A *dnaB*-like protein of *Pseudomonas aeruginosa*

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Received December 17, 1986; Accepted January 2, 1987

ABSTRACT

A dnaB-like protein from P.aeruginosa was purified to near homogeneity using as an assay the immunoprecipitation by E.coli dnaB antiserum in a solid-phase. In the chromatographic characteristics including the affinity to immobilized ATP the dnaB-like protein of P.aeruginosa is similar to the dnaB protein of E.coli with the exception that it does not bind to heparin-Sepharose. The dnaB-like protein has a native molecular weight of about 320,000 as determined by glycerol gradient sedimentation. It consists of several identical subunits of molecular weight of 56,000 as measured in a denaturing SDS gel. Associated with the enzyme is a DNA-dependent ATPase- and helicase activity. The dnaB-like protein is similar to the E.coli dnaB protein with regard to the binding of ATPγS and the formation of a ternary complex consisting of the enzyme, ATPγS, and φX174 DNA. However, the enzyme of P.aeruginosa is inactive in a φX174 DNA-dependent in vitro dnaB complementation assay using an E.coli dnaBts extract.

INTRODUCTION

Elucidation of the enzymology of the DNA replication machinery of E.coli was mainly possible by the availability of E.coli dnats mutants and small DNA phages the replication of which greatly depends on the replication apparatus of its host (1). With regard to P.aeruginosa such a favorable situation does not exist. Although DNA phages of P.aeruginosa have been isolated, the lack of suitable dnats mutants renders replication studies with this bacterial species more difficult.

A crude soluble enzyme system has been prepared from P.aeruginosa which is capable of converting the single-stranded viral DNA of the E.coli phages M13 and φX174 to the respective double-stranded replicative form (2). Furthermore, replication of exogenous DNA of the broad host range plasmid RSF1010 can be carried out by this system to some extent provided that the extract is prepared from an RSF1010-plasmid containing Pseudomonas strain (2). These findings provided evidence for the existence of at least one plasmid-encoded replication function and recently three RSF1010-encoded replication genes and the corresponding proteins have been identified (3).

These studies prompted us to learn more about the replication apparatus of P.aeruginosa. We began with a search for a dnaB-like protein because a quick purification procedure for dnaB- and dnaB-analog proteins is available by the use of ATP-agarose affinity

chromatography (4,5). In using immunoprecipitation by E.coli dnaB antiserum in a solid phase we have found and isolated such a protein from P.aeruginosa bacteria. Although it is very similar to the E.coli dnaB protein, a functional test for its participation in DNA replication is not yet available. Therefore, we use the term "dnaB-like protein" in the following text.

MATERIALS AND METHODS

Chemicals.

ATP-Agarose Type 4 (P-L Biochemicals, USA), ATPγS (Boehringer, Mannheim, FRG), [^{35}S]ATPγS (650 Ci/mmol), [γ-^{32}P]ATP, and [^{3}H]dTTP (43 Ci/mmol) (Amersham Buchler). Other chemicals were as described (4,6).

Bacteria, phage and phage DNA.

Pseudomonas aeruginosa 280 met was used for the preparation of extracts. φX174am3, a lysis deficient mutant was used as source of φX174 DNA (abbreviated φX DNA). For radioactive labelling of the DNA E.coli HF4704 thy$^-$ su$^-$ was grown at 37^{o}C in Frazer medium with 10 μg/ml of thymine. Bacteria were infected at 2 x 10^{8} cells/ml with φX phage (moi = 5) in the presence of 1 μCi/ml of [^{3}H]thymidine. [^{3}H]φX DNA has a specific radioactivity of 6 x 10^{6} cpm/μmol. Phages Pf1 and Pf3 were propagated at 37^{o}C in P.aeruginosa strain K and PAO1(RP1), respectively. Phages were purified by a CsCl step gradient. DNA was extracted from φX174, Pf1 and Pf3 by hot phenol.

Assays of dnaB protein.

