Reconstitution Studies Show That Rifampicin Resistance Is Determined by the Largest Polypeptide of Bacillus subtilis RNA Polymerase*

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A procedure has been developed to separate the subunits of Bacillus subtilis RNA polymerase rapidly and in good yield. The method involved the use of a blue dextran-Sepharose column which bound the β' subunit. A phosphocellulose column was used to separate the α and β subunits. During purification, the enzyme eluted from the DNA-cellulose column in three separate forms in the order αββ'ωα', αβωα' and αβω'α. Subunit reconstitution studies with RNA polymerase subunits from wild type and a rifampicin-resistant mutant indicated that the largest polypeptide was responsible for rifampicin resistance. Thus, this subunit is referred to as β. The mobility of the subunits in sodium dodecyl sulfate-polyacrylamide gel electrophoresis cannot be used as the sole criterion for designating the functions of the subunits of RNA polymerase.

DNA dependent RNA polymerase core of Bacillus subtilis (1–3) and Escherichia coli (4) is an oligomer composed of two large nonidentical polypeptides labeled β and β' and two smaller identical subunits labeled α. The largest polypeptide of the core enzyme from E. coli is labeled β' and functions by binding to DNA (5, 6). The second largest polypeptide, labeled as β, is responsible for the enzyme's sensitivity to rifampicin (7). Two other polypeptides, α and ω, have been found to be associated with core enzyme (4). Zillig et al. (8) have encouraged the labeling of the core subunits from prokaryotic species by function since the molecular weights of the large polypeptides have not been conserved during evolution. When the function of the largest polypeptide has not been determined it has become common practice to designate the largest polypeptide as β in a fashion analogous to E. coli. The functions of the largest polypeptides of B. subtilis have not been determined unambiguously.

Similarities between the RNA polymerase and its subunits from E. coli and B. subtilis have been established. The RNA polymerases from E. coli and B. subtilis have been shown to be metalloenzymes containing 2 zinc atoms per core enzyme (9, 10). Wu et al. (11) have shown that at least 1 zinc atom was associated with the β' subunit of E. coli core polymerase. Halling et al. (12) have shown that both zinc atoms were associated with the second largest polypeptide of the RNA polymerase from B. subtilis. The zinc data suggested that the second largest subunit from B. subtilis had properties similar to the β' subunit from E. coli. Further, elution profiles from the phosphocellulose column revealed a similarity between the β subunit of E. coli and Micrococcus luteus (12) and the largest polypeptide of B. subtilis core polymerase (13).

The RNA polymerase of B. subtilis was purified, separated into subunits, and reconstructed to determine which of the larger subunits was responsible for rifampicin sensitivity. The successful purification of RNA polymerase and the reconstitution of RNA polymerase core from isolated subunits were dependent on the development of methods to inactivate and remove proteases from enzyme preparations and to rapidly purify subunits of urea-dissociated RNA polymerase core. In contrast to other gel electrophoresis systems, considerable separation of the largest polypeptides was made possible by use of the urea-SDS-polycrylamide gel system of Wu and Bruening (14). Zillig et al. (8) have named the largest and second largest polypeptides of Bacillus cereus RNA polymerase as β and β' based on their charge densities. The reconstitution studies with RNA polymerase subunits from wild type and rifampicin-resistant mutants have revealed that the largest polypeptide of the polymerase from B. subtilis is responsible for the rifampicin-resistant phenotype. Thus, the largest polypeptide is referred to as β throughout these studies.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Medium—** Bacillus subtilis 168 wild type and a rifampicin-resistant mutant, W168 Ts-1 (15) were used in these studies. They were grown in a rich medium which contained 2.5% tryptose (Difco), 2.0% yeast extract (Difco), 0.5% K2HPO4, and 3.0% glucose at 37° with vigorous aeration and harvested at late log phase or equivalent to a Klett reading of 600 to 750 (10). The cells were resuspended and washed twice with Buffer A, then stored at -70° until needed.

**Buffers—** Buffer A: 0.1 M Tris, 10 mM MgCl2, 1 mM EDTA, 0.3 mM dithiothreitol, 1 mM KCl, 2 mM phenylmethylsulfonyl fluoride, 10% glycerol, pH 7.9, at 4°. Buffer B: 0.1 M Tris, 10 mM MgCl2, 1 mM EDTA, 0.3 mM dithiothreitol, 2 to 4 mM phenylmethylsulfonyl fluoride.

* The abbreviations used are: SDS, sodium dodecyl sulfate; TEMED, N,N,N',N'-tetramethylenediamine.

