Escherichia coli dnaB Protein

AFFINITY CHROMATOGRAPHY ON IMMobilIZED NUCLEOTIDES*

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The purification of the Escherichia coli dnaB protein by affinity chromatography on nucleotides bound to agarose is described. The dnaB protein, which contains an associated ribonucleoside triphosphatase activity (Wickner, S., Wright, M., and Hurwitz, J. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 783-787) binds to immobilized ATP, ADP, and UDP, but not to AMP. The type of linkage of ATP to agarose influences the adsorption, elution, and purification of the enzyme. Optimal purification is achieved using ATP bound to agarose via its oxidized ribose moiety. By this means, the dnaB protein can be obtained at least 95% electrophoretically pure after only three purification steps.

The enzyme can be eluted from immobilized nucleoside-5'-di- and -triphosphates by ATP, ADP, and pyrophosphate, but not by AMP or orthophosphate. ADP and pyrophosphate, as well as the substrate ATP in high concentration are at the same time inhibitors of the ribonucleoside triphosphatase.

The dnaB complementing and ribonucleoside triphosphatase activities could not be separated from each other by affinity chromatography, supporting the finding of others that they both reside on the same protein complex, namely a dnaB multimer. The results indicate that the dnaB protein binds to immobilized nucleotides by means of its ribonucleoside triphosphatase, and that at least the pyrophosphate moiety is essential for adsorption as well as elution of the enzyme.

The dnaB protein of Escherichia coli is required for replication of the bacterial chromosome (1, 2). The enzyme exerts its function during the initiation (3) and the elongation process (1, 2) of chromosomal replication. The dnaB protein is also required for conversion of φX174 single-stranded DNA to the duplex replicative form (4-6). Based on this requirement, the dnaB protein can be used to assay φX174 DNA-dependent incorporation of ['H]dTMP into DNA for 30 min at 30°C (6, 7, 18). One unit of dnaB complementing activity is defined

MATERIALS AND METHODS

The nucleotides immobilized on agarose are listed in Table I, and were obtained from P-L Biochemicals, Milwaukee, Wis. Nucleotides used as eluting agents were from Boehringer Mannheim. Pyrophosphoric acid, practicum crystallized, was from Fluka, Buchs, Switzerland.

Buffers—Generally a standard buffer of neutral pH (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol) was used, to which different eluting agents were added at the indicated final concentrations. The buffers were named according to the eluting agent added: AMP (5 and 10 mM), ADP (5 mM), ATP (5, 10, 12, and 20 mM), dATP (5 mM), UDP (5 mM), orthophosphate (5 mM), dithiothreitol (5 mM), and pyrophosphate (50 mM). In some experiments, a standard buffer was used in which the pH of the 20 mM Tris-HCl solution was adjusted to 5.5. In one experiment, an EDTA buffer was used, which was the same as standard buffer but contained 25 mM EDTA instead of MgCl₂.

Affinity Chromatography—All operations were carried out at 4°C. Columns packed with agarose-hexane-5'-nucleotides (Table I) were first washed with 5 to 10 bed volumes of standard buffer. Protein fractions (Fraction II, Table II) were diluted with standard buffer to a final concentration of approximately 1 mg/ml of protein and applied to the columns. These were then washed with 10 bed volumes of standard buffer followed by 10 bed volumes each of the buffers indicated. The bed volumes, fraction sizes, and flow rates are indicated in the legends to Figs. 1 and 2.

E. coli dnaB Protein—The E. coli K12 strains Q1710 and its P1 wild type lysogen, Q1710(P1) were used as enzyme source (17). Strain Q1/10 carries the dnaB266 amber mutation which is suppressed by suppressor supF leading to a temperature-resistant dnaB protein (12). DEAE-cellulose fractions of dnaB protein were prepared as described (Table II and Ref. 17) and stored at -10°C.

Assay for dnaB Protein—A dnaB complementation assay was used for measuring the activity of dnaB protein involving a φX174 DNA-dependent incorporation of ['H]dTMP into DNA for 30 min at 30°C (5, 7, 18). One unit of dnaB complementing activity is defined

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The abbreviations used are: ts, temperature-sensitive; SDS, sodium dodecyl sulfate.
**TABLE I**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Type*</th>
<th>mmol nucleotide/ml agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>4</td>
<td>3.4</td>
</tr>
<tr>
<td>ADP</td>
<td>3</td>
<td>8.9</td>
</tr>
<tr>
<td>UDP</td>
<td>2</td>
<td>4.9</td>
</tr>
<tr>
<td>ATP</td>
<td>4</td>
<td>3.3</td>
</tr>
</tbody>
</table>

*Type 2, N"-(6-aminohexyl)-ATP coupled to agarose via the 6-amino group; type 3, 8-(6-aminohexyl)amino-ATP coupled to agarose; type 4, the nucleotide is coupled to agarose via the 2',3'-periodate-oxidized hydroxyls of the ribose, using adipic acid dihydrazide as spacer.

