Structure and Function of *Escherichia coli* DnaB Protein: Role of the N-Terminal Domain in Helicase Activity†

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ABSTRACT: We have analyzed the contributions of specific domains of DnaB helicase to its quaternary structure and multienzyme activities. Highly purified tryptic fragments containing various domains of DnaB helicase were prepared. Fragment I lacks 14 amino acid (aa) residues from the N-terminal of DnaB helicase. Fragments II and III are 33-kDa C-terminal and 12-kDa N-terminal polypeptides, respectively, of fragment I. The single-stranded DNA-dependent ATPase and DNA helicase activities of DnaB helicase and its fragments were examined in detail. The ATPase activities of native DnaB helicase and fragment I were comparable; however, the ATPase activity of fragment II was somewhat diminished. Unlike the ATPase activity, the DNA helicase activity was totally abolished in fragment II and was not complemented by the addition of equimolar fragment III. Consequently, the N-terminal 17-kDa domain appeared to have an indispensable role in the DNA helicase action, but not in other enzymatic activities. Fragment I had a hexameric structure similar to that observed with DnaB helicase in both size exclusion HPLC (SE-HPLC) and chemical cross-linking studies. SE-HPLC analysis indicated that fragment II had an apparent hexameric form. However, a detailed chemical cross-linking analysis showed that it formed stable dimers but the formation of a stable hexamer was severely impaired. Thus, the N-terminal domain appeared to have a strong influence on the hexamer formation. Protein sequence analysis indicated that the DnaB protein has a putative "leucine zipper" [I.X6.L.X6.L.X6.L.X6.L] between amino acid residues 360 and 389 with a consensus basic region located between amino acid residues 322 and 331 at the N-terminal, which is a likely site for DNA binding. This site may form an appropriate dimerization site as well as a DNA binding site in the DnaB helicase.

The DNA helicases play important roles in DNA replication as well as various other cellular DNA transactions (Geider & Hoffman-Berling, 1981; Nossal, 1983; Matson & Kaiser-Rogers, 1990; Matson, 1991; Kornberg & Baker, 1992; Lohman, 1993). A number of DNA helicases have been characterized in *Escherichia coli* with diverse functions and mechanism of action (Nossal, 1983; Matson & Kaiser-Rogers, 1990; Kornberg & Baker, 1992; Yancey-Wrona et al., 1992). Several phage and viral DNA helicases that replicate the respective phage or viral DNA have also been characterized (Goetz et al., 1988; Matson et al., 1983; Venkatesan et al., 1982). However, among all prokaryotic and eukaryotic organisms, the DnaB helicase is still the only replicative DNA helicase that has been shown to be indispensable in the replication of the cellular chromosome (Lebowitz & McMacken, 1986; Funnell et al., 1987; Bramhill & Kornberg, 1988). The gene for the DnaB protein has been cloned, expressed, and sequenced (Ueda et al., 1978; Arai et al., 1981; Nakayama et al. 1984). Thus, DnaB helicase is a model cellular DNA helicase that could be used for analyzing the mechanism of various enzymatic actions and protein interactions that are involved in DNA unwinding in the replication fork. The mechanism of action of various helicases, including DnaB helicase, is complex, and detailed studies are required in order to understand their functions in the cellular DNA metabolic processes including DNA replication.

The *E. coli* DnaB helicase is a hexameric protein composed of six identical 52-kDa monomers (Kornberg & Baker, 1992; Arai & Kornberg, 1981a,b; Arai et al., 1981). It is a multifunctional enzyme that controls the formation and translocation of the replisome in *E. coli* and λ phage chromosomal DNA replication (LeBowitz & McMacken, 1986; Biswas & Biswas, 1987; Funnell et al., 1987; Bramhill & Kornberg, 1988). During replication of DNA it interacts with a host of other replication proteins such as DnaA protein, DnaC protein, DnaG protein or primase, λP protein, etc. (Biswas & Biswas, 1987; LeBowitz & McMacken, 1986; Arai & Kornberg, 1981c; Bramhill & Kornberg, 1988). The ability to physically interact and modulate the functions of other replication proteins endows DnaB helicase with a central role in the assembly of the primosome and subsequent movement of the replication apparatus (Kornberg & Baker, 1992; Arai & Kornberg, 1981c). DnaB helicase requires the aid of DnaC protein to form a $[DnaB{\cdot}DnaC]_6$ complex, with suppressed ATPase and helicase activities, which in turn delivers the DnaB helicase to the DnaA·*Ori*C complex for initiation of replication (Funnell et al., 1987).

Nakayama et al. (1984) have shown that the DnaB protein can be selectively cleaved by trypsin to generate specific fragments of DnaB protein. We have modified this procedure to develop a means of purification and isolation of the fragments: fragment I, which lacked 14 amino acid residues from the N-terminal of DnaB helicase; fragments II (33 kDa) and III (12 kDa), formed by the cleavage of fragment I and possessing the C-terminal and N-terminal regions of DnaB helicase, respectively. We have carried out a detailed analysis of the ATPase and helicase activities, as well as the quaternary structure, of each of these fragments in order to further define the functions of various domains of the DnaB protein. Using SE-HPLC and chemical cross-linking, the oligomerization characteristics of these fragments were examined. The

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primary structure of the DnaB protein was analyzed to delineate the putative locations of various activity domains.

MATERIALS AND METHODS

DnaB Protein. DnaB protein was purified to homogeneity as described (Arai et al., 1981) from YS1recA (pKA1) which was received as a kind gift from Dr. K. Arai. Some of the studies were done with DnaB protein obtained as a generous gift from Dr. Arthur Kornberg of Stanford University.

