaB protein preparations prepared in the presence (lanes 3−7) or absence (lanes 8−13) of Mg^{2+}. The assay itself was carried out as described in Materials and Methods in the presence of 5 mM Mg^{2+}. Lanes: 1, native substrate, no enzyme added; 2, heat-denatured substrate, no enzyme added; 3−7, enzyme, 25, 50, 100, 200, and 400 ng; 8−13, enzyme, 25, 50, 100, 200, and 400 ng. (B) Protein titration of the helicase activity of DnaB protein analogous to that in (A) except the enzyme was maintained at a protein concentration of 100 μg/mL following purification. Lanes: 1, native substrate, no enzyme added; 2, heat-denatured substrate, no enzyme added; 3−7, enzyme, 25, 50, 100, 200, and 400 ng; 8−13, enzyme, 25, 50, 100, 200, and 400 ng. (C) Protein titration of the ATPase activity of DnaB protein in the presence and absence of ssDNA using protein preparations purified with or without Mg^{2+}.

helicase activities. In an effort to delineate the functions of distinct domains of DnaB helicase, we have prepared selective deletion mutants that contain one or more of these domains. These regions were designed from the predicted secondary structures of the polypeptide chain that indicated

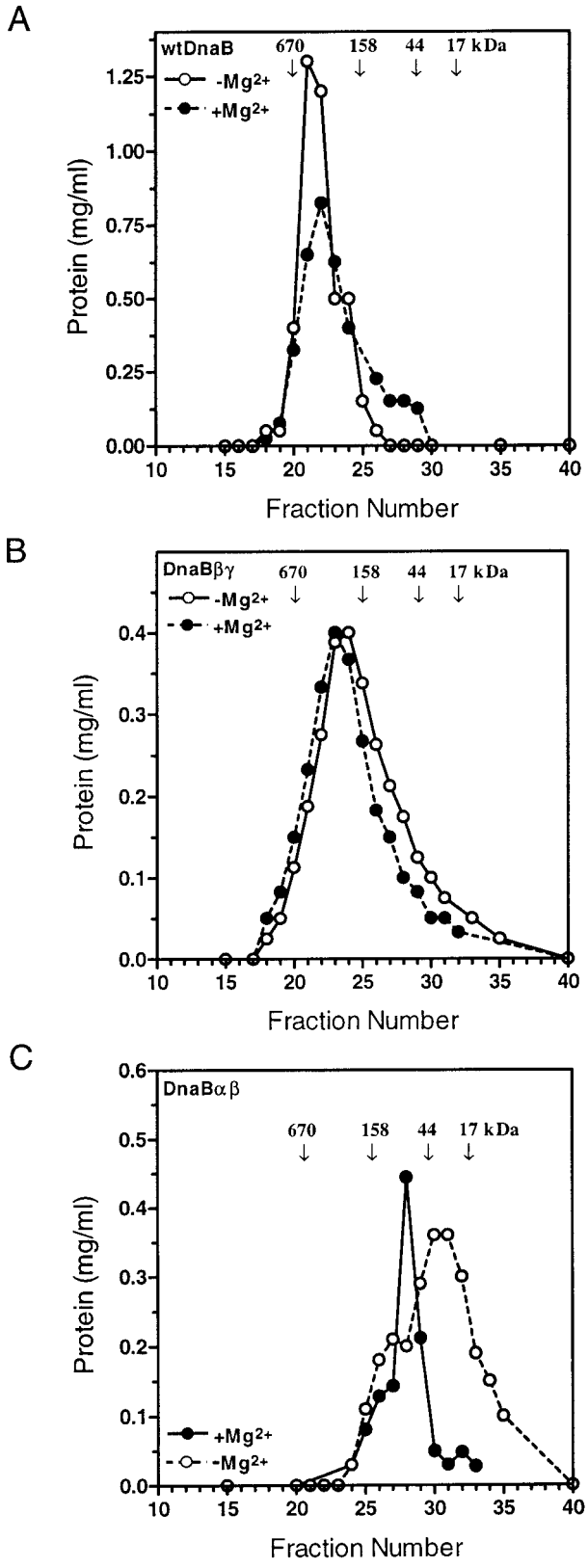

FIGURE 10: Effect of Mg^{2+} on the quaternary structure of DnaB protein. Size-exclusion HPLC of wtDnaB (A), DnaB$\beta\gamma$ (B), and DnaB$\alpha\beta$ (C) proteins. Details of the chromatography are given in Materials and Methods. Fractions were analyzed for protein content by the method of Bradford (*25*). Gel filtration standards were as described in the legend of Figure 3. Native molecular masses were estimated relative to a linear regression curve generated from the elution profile of the protein standards.

certain flexible regions similar to that described by Nakayama et al. (*30*).