**TABLE II**

Purification of the dnaB complementing activity from strain Q1710

As described previously (17), 1200 g of wet cell paste were lysed. The yield of crude extract was 44.3 g of protein. The ATP-agarose purification step was performed with only a part of Fraction II. The values reported assume that the yield and purification would be the same if the entire Fraction II was subjected to the purification procedure.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total units</th>
<th>Total protein mg</th>
<th>Specific activity units/mg</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Ammonium sulfate</td>
<td>31,400</td>
<td>6,460</td>
<td>4.86</td>
<td>100</td>
</tr>
<tr>
<td>II DEAE-cellulose</td>
<td>6,550</td>
<td>211.6</td>
<td>31.0</td>
<td>20.9</td>
</tr>
<tr>
<td>III ATP-agarose type 4</td>
<td>7,220</td>
<td>2.35</td>
<td>3.080</td>
<td>23.0</td>
</tr>
</tbody>
</table>

as the number of nanomoles of [3H]dTMP incorporated under these conditions (17).

**RESULTS**

Affinity Chromatography on ATP-Agarose Type 4 of E. coli dnaB Protein

Chromatography on ATP-agarose type 4 has been used for the purification of the dnaB and the dnaB Plbac protein multimers from several E. coli strains (16-18). After passing the proteins of DEAE-cellulose fractions (Fraction II, Table II) through an ATP-agarose column, four major proteins remained bound and were eluted by 5 mM ATP buffer: the dnaB protein (60,000- to 62,000-dalton polypeptide subunits), the Plbac protein (56,000 daltons, from E. coli Plbac lysogen), and two other hitherto unknown proteins (38,000 and 70,000 daltons). With an improved affinity chromatographic method described here, the latter two proteins can now be separated from the dnaB protein. After adsorption of the dnaB protein (Fraction II) of strain Q1710 to ATP-agarose, the contaminating proteins are eluted by AMP buffer prior to the elution by ATP buffer of the dnaB protein itself (Fig. 1A). Ninety-five per cent of the dnaB complementing activity could be adsorbed to the ATP-agarose and was recovered quantitatively in the ATP buffer eluate (Fig. 1A). The flow rate was 10 ml/h. Aliquots (0.05 ml of the loading material, 0.1 ml each of the nonadsorbed material, the AMP, and the ATP buffer eluate) were used for SDS gel electrophoresis. a, loading material; b, nonadsorbed material; β-Gal, β-galactosidase; BSA, bovine serum albumin; OVA, ovalbumin; CHYA, chymotrypsinogen A; RNASE A, ribonuclease A. B, elution by pyrophosphate. dnaB protein (3 ml) (Fraction II, 258 units, 4.68 g of protein) in standard buffer was applied to a 0.5-ml ATP-agarose column (0.4 x 4.1 cm). The column was washed with 5 ml of 0.1M AMP buffer (5.8 ml/fraction). The dnaB protein was eluted subsequently with 50 ml of 5 mM ATP buffer (3.9 ml/fraction). The flow rate was 1 ml/h. Aliquots (0.05 ml of the loading material, 0.2 ml each of the nonadsorbed material, the pyrophosphate and orthophosphate buffer) were used for SDS gel electrophoresis (abbreviations as in A).
the dnaB protein is reduced to only three purification steps (Table II). About 100 dnaB molecules \((M_r = 260,000)\) are calculated to be present per \(E.\ coli\) cell, not taking into account that the dnaB protein might not be recovered quantitatively by the ammonium sulfate precipitation (Fraction I, Table II). The dnaB protein is a multimer of 60,000-dalton polypeptide subunits (Fig. 1, A and B) and has a native molecular weight of approximately 260,000 as estimated by glycerol gradient centrifugation (17).

The ATPase activity associated with the dnaB protein (14) is inhibited by its substrate ATP at high concentrations as well as by ADP and pyrophosphate, but not by AMP or orthophosphate. In accordance with these findings, the calculated to be present per Table II). The protein bound to ATP-agarose type 4 can be eluted by pyrophosphate (Fig. 1B) and ADP buffer but not by AMP or orthophosphate buffer (see below). The dnaB protein eluted by pyrophosphate buffer is at least 90% pure and is recovered quantitatively (Fig. 1B). With pyrophosphate as eluting agent, the two proteins with polypeptide molecular weights of 38,000 and 70,000 remain almost quantitatively bound to the ATP-agarose and they can be eluted subsequently by 10 mM AMP buffer (data not shown). Elution by pyrophosphate buffer of the dnaB protein has the disadvantage that the eluted samples have to be diluted or dialyzed before being assayed since pyrophosphate inhibits both the dnaB complementing and the dnaB ATPase activity.