Nucleic Acids, Enzymes, and Other Reagents. Ultrapure deoxy- and ribonucleotides were obtained from Pharmacia and were used without further purification. [α-^{32}P]ATP and [γ-^{32}P]ATP were obtained from Amersham. *E. coli* SSB was purchased from Promega Biotech, and its purity was verified by SDS–PAGE (Milwaukee, WI). T4 polynucleotide kinase was obtained from New England Biolabs Inc. (Beverly, MA). Ultrapure trypsin was from Worthington Biochemicals. All chemicals used to prepare buffers and solutions were reagent grade and were purchased from Fisher Chemical Company (Pittsburgh, PA). Polyethyleneimine-cellulose TLC strips were from J. T. Baker Chemical Co. (Pittsburgh, PA). Oligonucleotides were synthesized by Oligos Inc. (Corvallis, OR). The oligonucleotides were analyzed and further purified by 20% PAGE–8M urea. The final purity of the oligonucleotides were >99% as determined by autoradiography of the phosphorylated products.

Buffers. 1× TBE contained 89 mM Tris-borate[1] (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.

ATPase Assays. The ATPase assays were carried out as previously described (Biswas et al., 1986, 1993b; Biswas & Biswas, 1987). The amount of DnaB protein 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 (1000–2000 cpm/pmol), and enzyme 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 polyethylenimine-cellulose strips which were prespotted with ADP–ATP marker. The strips were developed with 1 M formic acid, 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.

In kinetic analyses a given set of reactions were carried out in a single tube and were initiated by the addition of DnaB protein. At the indicated time points, 10-μL aliquots were removed placed in tubes containing 2 μL of 200 mM EDTA and held on ice until completion of the last time point. The remainder of the assay was carried out as described above.

Helicase Assays. The helicase assays were based on the methods described by Biswas et al. (1993c). The substrate was prepared by hybridizing a synthetic 60-mer oligonucleotide to M13mpl9 ssDNA as previously described (Biswas et al., 1993b). 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 (10000–20000 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, 100 μg/mL each BSA and *E. coli* SSB, 17 fmol (10000–20000 cpm/μL) of substrate, and the indicated amount of enzyme 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 volts. Following electrophoresis, the gels were dried and exposed to Fuji RX film at −80 °C for 12 h. Helicase activity was quantitated by scintillation counting of excised substrate/product bands from the dried gels as described by LeBowitz and McMacken (1986).

Preparation of Tryptic Fragments of DnaB Protein: Fragment I. The 50.7-kDa fragment I was prepared under conditions similar to those described by Nakayama et al. (1984). In order to maximize the amount of fragment I obtained from this procedure kinetic analyses were carried out to determine the optimal trypsin/DnaB protein ratio and digestion time (data not shown). The optimized reaction conditions were as follows. DnaB protein (500 μg) was digested with trypsin at a 1:20 ratio in 0.5 mL of a solution containing 50 mM Tris-HCl (pH 7.5), 25% glycerol, 1 mM ATP, and 10 mM $MgCl_2$. The reaction was carried out at 0 °C for 9 min and was terminated by the addition of 5 μL of 2.5 mg/mL TLCK and 1 mg/mL leupeptin. The mixture was immediately fractionated by SEHPLC (Pharmacia Superdex 200 HR) using buffer B as eluant. Fractions were analyzed for protein content and by SDS–PAGE. The HPLC chromatogram is presented in Figure 1. The appropriate fractions were pooled and concentrated by ultrafiltration using a Spectrapor C50 membrane. The purified fragments were stored in aliquots at −80 °C until further use. Fragment I was stable for at least 6 months at −80 °C although repeated freezing and thawing was avoided.

Fragment II and Fragment III. Pilot kinetic studies were carried out to determine appropriate conditions for trypsin digestion and the following conditions were used for the preparation of fragments II and III: DnaB protein (500 μg) was digested with trypsin at a 1:100 ratio in 0.5 mL of a solution containing 25 mM Tris-HCl (pH 7.5), 5% glycerol, 2 mM ATP, and 1 mM $MgCl_2$. The reaction was carried out at 0 °C in ice-water for 80 min and was terminated by the addition of 5 μL of 2.5 mg/mL TPCK and 1 mg/mL leupeptin and immediately fractionated. Under these conditions the DnaB protein was completely digested into a mixture of fragment II and III which were resolved by SEHPLC as described for fragment I (Figure 1). The fractions corresponding to fragment II and III were pooled separately and concentrated by ultrafiltration using a Amicon YM-10 membrane. The purified fragments were stored in aliquots at −80 °C until further use. The peptides were observed to be stable for at least 6 months at −80 °C although repeated freeze-thawing was avoided.

SE-HPLC Fractionation of DnaB Protein. In order to rule out any detrimental effects of the chromatographic and ultrafiltration procedures on enzymatic activities, DnaB

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; TPCK, *p*-tosylphenyl chloromethyl ketone; TLCK, *p*-tosyl lysyl chloromethyl ketone.

protein was also treated and purified by HPLC under conditions identical to those described for fragment II except that trypsin was omitted. SE-HPLC was carried out on a Pharmacia Superdex 200 HR column using buffer B as eluant.

Glutaraldehyde Cross-Linking. Glutaraldehyde cross-linking was carried out essentially as described by Hermann et al. (1981). Proteins in 25 mM Hepes (pH 7.5), 10% glycerol, 5 mM $MgCl_2$, 0.1 M NaCl, and 1 mM DTT were treated with varying amounts (as indicated) of glutaraldehyde. The reactions were allowed to proceed at room temperature for 5 min (unless otherwise indicated). Reduction of the reaction mixtures was then carried out by adding sodium borohydride to the reaction mixtures in an amount equimolar to the amount of glutaraldehyde present. The reaction mixtures were chilled on ice for 20 min and neutralized by the addition of Tris-HCl (pH 6.8). The products were then analyzed by SDS–PAGE followed by Coomassie staining.

Peptide Sequencing. Peptide sequencing was carried out in an Applied Biosystems Inc. (Foster City, CA) gas phase peptide sequencer.

Other Methods. Protein concentrations were estimated according to the method of Bradford (1976), using bovine serum albumin as a standard.