The Overall Quaternary Structure of DnaB Helicase Requires Two Distinct Protein−Protein Interactions. The DnaB helicase is a member of the hexameric family of DNA helicases, a number of which are known to be involved in DNA replication. As shown in Figure 3, gel filtration studies of wtDnaB, DnaB$\beta\gamma$, and DnaB$\alpha\beta$ proteins were used to determine the native size of each of these proteins. WtDnaB and DnaB$\beta\gamma$ proteins eluted at volumes that were consistent with hexameric structures, while DnaB$\alpha\beta$ formed only dimeric structures in a Mg^{2+}-dependent manner. Hexamer formation by mutant DnaB$\beta\gamma$ and dimer formation by mutant DnaB$\alpha\beta$ suggest that one of the protein interaction sites required to form hexamers most likely resides within domain γ, while the other site is located in a region shared by domains α and β. Domains α and β, when expressed individually, formed dimers in both cases (data not shown), which suggests that the N-terminal dimerization site 1 is shared between these two domains.

The Central Catalytic Site for ATPase Is Located within Domain β. Previous studies, including the presence of a Walker type I motif (*14*), pointed to the existence of a nucleotide binding site within domain β. We have attempted to delineate whether domain β, composed of 143 amino acid residues, is sufficient for ATP hydrolysis or does so in conjunction with another domain. In this regard, analysis of the ATPase activity of highly purified DnaBβ protein showed that the structural features required for ATP hydrolysis are indeed found within this domain (Figure 6). The ATPase activity was DNA independent, which indicated that the DNA binding domain was located outside of domain β. Comparison of the ATPase activity of DnaBβ protein (Figure 6) with that of the wtDnaB protein (Figure 4) indicated that there was only a small difference (~2-fold) in the affinity of nucleotide binding (K_m), although there was a ≤10-fold difference in the rate of ATP hydrolysis (V_{max}) between DnaBβ and wild-type DnaB protein in the absence of DNA. Thus, the results indicated that domain β contained the complete ATPase active site and was capable of hydrolyzing ATP and the rate of ATP hydrolysis was, however, much slower than the wtDnaB protein. The V_{max} of ATP hydrolysis of DnaB$\alpha\beta$ was nearly identical to that of DnaBβ (Figures 5 and 6). The ATP binding affinity of DnaB$\alpha\beta$ (as indicated from K_m of ATP hydrolysis) was comparable to that found with wild-type DnaB protein and higher than the DnaBβ protein (Figures 4−6). Thus domain α influenced the ATP binding by domain β but not the rate of ATP hydrolysis. On the other hand, domain γ played a very different role on the kinetics of ATP hydrolysis (Figure 7). In DnaB$\beta\gamma$, domain γ increased the V_{max} of ATP hydrolysis ≥35-fold in the absence of DNA when compared to DnaBβ. In the presence of DNA, the V_{max} of DnaB$\beta\gamma$ increased another 5.5-fold, and this rate was then comparable to that observed with wild-type DnaB helicase (Table 1). The ATP binding affinity, however, did not appear to be altered by domain γ at least to any significant level.

Single-Stranded DNA Binding Is Mediated through Domain γ. The results, presented above, indicated that the DNA binding is associated with domain γ and ruled out any involvement of domains α and β in this process. We have described earlier (*16*) the presence of a leucine zipper motif (*34*, *35*) in the C-terminus of DnaB protein, defined here as domain γ (*16*). DNA stimulation of ATPase activity was observed only with wtDnaB and DnaB$\beta\gamma$ proteins (Figures 4 and 7). The rate of ATP hydrolysis was stimulated in the presence of DNA, to the level of the wild-type protein. Further analysis of the DNA binding affinity, as determined by the K_d of oligo$(dT)_{50}$ binding, demonstrated that these two polypeptides bind DNA with comparable affinity as determined by gel shift assays and DNA titration of ATPase activity (data not shown).

Domain α Plays an Essential Role in DnaB Helicase Action. The primary function of a replicative DNA helicase is the unwinding of the double-stranded DNA in the replication fork during replication. The hydrolysis of ATP supports this activity by providing the energy required to disrupt the hydrogen bonds of adjacent base pairs in a double helix. Thus, it was of great interest to evaluate the DNA unwinding abilities of various deletion mutant proteins to determine the roles of various domains in the energy transduction mechanism (Figure 8). None of the truncated proteins were capable of unwinding DNA. These results strongly indicated an essential role of domain α in the helicase activity of DnaB protein, as DnaB$\beta\gamma$ was incapable of DNA unwinding despite the fact that it had a DNA-dependent ATPase activity comparable to that of the wild-type DnaB helicase and it was hexameric in structure. Consequently, a hexameric structure and a DNA-dependent ATPase activity are obvious prerequisites but are not sufficient for the ability to unwind DNA. Domain α is thus likely involved in direct transduction of the energy derived from ATP hydrolysis into unwinding of the DNA strands and translocation of the helicase in addition to its contribution to dimerization.