### Binding of dnaB Protein to Immobilized Nucleotides

#### Effective Nucleotides and Eluting Agents—Various immobilized nucleotides and eluting agents were investigated in order to learn more about the interaction of the dnaB ATPase with its substrate (Fig. 2). Best results with regard to purity and recovery of the dnaB protein were obtained with ATP- and ADP-agarose of type 4 (Fig. 2, A to D). The dnaB protein adsorbs to ADP- and ATP-agarose equally well, and ADP buffer is as effective as ATP buffer in eluting the enzyme (Fig. 2, A, E, and F). However, ATP in concentrations up to 20 mM does not desorb the dnaB protein at pH 5.5 (Fig. 2C). The nature of the immobilized nucleotide base seems to be of minor importance since the dnaB protein also binds to UTP-agarose (Fig. 2F) and can also be eluted by UDP buffer (Fig. 2B). Likewise, no specificity is observed with regard to the sugar moiety of the nucleotide since dATP buffer is as effective as eluting agent as ATP buffer (Fig. 2C). However, a pyrophosphate group is essential for adsorption and elution of the dnaB protein for the following reasons. The enzyme can be eluted by pyrophosphate buffer from ATP- (Fig. 1B) or ADP-agarose of type 4 (Fig. 2D). It is not adsorbed onto AMP-agarose type 4 (data not shown) nor is it eluted from ADP- or ATP-agarose of type 4 by orthophosphate (Fig. 2D) or AMP buffer of pH 7.5 (Fig. 2, A and B) or pH 5.5 (Fig. 2C). The inability of the enzyme to bind to AMP-agarose indicates that no unspecific adsorption to some other part of the nucleotide-agarose occurs. Besides ADP (see above), adenyl-

### Influence of the Type of Linkage of ATP to Agarose—

Purine nucleotides (ATP and ADP) can be replaced by a pyrimidine nucleotide (UDP) as immobilized nucleotide of agarose type 4 without impairing the binding of the dnaB protein (see above). Rather than the nature of the purine or pyrimidine base, the type of linkage of ATP to agarose

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#### Table II

| Column | Buffers | Purity of dnaB protein (%)
|---|---|---
| A | 5 mM AMP, 5 mM ADP | 74 |
| B | 10 mM AMP, 5 mM UDP | 78 |
| C | 10 mM AMP, pH 5.5, 5 mM ATP, pH 5.5 | 76 |
| D | 20 mM ATP, pH 5.5, 5 mM standard, pH 5.5, 5 mM dATP | 86 |
| E | 20 mM orthophosphate, 20 mM pyrophosphate, 10 mM ATP | 95 |
| F | 10 mM AMP, 5 mM ADP | 29 |
| G | Standard buffer, 10 mM AMP, 5 mM ADP | 27 |
| H | 5 mM ATP, 12 mM ATP, 12 mM ATP + 550 mM NaCl | 74 |

The pH of the buffers was 7.5 unless otherwise noted. Columns C and G were washed with standard buffer of pH 5.5 after the protein fraction had been loaded onto the column at the same pH. The agent eluting the dnaB protein is underlined. Aliquots of these fractions containing 5 to 12 units of dnaB protein were used for SDS gel electrophoresis. (Among the different ATP buffers of Column H, the 12 mM ATP fraction was used.) The dnaB protein from strain Q1710 (Pl) was applied to Column A. All other columns were loaded with dnaB protein from strain Q1710, of which also the loading material (a) is shown. Between 37 and 95% of the dnaB complementing activities applied to the columns were recovered. The purity of the dnaB preparations was determined by scanning the SDS gels. For further explanations see text. Abbreviations as in Fig. 1.

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strongly influences binding and elution of the dnaB protein. The enzyme binds only weakly to ATP-agarose type 3 at pH 7.5. Eighty-nine per cent of the dnaB complementing activity is not retained in the column. The fraction of the dnaB protein bound to the column can be eluted by 5 mM ADP buffer (data not shown). Binding of dnaB protein can be increased if Fraction II is applied at pH 5.5 to the column equilibrated at the same pH. Under these conditions, 73% of the dnaB complementing activity is bound to the column and can be eluted by ADP buffer (Fig. 2G). The dnaB protein, on the
other hand, binds strongly to ATP-agarose type 2. Thirty-three per cent of the dnaB complementing activity is recovered by elution with 5 mM ATP (13%), 12 mM ATP (11%), and 12 mM ATP + 500 mM NaCl buffer (9%) (Fig. 2I). The dnaB protein eluted from either agarse type 2 or 3 is less pure than that from ATP-agarose type 4 (Fig. 2).