RESULTS

Preparation of Tryptic Fragments of DnaB Protein. In order to study the functions of various domains of the DnaB protein, we have prepared highly purified tryptic fragments by limited proteolysis under controlled conditions followed by HPLC purification. As indicated under Materials and Methods, we have used the procedure of Nakayama et al. (1984) to prepare the 50 690-Da fragment I of DnaB protein, which lacks 14 N-terminal amino acid residues. DnaB was digested with trypsin at a 1:20 ratio for 9 min at 0 °C to form fragment I, and it was purified by size exclusion HPLC (Figure 1A). Our studies indicated that these proteolysis conditions were not suitable for the formation fragment II containing the C-terminal 33-kDa domain of fragment I. However, digestion of DnaB protein using a 1:100 trypsin:DnaB protein ratio for 80 min at 0 °C was just sufficient for complete proteolysis of DnaB protein to fragments II and III, as determined by kinetic studies. The HPLC chromatogram of the final digestion product containing fragments II and III is shown in Figure 1A. An SDS–PAGE of all of the purified fragments utilized in this study is shown in Figure 1B. Our analysis indicated that each of these fragments was essentially homogeneous (>95%). As the proteolysis conditions for generating fragment II were different than that used by Nakayama et al. (1984), we have also determined the N-terminal amino acid sequence of fragment II by protein sequencing. The N-terminal peptide sequence was NH_2-Ala-Asn-Lys-Asp-Glu-, indicating that despite the altered reaction conditions the cleavage occurred at exactly the same site as observed by Nakayama et al. (1984).

Single-Stranded DNA-Dependent ATPase Activities of DnaB Protein and Its Tryptic Fragments. In order to delineate the function(s) of the N-terminal domain of DnaB protein in the ssDNA dependent ATPase activity, we have extensively analyzed the kinetic parameters of the ATP hydrolysis by DnaB protein and its highly purified tryptic fragments I and II.

DnaB protein, fragment I, and fragment II had strong DNA-dependent ATPase activity (Figure 2A). These three proteins were compared in parallel ATPase assays using equimolar

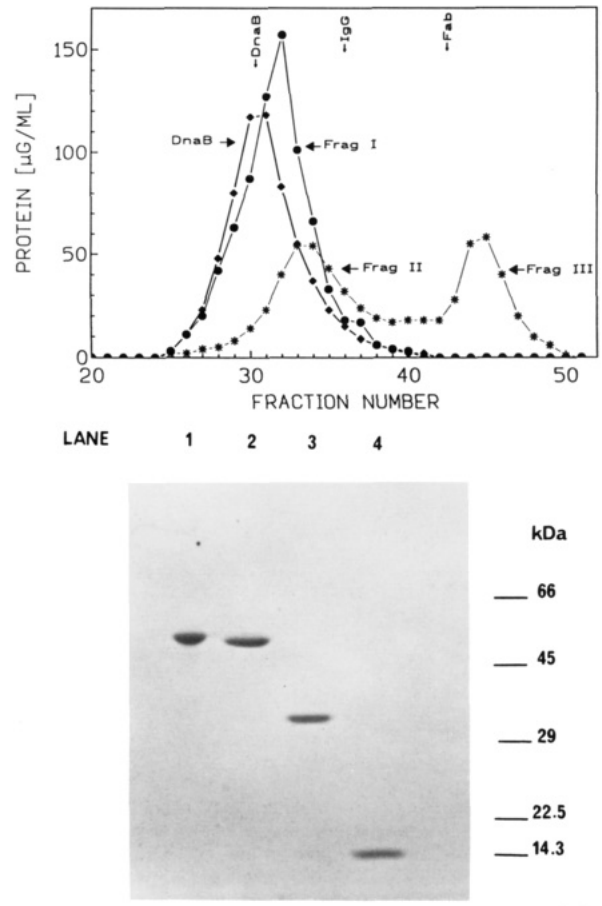

FIGURE 1: (A, top) Size estimation of tryptic fragments of DnaB protein by SE-HPLC analysis. Chromatogram from SEHPLC purification of DnaB helicase (◆), fragment I (●), and fragments II (*) and III (*) using Pharmacia Superdex 200 HR chromatography column in buffer B as described under Materials and Methods. Marker proteins bovine immunoglobulin G (IgG) and its Fab fragment were chromatographed separately under identical conditions. (B, bottom) SDS–PAGE analysis of tryptic fragments I, II, and III. Fragments were prepared and purified as shown in panel A. Five micrograms of each protein was analyzed on a 5–15% SDS–PAGE gel which was stained with Coomassie Blue R250. (Lane 1) DnaB protein; (lane 2) fragment I; (lane 3) fragment II; (lane 4) fragment III. Markers were BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (22.5 kDa), and lysozyme (14.3 kDa).

amounts of each enzyme in the presence and absence of M13mp18 ssDNA. All three enzymes showed significantly lower levels of ATPase activities in the complete absence of DNA cofactor (Figure 2). However, in each case the ATPase activities were stimulated approximately 5-fold in the presence of ssDNA. Although DnaB protein and fragment I were very comparable in ATPase activities in the presence or absence of ssDNA, the extent of ATP hydrolysis was in general higher in the case of DnaB protein at subsaturating levels. The extent of ATP hydrolysis by fragment II, in the presence or absence of DNA, was somewhat lower than DnaB protein and fragment I. The N-terminal domain, fragment III, did not have any ATPase activity (data not shown).

A time course analysis of ATP hydrolysis by fragment II (1 pmol) indicated that the rate of hydrolysis was linear for approximately 10 min (Figure 2B). The kinetics of ATP hydrolysis by fragment II and the DnaB protein were parallel; however, the extent of hydrolysis was diminished with fragment II similar to that observed earlier (Figure 2A). A ssDNA titration of fragment II demonstrated that the stimulation by ssDNA was comparable with both DnaB protein and fragment II (Figure 2C).