E.coli YS1 recA harboring the high copy-number plasmid pKA1 carrying the E.coli dnaB gene (7) was used as source for the dnaB protein. Purification of dnaB protein (ATP agarose fraction, spec. activity 740 units/mg) and preparation of E.coli dnaB antiserum was described elsewhere (4). E.coli dnaB518::Tn10 (P1 bac crr, λcI857) harboring the multicopy-plasmid pPLc28 carrying the ban gene (8) was used for the induction of P1 ban protein synthesis. The purification and characterization of ban- in the absence of dnaB protein will be described elsewhere (Riedel,H.D., Heisig,A. and Schuster,H., in preparation).

Solid-phase immunoassay. Proteins were transferred electrophoretically to nitrocellulose membrane as described (9,10). Antiserum against E.coli dnaB protein and fluorescein-conjugated rabbit anti-sheep IgG (Miles-Yeda) were diluted 1 : 250 and 1 : 50, respectively.

dnaB complementation. Complementation of inactivated E.coli dnaBts extracts (strain BT1071, 90 sec, 37^{o}C) using φX DNA as template was measured as described (4). DNA synthesis was followed by [^{3}H]dTMP incorporation into TCA insoluble material for 30 min at 30^{o}C. DNA synthesizing activity of P.aeruginosa protein fraction per se was measured in the same way but omitting the E.coli dnaBts extract.

ATPase. ATPase activity was determined according to Conway and Lipmann (11). Hydrolysis of [^{32}P]γATP (0.5 μCi/test) was followed in the presence of N-ethylmaleimide (10 mM) and

rifampicin (20 μg/ml) for 30 min at 30°C. DNA stimulated ATPase activity was determined in the presence of 1 nmol DNA.

Helicase. Helicase activity was determined essentially as described by LeBowitz and McMacken (12). The reaction mixture (25 μl) contained 40 mM Hepes/NaOH, pH 7.6, 10 mM magnesium acetate, 1 mM DTT, 2 mM ATP, 50 μg of bovine serum albumin/ml, 10 mM creatine phosphate, 80 μg of creatine kinase/ml, 0.1 μg of helicase substrate (described in the legend to Fig. 4) and up to 1 μg of the protein to be tested.

dnaB protein·nucleotide complexes. The binding of [^{35}S]ATPγS (300 cpm/pmol, 100 μM) to dnaB protein, and the binding of dnaB protein to [^{3}H]φX DNA (6000 cpm/nmol, 6 nmol as nucleotide) in the presence of ATPγS was determined by adsorption to nitrocellulose membrane filters as described (13,14). The reaction mixtures (30 μl each) were incubated at 0°C for 5 min.

Isolation procedure for the dnaB-like protein.

Bacteria were grown to about 6 x 10^8 cells/ml in TY medium at 37°C at a constant pH (7.5) in a 100 l Bioengineering fermenter (6). Lysis, crude extract preparation, streptomycin sulphate precipitation and ammonium sulphate (243 mg/ml) fractionation were described elsewhere (4,6). The ammonium sulphate fraction was dialyzed against buffer A (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 12% glycerol) and applied to a DEAE-Sephacel column (Pharmacia). Details are described in the legend to Fig.1. The pooled fractions were precipitated with ammonium sulphate (390 mg/ml), the precipitate dissolved, dialysed against buffer A and applied to a heparin-Sepharose CL 6B column (10 mg protein/ml resin; flow rate 1 bed vol./h). The dnaB-like protein was found in the flow-through and directly applied to an ATP-agarose column (for details see the legend to Fig.2). The purified enzyme was frozen in liquid nitrogen and stored at -70°C.

Other methods.

15% polyacrylamide SDS slab gels were performed as described (15). Glycerol gradient sedimentation is described in the legend of Fig. 3. Protein was determined according to Miller (16).

RESULTS AND DISCUSSION

Strategy for the isolation of a dnaB-like protein of P.aeruginosa.