Role of Mg^{2+} in the Structure and Function of DnaB Protein. Mg^{2+} has been shown to influence the stability of the hexameric state of DnaB protein (*2*). Wild-type and truncated DnaB proteins were used to probe the role of Mg^{2+} in the structure and function of the DnaB helicase, as well as the locus of Mg^{2+} interaction. There were no observed differences in the helicase activities of wild-type DnaB protein (at 1 mg/mL concentration) prepared in the presence or absence of Mg^{2+} (Figure 9A). There were differences observed in the DNA unwinding ability of dilute (100 μg/mL) solutions of the protein prepared in the absence of Mg^{2+} (Figure 9B). Similar results were obtained for the effect of Mg^{2+} on the ATPase activity of DnaB protein (Figure 9C). Because Mg^{2+} is known to be involved in stabilizing the DnaB helicase (*2*), we examined the role of Mg^{2+} on the oligomerization of the protein and the site of its interaction. Experiments, as described above, examining the structures of the truncated fragments had suggested that two different protein−protein interactions were responsible for formation of the DnaB hexamer. The oligomeric structures of wild type, DnaB$\alpha\beta$, and DnaB$\beta\gamma$, prepared in the presence or absence of Mg^{2+}, were examined and are shown in Figure 10. Wild-type protein and DnaB$\beta\gamma$ appeared to retain the hexameric structures in the presence and absence of Mg^{2+} in the concentration range we have studied. However, as shown in Figure 10C, Mg^{2+} clearly exerted an effect on the oligomeric structure of DnaB$\alpha\beta$. As shown previously (Figure 3), DnaB$\alpha\beta$ had an elution volume consistent with that of a dimeric structure in the presence of Mg^{2+}. In the absence of Mg^{2+}, this elution profile changed to that which corresponded

Table 2: Summary of Function(s) of Each of the Three Domains of DnaB Helicase

domain α residues 1−156	domain β residues 157−302	domain γ residue 303−471
energy transduction	ATPase active site	DNA binding site
protein−protein interaction site for hexamer formation shared with domain β	nucleotide binding site	ATPase regulatory site
	protein−protein interaction site for hexamer formation shared with domain α	protein−protein interaction site for hexamer formation

to a monomer (Figure 10C). Therefore, it appears that the locus of interaction of DnaB protein with Mg^{2+} lies within site 1 and is involved in the dimer formation. The presence of domain γ as well as higher protein concentration appears to minimize the role of the Mg^{2+} effect on stabilization of the hexamer.

The design and construction of a variety of truncated DnaB proteins and their subsequent analysis allowed us to test their enzymatic and structural features. Overall, the results showed that certain enzymatic functions such as ATPase and DNA binding reside within a single autonomous domain, while others such as DNA unwinding required the concerted actions of some or all of its domains. Domain β, which contains a nucleotide binding motif, was found to be capable by itself of ATP hydrolysis. On the other hand, domain γ was found to contain the determinants required for DNA stimulation of ATPase activity and was required for hexamer formation. Despite the lack of any detectable enzymatic activities, domain α was essential for DNA helicase activity, and its likely role is in the transduction of the energy of ATP hydrolysis to unwinding of duplex DNA. A summary of the findings pertaining to the function(s) of each domain is presented in Table 2.

Questions that remain to be answered include the mechanism of DNA binding mediated through domain γ and the exact nature of the role played by domain α in DnaB function. Nevertheless, the studies presented here have clearly defined domains within which the determinants required for specific activities of this protein reside and help to further define its structure and function. In the following paper, we have presented results concerning the mechanism of domain γ mediated DNA binding.

ACKNOWLEDGMENT

The authors thank Drs. G. Bailin and R. Sharma, University of Medicine and Dentistry of New Jersey, for critical reading of the manuscript and Mr. Kim Sokoloff, University of Medicine and Dentistry of New Jersey for help with illustrations and photography. The authors also thank Mr. William Fricke for technical assistance with various assays, Dr. Fanxiu Zhu for helpful discussions, and Mr. Ananda Ray and Mr. Koustav Mukherjee for help with protein assays.

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BI990048T