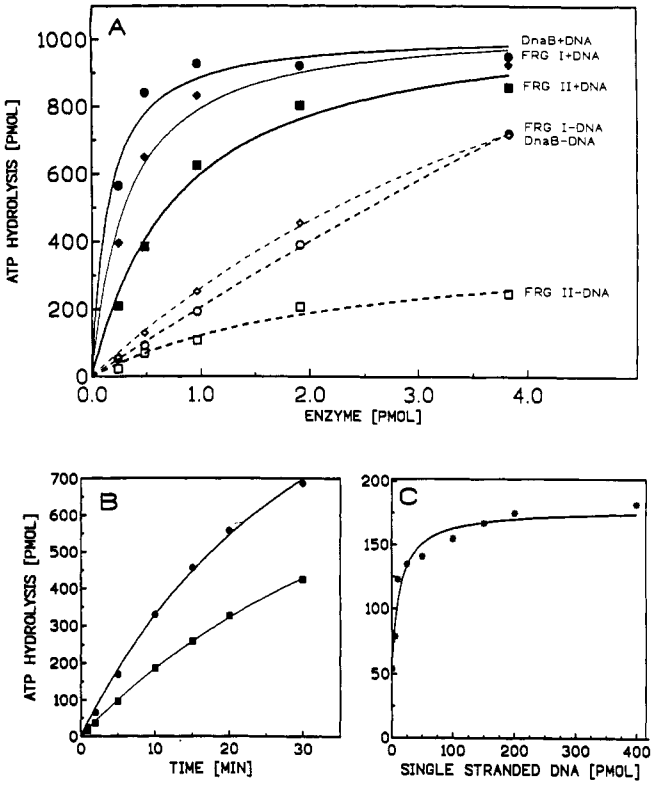

FIGURE 2: (A) Comparison of ATPase activities of DnaB protein and its tryptic fragments. Standard ATPase assays were carried out as described under Materials and Methods at 37 °C for 30 min, in the presence and absence of 200 pmol of M13mp18 ssDNA with indicated amounts of the following enzymes: DnaB protein, –DNA (○), +DNA (●); fragment I, –DNA (◇), +DNA (◆); fragment II, –DNA (□), +DNA (■). Each point represents the average of duplicate experiments. The curves in all plots were generated by nonlinear least-squares regression analysis of individual sets of data using INPLOT IBM-PC program. (B) Time course of ATP hydrolysis by fragment II. Standard ATPase assays were carried out as described for panel A at 37 °C for the indicated period in the presence of 200 pmol of M13mp18 ssDNA and 0.5 pmol of fragment II (■) or DnaB protein (●). Aliquots were taken at the indicated time and were analyzed for ATP hydrolysis. (C) DNA titration of the ATPase activity of fragment II. The ATPase assay was carried out as described for A at 37 °C for 5 min with the indicated amounts of DNA and 0.5 pmol of enzyme.

The results presented in Figure 2 demonstrated that there were some minor differences in the ssDNA-dependent ATPase activities among DnaB protein and fragment I. The differences were more pronounced between DnaB protein and fragment II. Consequently, the steady-state kinetic parameters of ATP hydrolysis by DnaB protein and fragment II were determined in order to critically evaluate the alterations in substrate binding and hydrolysis resulting from the deletion of the N-terminal region of DnaB protein. Analysis was carried out in the concentration range of 5–100 μM for ATP with 0.2 pmol of DnaB protein or fragment II, and the reactions were allowed to proceed for 2 min (Figure 3). The values for K_m of DnaB protein and fragment II were 22 and 33 μM, respectively. The difference in the K_m values were not significant, and the results indicated that the affinity for ATP remained relatively unchanged in fragment II. The only notable difference observed was in the values of the V_{max}. The V_{max} values indicated that fragment II (V_{max} = 6.8 pmol/min) had an attenuated rate of ATP hydrolysis in comparison to the DnaB protein (V_{max} = 10.2 pmol/min). Although these results clearly indicated a specific difference in the kinetics of ssDNA-dependent ATP hydrolysis between DnaB protein and its truncated form, fragment II, the difference was only

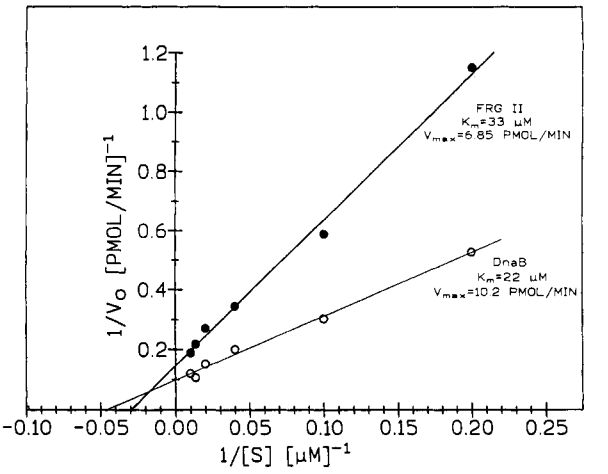

FIGURE 3: Kinetics of ATP hydrolysis by DnaB protein and fragment II. Influence of substrate concentration on the initial rate of ATP hydrolysis by DnaB helicase (○), and fragment II (●). The ATPase assays were carried out at 37 °C for 5 min with 0.2 pmol of the indicated enzymes and varied concentrations of [α-^{32}P]ATP.

very limited. Fragment II appeared to retain most of the major enzymatic characteristics of the ATP hydrolysis of DnaB protein despite the lack of 171 N-terminal amino acid residues. The addition of the purified N-terminal fragment III did not alter the ATPase activity of fragment II (data not shown). The N-terminal domain appeared to be dispensable as far as the DNA binding, ATP binding, and the ssDNA-dependent ATPase activities were concerned.