The dnaB protein of E.coli has been purified to near homogeneity by ATP-agarose affinity chromatography using as an assay the stimulation of φX DNA-dependent DNA synthesis in inactive extracts of E.coli dnaBts mutants (4,5). We applied the same purification scheme for the isolation of a dnaB-like protein from P.aeruginosa. To set up an assay we tested the capacity of an ammonium sulphate fraction from P.aeruginosa for DNA synthesis with the single-stranded DNA of phage Pf1, Pf3, and φX as templates. In the absence of rifampicin

Table 1
DNA synthesis in an extract of P. aeruginosa

DNA	dTMP incorporation (pmol) *rifampicin*	
	-	+
Pf1	122.1	9.8
Pf3	13.5	2.4
ϕX174	80.2	64.0

DNA synthesis was measured as described in Materials and Methods using Fraction II (see Table 2). 0.15 μg of DNA and 750 pmol of [^{3}H]dTTP were used per assay.

stimulation of DNA synthesis was observed with all three phage DNAs. However, in the presence of rifampicin only ϕX DNA was active as DNA template (Table 1) confirming earlier reports (2). ϕX DNA was converted to the replicative form in the rifampicin-resistant reaction (data not shown). These results suggested that replication of Pf1 and Pf3 DNA is primed by RNA polymerase whereas ϕX complementary strand synthesis might depend on a priming process mediated by a dnaB-like protein. Therefore we tried to use dnaB complementation as an assay (4). However, an inactive extract of an E.coli dnaBts mutant could not be complemented by extracts of P.aeruginosa to stimulate ϕX DNA synthesis, although the latter contained a dnaB-like protein as indicated by a positive reaction with antiserum against E.coli dnaB protein (data not shown). Therefore, at first we had to rely on an immunoassay for tracing the dnaB-like protein of P.aeruginosa. In using this method the protein was purified to near homogeneity. The purification steps are summarized in Table 2. The procedure will be described in the following section.

Isolation of a dnaB-like protein of P.aeruginosa by ATP-agarose affinity chromatography. Purification to near homogeneity of E.coli dnaB protein was achieved in three steps starting with a crude cellular extract: (i) ammonium sulphate (43%) fractionation, (ii) DEAE-Sephacel , and (iii) ATP-agarose chromatography (4). In following this procedure and in using

Table 2 Purification of a dnaB-like protein from P.aeruginosa

Fraction		Protein	
		total (mg)	%
I	crude extract	15470	100
II	ammonium sulphate	3270	21.1
III	DEAE-Sephacel, peak II	328	1.5
IV	heparin-Sepharose	56	0.36
V	ATP-agarose	0.65	0.004

Purification was started from 375 g of wet cell paste (corresponding to a total of 2 x 10^{14} cells). Cells were lysed and the crude extract fractionated as described (4,6).