**DISCUSSION**

Affinity chromatography on ATP-agarose type 4 leads to a quick and efficient purification of the dnaB protein. This enzyme is at least 95% electrophoretically pure after only three purification steps. The number of dnaB protein molecules calculated to be present per cell of strain Q1710 is unexpectedly large when compared to the corresponding number reported for other E. coli strains (9, 14). However, it should be pointed out that this large number is unique for strain Q1710. Most probably it has something to do with the suppression of the dnaB amber mutation by suppressor supF, since strain Q1610 in which the same dnaB mutation is suppressed by suppressor supK in an otherwise isogenic background yields only about 10 dnaB protein molecules/cell. In addition, a value of about 15 dnaB molecules/cell of strain H560 was calculated from the amount of dnaB isolated by the affinity chromatographic procedure described here. The latter value is close to the number 20 reported for the same strain (9).

The dnaB protein has a polypeptide weight of 60,000 (Fig. 1, A and B, and Ref. 17). This value is considerably larger than the 55,000- and 48,000-dalton polypeptide subunits of the dnaB protein reported by Ueda et al. (9) and Wickner et al. (14). This discrepancy is most probably due to different degrees of cross-linking in the SDS gel system. When the dnaB protein of strain Q1710 was subjected to SDS gel electrophoresis in 10% (w/v) polyacrylamide and 0.29% N,N'-methylenebisacrylamide, a value of 52,000 daltons was calculated (data not shown).

Recovery of the dnaB complementing activity after affinity chromatography on ATP-agarose type 4 is very often 100% or even higher (Table II and Refs. 16 and 1I), a fact which indicates a regeneration and stabilization of the activity during the interaction of the enzyme with its immobilized substrate molecule. Therefore, this method is especially suitable for the isolation and characterization of thermolabile dnaB protein molecules from E. coli dnaBts mutants (16-18). Affinity chromatography may also facilitate the biochemical analysis of other biologically interesting dnaB mutants. In addition, it may be applicable for the isolation of other DNA replication proteins with inherent ATPases.

Among the different types of ATP-agarose, type 4 was particularly suitable for the purification of the dnaB protein (Fig. 1 and Fig. 2A to C). Therefore, this type has been used exclusively for the isolation of heteromultimers composed of dnaBts and P1B protein subunits (16-18). For the same reason, most of the studies described in this paper have been made with ATP-agarose type 4. The results provide a first insight into the nature of the interaction of the enzyme with its immobilized substrates or competitive inhibitors. The dnaB protein binds to ADP- and ATP- but not to AMP-agarose of type 4, indicating that a pyrophosphate group is essential for binding. There seems to be no specificity with regard to the base (purine or pyrimidine) since the enzyme binds to UDP-agarose as well. This is in accordance with the finding that the ATPase associated with the dnaB protein hydrolyzes all four ribonucleoside triphosphates (14). On the other hand, coupling of the spacer to the C-8 atom of the adenine moiety of the ATP molecule (agarose type 3) strongly inhibits binding at neutral pH, whereas coupling to the N-6 atom (agarose type 2) leads to a higher affinity of the dnaB protein to the ligand. Model building revealed that in agarose type 3, the spacer molecule is much closer to the pyrophosphate group than in type 2. Steric hindrance might therefore be the reason for the inability of the enzyme to bind to ATP-agarose type 3.

Elution of the dnaB protein from immobilized nucleotides of agarose type 4 by pyrophosphate or ADP but not by orthophosphate or AMP is again in accordance with the inhibitory effect of the former but not of the latter compounds on the ATPase activity. It would be interesting to see whether an immobilized ribose-pyrophosphate or pyrophosphate molecule is sufficient for binding the dnaB protein; attempts are being made to synthesize these compounds.

Aliquots of the dnaB protein samples eluting from Columns A to H (Fig. 2) were tested for dnaB complementing and ATPase activity. The ratio of the two activities was 13 to 17 for all samples tested (data not shown). These results support the finding of others (14) that both enzyme activities reside on the same protein complex.

The dnaB protein of E. coli interacts with the E. coli dnaC protein, resulting in an inhibition of the ATPase activity associated with the dnaB protein (14, 23). The existence of such an interaction is supported by the observation that dnaC protein when present in about a 1:1 ratio (based on dnaB and dnaC units measured by the complementation assay) prevents the binding of most of the dnaB protein to ATP-agarose type 4. Likewise this observation strengthens the idea that the dnaB protein binds to nucleotide-agarose by its ATPase site.

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