DNA Helicase Activities of DnaB Protein and Its Tryptic Fragments. The DnaB protein and its tryptic fragments I and II have varying but relatively strong ATPase activities. A ssDNA-dependent ATPase activity is an important prerequisite for DNA helicase activity. Consequently, we have analyzed the helicase activity of these DnaB fragments. In order to eliminate any diminution of activity during digestion and purification of the fragments, the DnaB protein, used as the control, was treated and purified by HPLC under conditions identical to that described for fragment II except that trypsin was omitted. Thus, any difference in the activities did not reflect alteration in the structure and function as a result of extensive purification. All of the fragments were compared on a equimolar levels of 2.3, 4.6, and 9.2 pmol per assay. The DnaB protein retained full DNA helicase activity before and after mock digestion and purification (lanes 3–6, Figure 4) as well as ATPase activity (data not shown). Consequently, comparison of relative helicase activity, at equimolar levels, was meaningful, and any alterations in the activity were not artifacts of the purification procedure. The DNA helicase activity observed with DnaB protein (lanes 4–6) was essentially comparable to that observed with fragment I (lanes 7–9). Analysis of the ATPase activities of these two proteins (Figure 2) showed that the ATPase activities were comparable although not identical. Thus, comparison of ATPase and DNA helicase activities of the two polypeptides indicated that the deletion of the N-terminal 14 amino acid residues did not significantly alter these two enzymatic activities of DnaB protein. However, it should be noted that the N-terminal 14 amino acid residues may play a role in other functions of DnaB protein such as the protein–protein interactions required for the formation of the replisome (Nakayama et al., 1984; Kornberg & Baker, 1992). Studies with the ATPase activities, as shown above, indicated that despite the lack of a major portion of the N-terminal domain, fragment II retained in large part (≥60%) of the ssDNA-dependent ATPase activity of the DnaB protein (Figure 2);

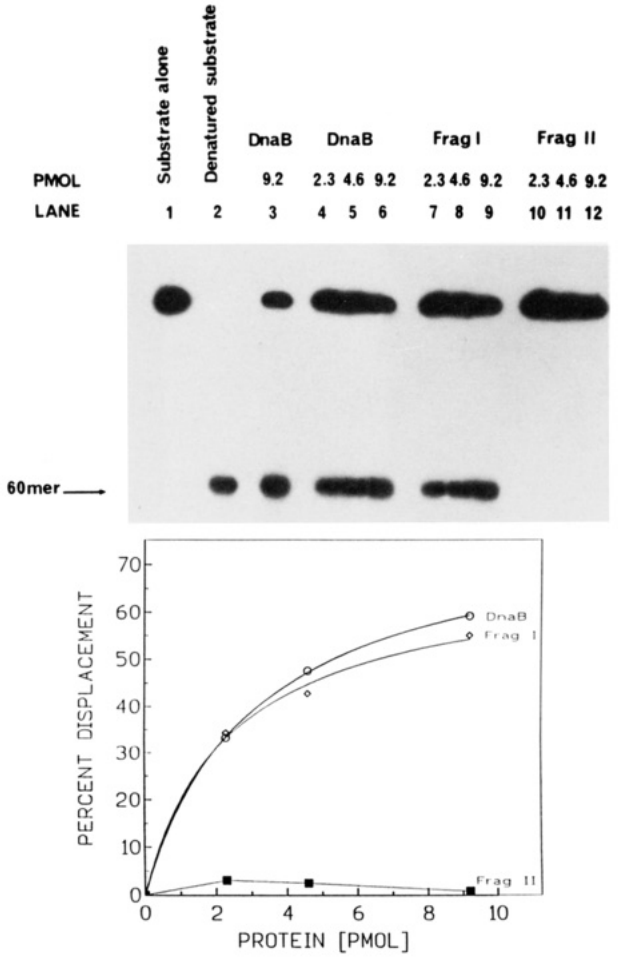

FIGURE 4: (A, top) Helicase activities of DnaB protein and its fragments. A standard helicase assay was carried out utilizing the 60 bp partial duplex substrate and described under Materials and Methods. (Lane 1) Substrate and SSB alone. (Lane 2) Heat denatured substrate. (Lane 3) 9.2 pmol of DnaB protein which had not been subjected to SE-HPLC. (Lanes 4–6) DnaB protein, 2.3, 4.6, and 9.2 pmol, respectively. (Lanes 7–9) Fragment I, 2.3, 4.6, and 9.2 pmol, respectively. (Lanes 10–12) Fragment II, 2.3, 4.6, and 9.2 pmol, respectively. (B, bottom) Quantitative analysis of the helicase activity of DnaB helicase and its fragments. The gel fragments corresponding the appropriate signals in the autoradiogram, shown in panel A, were excised counted by scintillation counting as described under Materials and Methods. The curves were generated by nonlinear least-squares regression analysis of each set of data for DnaB helicase (○), fragment I (◇), and fragment II (■).

including the essential dependence on an ssDNA cofactor. Thus, it was anticipated that fragment II would have some DNA helicase activity. The results presented in Figure 4 clearly demonstrated that fragment II did not have measurable DNA helicase activity (lanes 10–12, Figure 4). The helicase substrate used in the above comparison contained a 50 bp duplex, and it was possible that helicase activity of fragment II could be detected using shorter duplexes. Therefore, we also tested substrates with shorter duplex regions of 30 and 20 bp. No displacement was observed by fragment II even with a substrate containing a 20 bp duplex (data not shown). Neither fragment III had any helicase activity of its own nor did it complement the helicase activity of fragment II (data not shown). However, the N-terminal 17-kDa domain is indispensable in the helicase activity of DnaB protein, since fragment II differs from fragment I by removal of this N-terminal domain.