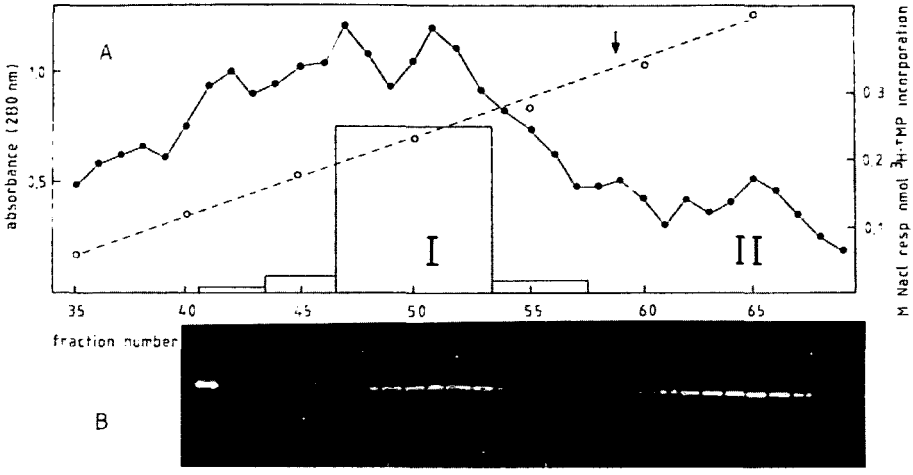

Figure 1. DEAE-Sephacel column chromatography of the dnaB-like protein of P.aeruginosa. A. Fraction II (3270 mg of protein) was diluted in buffer A to a concentration of 10 mg/ml and applied to a DEAE-Sephacel column (5 x 15.5 cm). The column was washed with 5 1/2 bed volumes of buffer A. Protein was eluted with a linear NaCl gradient (15 bed volumes of 50 - 600 mM NaCl in buffer A). Fractions of 90 ml were collected at a flow rate of 180 ml/h. A set of subsequent fractions were pooled, and the protein precipitated with 0.37 g/ml ammonium sulphate. An aliquot of the pooled fractions was tested for its ability to incorporate [^{3}H]dTMP into ϕX DNA (shown by the boxes in A in arbitrary units). (o--o), molarity NaCl, (•--•), absorbance at 280 nm. The arrow (↓) indicates the position of elution of E.coli dnaB protein under otherwise identical conditions. B. An aliquot (4 μl) of each fraction was subjected to SDS gel electrophoresis and tested in a solid-phase immunoassay using E.coli dnaB antiserum as described in Materials and Methods. E.coli dnaB protein (left lane) was run as a control.

an immunoassay, the dnaB-like protein of P.aeruginosa eluted from DEAE-Sephacel in two peaks (I and II) at approx. 220 mM and 400 mM NaCl, respectively (Fig.1, A and B). Fractions of peak II did not complement an E.coli BT1071 dnaBts extract, but fractions of peak I either per se or in the presence of BT1071 extract stimulated ϕX DNA synthesis in a rifampicin-resistant reaction (Fig. 1A).

Using the same conditions the dnaB complementing activity of E.coli dnaB protein eluted at about 340 mM NaCl at a position intermediate between peak I and II (Fig. 1A, arrow). Tracing E.coli dnaB protein with dnaB antiserum revealed the major fraction of the protein coinciding with the dnaB complementing activity. Also here, a minor fraction of dnaB protein, below the level of detection by the complementation assay, eluted ahead of the dnaB activity peak (data not shown). We assume that this minor fraction of the E.coli dnaB protein as well as the peak I fraction of the dnaB-like protein of P.aeruginosa represent molecules contained in an still undissolved complex of several replication proteins as suggested earlier for the dnaB protein of E.coli (17).

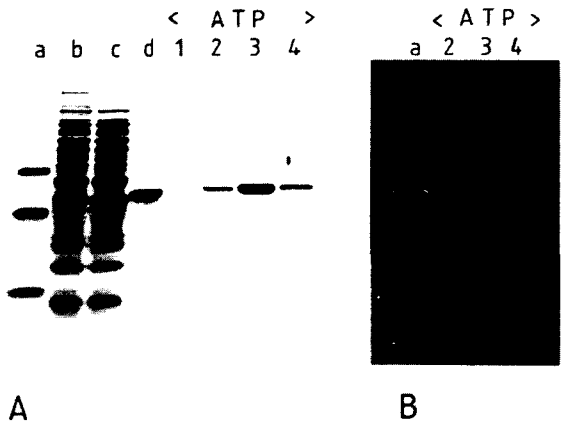

Figure 2. Affinity chromatography on ATP-agarose Type 4 of the dnaB-like protein of P.aeruginosa. Fraction IV (56 mg protein) was applied to an ATP-agarose column (1 x 8 cm). The column was washed with 50 ml of buffer A, followed by 50 ml of buffer A containing 5 mM AMP. The dnaB-like protein was eluted with 50 ml of buffer A containing 10 mM ATP (1.5 ml/fraction). The flow rate was 6 ml/h. A. Aliquots were used for electrophoresis in 15% acrylamide gels. a) Marker in descending order: bovine serum albumin, ovalbumin, chymotrypsinogen A, b) loading material (Fraction IV, 3 μl), c) flow-through (3 μl), d) E.coli dnaB protein (3 μg), 1-4: ATP eluate (20 μl each). B. Aliquots (5 μl) of the ATP eluate were tested in a solid-phase immunoassay using E.coli dnaB antiserum as described in Materials and Methods. a. E.coli dnaB protein (0.3 μg).