Cross-Linking Studies To Determine the Quaternary Structures of Tryptic Fragments of DnaB Protein. HPLC size exclusion chromatographic studies indicated that both

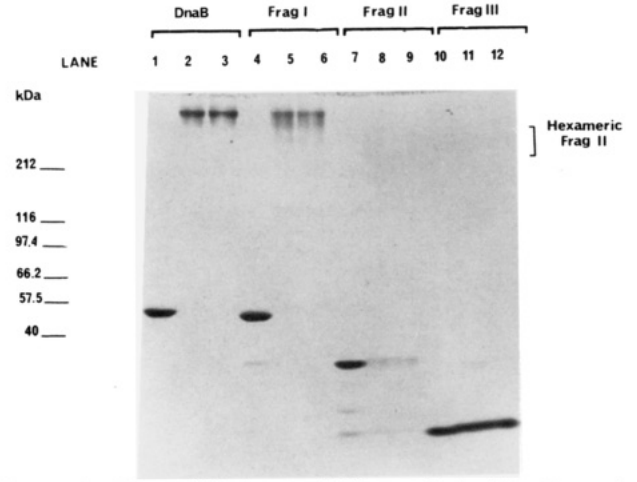

FIGURE 5: Glutaraldehyde cross-linking analysis of DnaB protein and its fragments. The experimental details of the procedure are described under Materials and Methods. The cross-linking was carried out for 5 min using 5 μg of the respective polypeptides and the indicated amounts of glutaraldehyde. Following cross-linking the samples were analyzed on 3–18% SDS–PAGE followed by Coomassie staining. Molecular weight standards (from Promega) were as follows: myosin, β-galactosidase, phosphorylase B, bovine serum albumin, catalase, and aldolase. (Lanes 1–3) DnaB protein; (lane 1) 0 mM glutaraldehyde, (lane 2) 0.1 M glutaraldehyde, (lane 3) 0.2 M glutaraldehyde. (Lanes 4–6) Fragment I; (lane 4) 0 mM glutaraldehyde, (lane 5) 0.1 M glutaraldehyde, (lane 6) 0.2 M glutaraldehyde. (Lanes 7–9) Fragment II; (lane 7) 0 mM glutaraldehyde, (lane 8) 0.1 M glutaraldehyde, (lane 9) 0.2 M glutaraldehyde. (Lanes 10–12) Fragment II; (lane 10) 0 mM glutaraldehyde, (lane 11) 0.1 M glutaraldehyde, (lane 12) 0.2 M glutaraldehyde.

fragment I and fragment II retained the homohexameric quaternary structure of DnaB protein (Figure 1). Therefore, our results indicated that the sites for hexamer formation of DnaB protein remained intact in fragment II. As fragment II showed no helicase activity, we decided to investigate further any alterations in the quaternary structure of fragment II. It was hoped that further information regarding structural aspects of fragment II might help to explain the loss of helicase activity. We used several cross-linking agents; however, glutaraldehyde cross-linking appeared to be the most efficient (data not shown). Glutaraldehyde has been used efficiently to cross-link multimeric proteins, and reduction of the imine moieties with $NaBH_4$ results in the formation of a permanent complex (with nonhydrolyzable covalent bonds) which can be analyzed by SDS–PAGE (Hermann et al., 1981). The cross-linking of DnaB protein with glutaraldehyde/$NaBH_4$ resulted in the formation of a single hexameric band of 300 kDa (Figure 5, lanes 2 and 3) at both 100 and 200 mM glutaraldehyde. In the case of fragment I (lanes 5 and 6), the predominant species was the hexamer, but a small amount of pentamer was also visible. Thus, there appears to be a decrease in the cross-linking efficiency in fragment I. This small decrease in cross-linking could be due to several factors including the conformational perturbations resulting from the removal of the 14 amino acid residues. The most important finding was that in the case of fragment II, under identical reaction conditions, no discreet hexamer formation was observed (lanes 8 and 9). The observed cross-linked products were highly diffused bands (>212 kDa) marked as "Hexameric Frag II". Because of the high concentrations of glutaraldehyde, these diffuse bands could be due to random cross-linking of glutaraldehyde to fragment II. Fragment III did not undergo any measurable cross-linking (lanes 11 and 12).

We have carried out a kinetic experiment in the 0–30-min range with a lower concentration of glutaraldehyde with DnaB

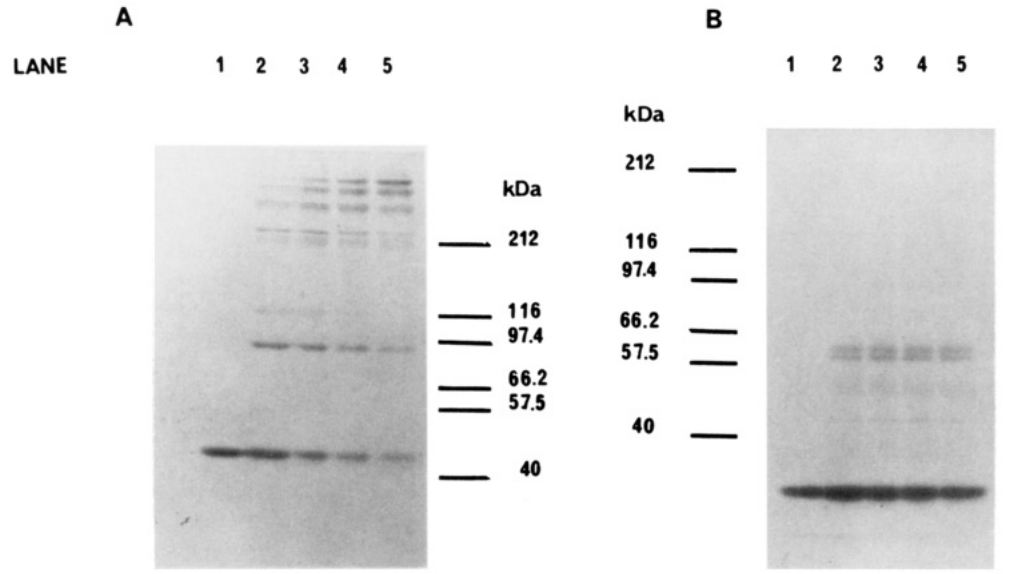

FIGURE 6: Time course analysis of glutaraldehyde cross-linking of DnaB protein and fragment II. (A) Five micrograms of DnaB protein was crosslinked using 2 mM glutaraldehyde for the times indicated, followed by analysis by 3–18% SDS–PAGE. (Lane 1) 2.5 μg of DnaB protein, no glutaraldehyde. (Lanes 2–5) Glutaraldehyde cross-linked DnaB protein; (lane 2) 5 min, (lane 3) 10 min, (lane 4) 15 min, (lane 5) 30 min. (B) Ten micrograms of fragment II were cross-linked using 5 mM glutaraldehyde for the times indicated, followed by analysis by 5–15% SDS–PAGE. (Lane 1) 5.0 μg fragment II, no glutaraldehyde. (Lanes 2–5) Glutaraldehyde cross-linked fragment II; (lane 2) 5 min, (lane 3) 10 min, (lane 4) 15 min, (lane 5) 30 min. Molecular weight standards for both panels A and B were as given in the legend to Figure 5.

protein (Figure 6A) and fragment II (Figure 6B). In the case of DnaB protein, at 5 min (Figure 6A, lane 2) dimer was the predominant product and formation of higher oligomers increased steadily with time (Figure 6A, lanes 3–5), and at 30 min (Figure 6A, lane 5) the predominant product was hexamer followed by the pentamer and tetramer. Thus, under these experimental conditions it is possible to identify all possible oligomeric species resulting from a hexameric protein complex.

When an analogous experiment was carried out with fragment II, the distribution of species formed differed significantly from that observed with DnaB protein. The major cross-linked product was a pair of dimers of 66 kDa (Figure 6B, lanes 2–4). The dimer formation was rapid, and cross-linking longer than 5 min resulted in minor bands larger than the dimer (Figure 6B, lanes 3–5). Cross-linked species that appeared to be trimer, tetramer, and pentamer were formed only in very small quantities (<1%). Hexamer was not observed at any time point in this study. The formation of the dimer doublet was likely due to heterogeneous sites of cross-linking that resulted in slightly different mobilities in a high resolution gradient gel. Doublets were also observed, although less pronounced, with DnaB protein cross-linking.

Thus, our crosslinking studies clearly indicated that a stable dimer formation is evident in fragment II. However, the hexamer formed from three dimers was likely weak and not amenable to glutaraldehyde cross-linking. DnaB protein must have more than one protein–protein interface that leads to the formation of a hexamer. It is possible that rearrangement of the reactive amino groups occurs during trypsin digestion, and, as a result, one of the protein–protein interfaces was impaired so that the hexamer as such could not be cross-linked. Increasing glutaraldehyde concentration led to more incorporation of glutaraldehyde in fragment II and its dimer without further appreciable intermolecular cross-linking (data not shown).

Analysis of the DnaB protein sequence for various structural motifs indicated the presence of a unique "leucine zipper motif" in the C-terminal end of the molecule (Kozarides & Ziff, 1989; O'Shea et al., 1991; Turner & Tijan, 1989; Vinson et al., 1989, 1993). The leucine zipper motif, present in the DnaB helicase sequence, is shown in Chart 1. With the exception of an isoleucine at position 360, this is a dimerization domain of a "leucine zipper" (Vinson et al., 1989, 1993). In addition, this region displays a 4–3 heptad repeat similar to that observed with the leucine zipper region of GCN4 (O'Shea et al., 1989; Ellenberger et al., 1992). Perhaps this is one of the dimerization sites for DnaB protein, which incidentally remains intact in fragment II. The dimer formation observed with fragment II in the cross-linking studies could well be due to this sequence motif. A basic region is frequently observed in eukayotic sequence specific DNA binding proteins at the N-terminal upstream (–1 to –25 aa residue) of the leucine zipper, and the two basic sites of the dimer are normally thought to be associated with DNA binding. We found a basic region (Chart 1) in the N-terminal upstream region of the putative leucine zipper, which may be involved in DNA binding. O'Neil et al. (1990) have designed a synthetic leucine zipper peptide that binds DNA and dimerizes *in vitro* similar to that observed with leucine zipper proteins. The basic region between 322-Val and 331-Arg with sequence VRSRARRIAR in DnaB protein is homologous to the major basic region of the synthetic DNA binding leucine zipper model peptide described by O'Neil et al. However, this sequence in DnaB is located further upstream (–36 aa residue) of the putative leucine zipper than those associated with eukaryotic leucine zippers. The role(s), if any, of this leucine zipper motif in the structure and function of DnaB helicase would require further investigation.

Chart 1. Amino Acid Sequence of the Leucine Zipper and the Proximal Basic Region in DnaB Helicase

Basic Region

320 T E V ***R S R A R R I A R***

332 E H G G I G L I M I D Y L Q L M R V P A L S D N R T L E

360 ***I A E I S R S L K A L A K E L N V P V V A L S Q L N R S L*** E Q

←----------→

Putative Leucine Zipper

A second protein–protein interaction site is probably located further upstream in the molecule as the deletion of the N-terminal 170 amino acid residues resulted in a severe weakening of this site, rather than its total elimination. Perhaps, because of these reasons, fragment II formed strong dimers, and three of these dimers may interact weakly to form a feeble hexamer and under our cross-linking conditions could not be cross-linked as well.

DISCUSSION

The DnaB protein of *E. coli* is the only well established cellular replicative DNA helicase (Kornberg & Baker, 1992). It is a pivotal replication protein in *E. coli* that has been shown to be involved in the initiation, priming, and elongation stages of DNA replication. It has a number of enzymatic activities that uniquely enable it to interact with other DNA replication proteins that are required for the proper assembly of the replisome and its entry into DNA replication. We have analyzed the function(s), especially the DNA helicase activity, of various structural domains of the DnaB helicase in order to develop a model for its mechanism of action.

Enzymatic Activities of the DnaB Protein and Its Tryptic Fragments. Nakayama et al. (1984) identified two domains of DnaB protein that are involved in its various actions and separated them by controlled trypsin proteolysis. We have purified the tryptic fragments to homogeneity and verified the integrity by N-terminal amino acid sequencing. DnaB protein and its C-terminal fragments I and II retained the DNA binding, ATP binding, and ssDNA-dependent ATPase activities. These results correlated well with the reports of Nakayama et al. (1984). Kinetic studies show that DnaB protein and fragment I remained closely comparable in their ATPase activities. Fragment II retained approximately 60% of the ATPase activity of the DnaB protein. The K_m for ATP was not altered, but V_{max} of the ATPase was diminished by the removal of the N-terminal fragment (fragment III). Considering the fact that fragment II lacks approximately 30% of the DnaB protein sequence, which likely caused local and overall conformational changes in fragment II, a small diminution of ATPase activity was not surprising. We have reported earlier that an ATP binding motif, [(G/A).X.X.X.G.K.(T/S)], is present in DnaB protein between aa residues 229–237 which remains intact in fragment II and the cross-linked [α-^{32}P]ATP remains with fragment II on trypsin digestion (Biswas & Biswas, 1987). Consequently, retention of the ATPase activity in fragment II was in agreement with this report, and it is also in agreement with the findings of Nakayama et al. (1984).