Fractions of peak I and II (Fig. 1A) were pooled separately and aliquots of each applied to ATP-agarose. With both fractions the dnaB-like protein was adsorbed and could be eluted by ATP. A comparison of the ATP eluates by SDS gel electrophoresis and immunoassays revealed that the peak II fraction contained by far the larger amount of a protein of similar size as the E.coli dnaB protein (Fig.1, and data not shown). Moreover, the DNA-synthesizing activity of the peak I fraction was lost during the ATP-agarose chromatography. Therefore, we continued to purify and characterize the dnaB-like protein of peak II fraction only.

Peak II fraction eluting from ATP-agarose by ATP still contained two major proteins of which only the slower moving protein reacted with dnaB antiserum (data not shown). In preliminary experiments it was found that these proteins could be separated from each other by heparin-Sepharose chromatography. Therefore the major part of peak II fraction was first applied to a heparin-Sepharose column. Only the contaminating protein was adsorbed to heparin-Sepharose whereas the dnaB-like protein was recovered from the flow-through. This is in contrast to the chromatographic property of E.coli dnaB protein which under the same conditions is adsorbed to heparin-Sepharose and eluted with about 220 mM NaCl (unpublished results). Fraction IV (flow-through) was applied to ATP-agarose and eluted with ATP. The

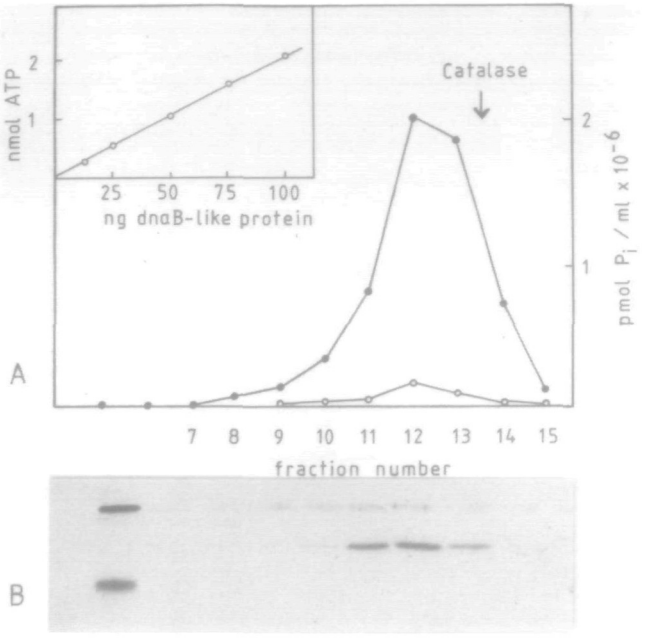

Figure 3. Glycerol gradient centrifugation of the dnaB-like protein from P.aeruginosa. Glycerol gradients [4.8 ml, 15-30% glycerol (w/v)] containing 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 1 mM ATP, 50 mM NaCl, 10 mM $MgCl_2$ were prepared in cellulose nitrate tubes. Fraction V of the dnaB-like protein (70 μl) was layered onto the gradient. Catalase as reference was run in a parallel gradient. Centrifugation was performed in a Beckman SW65 rotor for 15 h at 45,000 rpm and 2°C. Fractions (0.2 ml) were collected from the tube bottom. A. ATPase activity was measured as described with 3 μl of each fraction. (•), + ϕX DNA, (o), - ϕX DNA. The arrow indicates the position of catalase in the parallel gradient. B. Electrophoresis of 40 μl aliquots of fractions on a 15% acrylamide gel. Marker (left lane) in descending order: bovine serum albumin, ovalbumin.

dnaB-like protein was found to be at least 90% pure (Fraction V, Fig. 2A). Of the four minor proteins still contained in Fraction V, two slowly moving bands also reacted with dnaB antiserum (Fig. 2B). The relationship of these two polypeptides to the dnaB-like protein is unknown. From the data of Table 2 (presupposing 100% yield) about 35 dnaB-like monomer molecules per cell are calculated. A similar value is found for the dnaB protein of E.coli (1).