The N-Terminal Domain Is Required for the DNA Helicase Activity. The DNA binding, ATP binding, and ssDNA-dependent ATPase activities are required for a functional DNA helicase activity in most known DNA helicases. Therefore, we compared the DNA helicase activities of DnaB protein and its tryptic fragments. DnaB protein and fragment I had comparable DNA helicase activities, which is a result similar to that observed with their ATPase activities. However, fragment II was completely devoid of DNA helicase activity. Although we observed some diminution of the ATPase activity, this decrease could not account for the total loss of DNA helicase activity. The N-terminal 12-kDa fragment was definitely required for the DNA helicase activity although it is clearly dispensable for the ATPase activity as well as for DNA and ATP binding. One likely possibility is that the C-terminal domain is responsible for DNA binding as well as supplying energy to DNA unwinding and movement, whereas the N-terminal domain is also required for mechanical unwinding of the DNA. It is also possible that fragment II is impeded in translocation on the DNA in the absence of the N-terminal domain.

The N-Terminal Domain Is Required for a Stable Hexamer Formation. Our size exclusion HPLC analyses indicated that fragment II retained the hexameric structure of the DnaB protein, a result in agreement with earlier studies of Nakayama et al. (1984). Protein cross-linking analyses indicated that fragment II dimer formation was comparable to that observed with DnaB protein. However, larger oligomers were far less stable and less amenable to intermolecular cross-linking. Formation of hexamers could be easily demonstrated by cross-linking with both DnaB protein and fragment I. Extensive cross-linking studies failed to demonstrate even a small proportion of hexamer formation with fragment II. In order to cross-link a hexamer completely, a minimum of 12 protein–protein interfaces must be cross-linked. If half of the sites are somewhat altered in their stability of interaction and/or in the geometry of the side chains in the interface, a complete cross-linking of the hexamer would be difficult. Although no dimerization sites are found within the N-terminal 12-kDa fragment III itself, lack of this domain appears to result in an impairment of the dimerization of fragment sites of fragment II. Whether or not this defect in a stable hexamer formation is directly responsible for the lack of DNA helicase activity in fragment II will require further investigation and may be answered by *in vitro* mutagenesis studies in this region of DnaB protein.

A Putative Leucine Zipper in the C-Terminal May Contribute to the Dimerization. In the last few years a large number of sequence-specific DNA binding proteins have been identified (Kouzarides & Ziff, 1989; O'Shea et al., 1991; Turner & Tijan, 1989; Vinson et al., 1989, 1993). A class of these proteins requires dimerization for activation of DNA binding, and the dimerization is induced by parallel coiled-coil sequences of five heptad repeats containing characteristic hydrophobic residues (leucine) at six residue intervals and the consensus sequence is [L.X6.L.X6.L.X6.L.X6.L]. The sequence-specific DNA binding region is normally a basic region at the N-terminus of the leucine zipper. Our sequence analysis indicated that the DnaB protein contained a leucine zipper motif with an isoleucine at the first position located near the C-terminal (residues 360–390). The basic region very near the N-terminal (–1 to –25) of the leucine zipper was absent. However, a basic region, RSRARRIAR, is present further upstream at position 323–331, which closely resembled the core basic region of a model synthetic leucine zipper peptide designed by O'Neill et al. (1990). This peptide showed *in vitro* DNA binding as well as dimerization and contains a consensus sequence common to several leucine zipper-containing transcription factors such as GCN4, Fos, and Jun proteins (O'Neil et al., 1990; O'Shea et al., 1991; Vinson et al., 1993). The basic region of DnaB is not as elaborate as various other eukaryotic leucine zipper-containing proteins. However, GCN4 and other similar proteins are sequence-specific DNA binding/transcription factors that are required to bind the sequence with high affinity and sequence specificity. DnaB protein is not a sequence-specific DNA binding protein; on the contrary, it binds to both single- and double-stranded DNA without any sequence specificity. It should be noted that a DNA helicase such as DnaB protein could not bind DNA with high affinity for this would impede rapid and processive movement of the DNA-bound DnaB helicase as well as unwinding of the double helix. One may speculate

that the further upstream location of the basic region may likely be due to two factors: (i) DnaB helicase forms a hexamer rather than a dimer, which is a more rigid structure that requires the dimer interface to have a wide angle (~120°); such a rigid structure would place a limit on the proximity of the two basic regions to the zipper if they are still to be capable of DNA binding; (ii) the helicase action would require rapid twisting of the DNA strands which would be favored with a DNA binding region located away from the dimer interface such that the intervening spacer region would allow movement and twisting of the DNA binding region with bound DNA. Consequently, the location of the basic region further upstream than that observed with a typical sequence-specific DNA binding/transcription factor is perhaps reasonable. Our results showed that the fragment II formed a dimer in a manner similar to that of DnaB protein. The leucine zipper domain remained undisturbed during trypsin proteolysis of DnaB protein to fragment II. Therefore, it is possible that the leucine zipper may form one of the dimerization sites in the DnaB protein. Further studies will enable us to determine its function(s), if any, in DNA binding and perhaps helicase action.

Our results indicated that the N-terminal domain of DnaB protein is not absolutely required for DNA binding, ATP binding, and ATPase activity of DnaB protein. This domain is essential for (i) DNA helicase activity and (ii) for the maintenance of a stable hexameric structure in DnaB protein. In addition, the putative leucine zipper motif may play a role in the structure and mechanism of action of the DnaB helicase.

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