Properties of the dnaB-like protein of P.aeruginosa.

In this section the dnaB-like protein is compared to the E.coli dnaB protein with regard to its physical properties, its enzymatic activities, and the binding of ATP and ssDNA.

Physical properties. From the SDS gel electrophoresis of Fraction V a molecular weight of about 56,000 was estimated for the dnaB-like polypeptide of P.aeruginosa. In the same gel the corresponding value for the E.coli dnaB protein was found to be about 52,000 (Fig. 2A). A

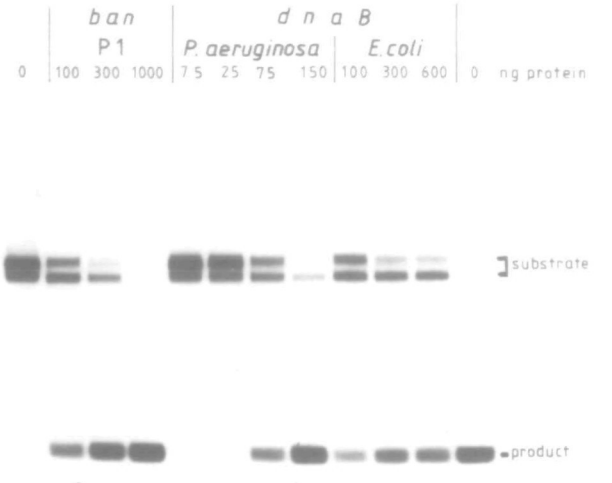

Figure 4. Helicase activity of the dnaB-like protein of P.aeruginosa. Helicase substrate, dnaB or ban protein in amounts as indicated were incubated for 30 min at 37°C as described in Materials and Methods and subjected to 1% agarose gel electrophoresis. The helicase substrate is composed of a 789-nucleotide long 5'-^{32}P-labeled DNA fragment hybridized to a 591-nucleotide long complementary sequence inserted into M13mp8. The complementary DNA fragments both derive from the Su^{r} region of plasmid RSF1010, the ^{32}P-labeled and the inserted DNA originating from a HincII- and an Alu-PstI fragment, respectively; of the annealed fragment 79 nucleotides at the 3'- and 119 nucleotides at the 5' terminus remain single-stranded (E.Scherzinger, to be published elsewhere). This substrate consists of two bands of which the faster moving band presumably is generated by linearization of M13mp8 DNA during preparation of the helicase substrate (12). Last lane on the right: nonhybridized ^{32}P-labeled 789-nucleotide HincII fragment.

similar correspondence followed from the determination of the molecular weight of the native molecules. Upon glycerol gradient centrifugation the dnaB-like protein sedimented with a velocity corresponding to a molecular weight of about 320,000 with catalase (molecular weight 250,000) as marker (Fig.3). In this experiment the dnaB-like protein was traced by (i) SDS gel electrophoresis and (ii) measurement of ATPase activity (see below).

Ribonucleoside triphosphatase activity. A N-ethylmaleimide (10 mM, 10 min, 30°C) -resistant DNA-dependent ATPase activity is found associated with the dnaB-like protein of P.aeruginosa. The corresponding activity in the absence of DNA is less than 10% of that obtained in the presence of ϕX DNA (Fig.3). From the rate of ATP hydrolysis dependent on the concentration of the dnaB-like protein, a turnover number of about 1200 ATP molecules hydrolyzed/min/molecule of enzyme was estimated (Fig.3, insert). A value of 2700 had been found for the dnaB protein of E.coli with poly(dT) as template (18).

The ATPase activity of the dnaB-like protein was inactivated by E.coli dnaB antiserum at a similar rate as the E.coli dnaB ATPase. Treatment with normal serum only had a marginal

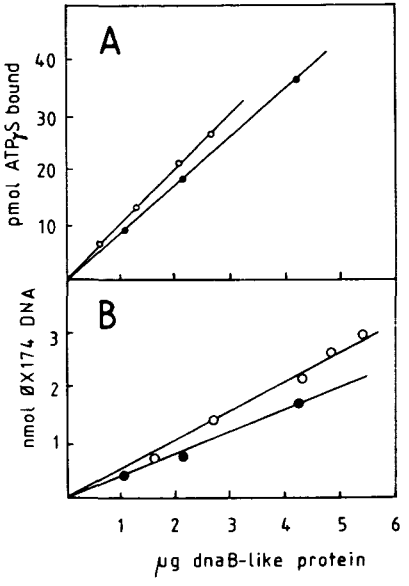

Figure 5. (A), binding of ATPγS to the dnaB-like protein of P.aeruginosa, and (B), binding of the dnaB-like protein to ssDNA. Details of the reaction are presented in Materials and Methods. (o), dnaB-like protein; (•) dnaB protein of E.coli.

effect on the activity of the E.coli dnaB protein, but inactivated the ATPase activity of the dnaB-like protein slightly less than the antiserum (data not shown). The reason for this is not yet understood.

Helicase activity. Purified dnaB-like protein of P.aeruginosa was tested for helicase activity by determining its capacity to unwind a partially duplex DNA, the helicase substrate (see legend to Fig.4). In the assay the unwound radioactive product is separated electrophoretically from unreacted helicase substrate (12). A helicase activity is found associated with the dnaB-like protein of P.aeruginosa. Its specific activity is even stronger than the corresponding activity of the dnaB protein of E.coli and of the ban protein of phage P1 under otherwise identical conditions (Fig. 4; Riedel,H.D., Heisig,A., and Schuster,H., in preparation).

Complex formation with ATPγS. ATPγS, a nonhydrolyzed analog of ATP, binds tightly to the dnaB-like protein of P.aeruginosa as it does with the E.coli dnaB protein (Fig.5 and references 13,14). From the amount of ATPγS bound to the dnaB protein as a function of the enzyme concentration, 0.57 and 0.47 molecules ATPγS per monomer of the dnaB-like protein of P.aeruginosa and the dnaB protein of E.coli, respectively, were found. For the E.coli enzyme a value of 0.94 had been found by others (13).

Binding to ssDNA. Binding of ATP to E.coli dnaB protein is very weak with ssDNA (13).

Therefore, the dnaB protein also does not bind to a ssDNA agarose column, although its appearance in the flow-through from that column is retarded if the operation proceeds in the presence of 1 mM ATP (unpublished results). Formation of a ternary complex was indicated by binding of ssDNA to E.coli dnaB protein in the presence of a nonhydrolyzed analog of ATP (18). The dnaB-like protein of P.aeruginosa also binds to ϕX DNA in the presence of ATPγS and the kinetics of the complex formation is similar to that observed with the E.coli dnaB protein (Fig. 5B).

Summarizing the results obtained so far we have shown that the dnaB-like protein of P.aeruginosa is very similar to the E.coli dnaB protein with regard to particular enzymatic functions and physical properties. Therefore, it is almost certain that it fulfills the same function in P.aeruginosa as the dnaB protein does in E.coli. Both enzymes are also related immunologically. Nevertheless the dnaB-like protein cannot replace the dnaB protein in an in vitro replication system using an extract of an E.coli dnaBts mutant. This suggests that it still differs from the E.coli enzyme in its ability to interact with other replication proteins of E.coli. It is worthwhile to mention here that another dnaB-like protein, the ban protein of P1, although very similar to the dnaB protein of E.coli, also still reveals a difference. Its ability to replace the dnaB protein in vivo is restricted to temperatures of $\geq 30^{\circ}C$ because of the coldsensitivity of the ban protein molecule (19).

ACKNOWLEDGEMENTS

We thank A.Kornberg for making available to us E.coli strain YS1recA(pKA1), L.Day and V.Stanisich for phage Pf1 and Pf3 and their respective bacterial hosts. We are much indebted to E.Scherzinger for providing us with the helicase substrate. Helpful suggestions by E.Lanka in the initial experiments are gratefully acknowledged. We thank M.Schlicht for preparing purified phages and phage DNA and I.Severin and M.Warmuth for expert technical assistance.

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