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**Fluoride, 10% glycerol, pH 7.9, at 4°.** Buffer C: 20 mM Tris, 10 mM MgCl$_2$, 1 mM EDTA, 0.2 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 20% glycerol, pH 7.9, at 4°. Buffer E: 50 mM Tris, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, pH 7.9, at 4°. Buffer F: 20 mM Tris, 10 mM MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM NaCl, 20% glycerol, pH 7.9, at 4°. Buffer G: 20 mM Tris, 10 mM MgCl$_2$, 0.1 mM EDTA, 10 mM dithiothreitol, 6.5 mM urea, 10% glycerol, pH 7.9, at 4°. Buffer H: 60 mM Tris, 0.1 mM dithiothreitol, 0.2 M KCl, 90% glycerol, pH 7.9, at 4°. Buffer I: 20 mM Tris, 0.1 mM EDTA, 1 mM dithiothreitol, 7 mM urea, 20% glycerol, pH 7.9, at 4°. Buffer K: 20 mM Tris, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl$_2$, 0.3 M KCl, 35% glycerol, pH 7.9, at 4°.

**Partition of RNA Polymerase—** All procedures were carried out at 0-4° unless noted otherwise. One hundred grams (wet weight) of cells were resuspended in 100 ml of Buffer B. After two passages through a modified French pressure cell (17) at 7,000 p.s.i., another 100 ml of Buffer B was added. The crude extract was stirred for 10 min and then centrifuged at 30,000 × g for 30 min to remove cell debris. In addition to including EDTA and phenylmethylsulfonyl fluoride in all of the buffers to inhibit proteases, the crude extract was treated further, at this time, with diisopropylfluorophosphate and hemoglobin-Sepharose (18) to prevent proteolysis of the enzyme. The supernatant was adjusted to 25 mM diisopropylfluorophosphate and loaded onto the Blue dextran-Sepharose 4B (17) at 7,000 p.s.i., and stirred for 20 min. Hemoglobin-Sepharose was removed from the buffer containing the protein by washing with Buffer C. The enzyme was further purified by partitioning between polyethylene glycol (6,000 M weight) and DNA-cellulose.

**Purification of RNA Polymerase—** The enzyme was further purified by chromatography on blue dextran-Sepharose (17) at 7,000 p.s.i., and stirred for 20 min. Hemoglobin-Sepharose was removed from the buffer containing the protein by washing with Buffer C. The enzyme was further purified by partitioning between polyethylene glycol (6,000 M weight) and DNA-cellulose.

**Preparation of Core from Holoenzyme—** The holoenzyme obtained from DNA-cellulose column chromatography was dialyzed against Buffer F + 0.02 M KCl for 24 h. The enzyme was applied to a phosphocellulose column (2.6 × 17 cm) which had been equilibrated with Buffer E + 0.02 M KCl. The column was then washed with the application buffer until the A$_{280}$ fell to less than 0.03 and then the core fraction was eluted with Buffer E + 0.5 M KCl. The fractions containing the initial effluent containing the α and β subunits were applied at 17 ml/h to a column (1.6 × 15 cm) of blue dextran-Sepharose 4B (24) equilibrated with Buffer G. The column was washed with Buffer G until a protein measured by Bradford (25) was no longer eluted. The initial flow-through contained the α and β subunits. The β subunit which was bound to the column was eluted with Buffer G + 1 M NaCl.

The peak fractions of the initial effluent containing the α and β subunits were applied at 17 ml/h to a phosphocellulose column (1.6 × 16 cm) equilibrated with Buffer G. The column was washed with Buffer G until a protein measured by Bradford (25) was no longer eluted. The initial flow-through contained the α and β subunits. The β subunit which was bound to the column was eluted with Buffer G + 1 M NaCl.

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Identification of the largest polypeptide as the rifampicin-sensitive subunit was not due to an anomaly in the gel electrophoresis system used. Slab gels were run on the purified subunits using three different systems.

The system of Neville (27) was used with several modifications. The upper reservoir buffer was modified to maximize separation of the β and β′ subunits, and the concentration of persulfate was reduced to allow slower polymerization. Also, the concentrations of acrylamide and N,N'-methylenebisacrylamide were altered in the stacking gel to allow proper formation of sample wells. The system we used is as follows.

**Running gel:** 8.5% acrylamide, 0.077% N,N'-methylenebisacrylamide, 0.030% N HCl, 0.424% Tris, 0.13% SDS, 0.10% Temed, 0.025% persulfate, pH 9.18, running pH 9.50. **Stacking gel:** 4.5% acrylamide, 0.15% N,N'-methylenebisacrylamide, 0.0267 M H$_2$SO$_4$, 0.0541 M Tris, 0.1% SDS, 0.15% Temed, 0.0255% persulfate, pH 6.1, running pH 8.64. Lower reservoir buffer: 0.0308 M HCl, 0.4244 M Tris, pH 9.18. Upper reservoir buffer: 0.04 M boric acid, 0.0034 M Tris, 0.1% SDS, pH 8.45.

The protein samples were dialyzed against 0.0267 M H$_2$SO$_4$, 0.0641 M Tris, 15% glycerol, 0.1% SDS, 0.06% dithiothreitol, pH 6.1; then mixed with an equal volume of 23% glycerol, 2% SDS, 0.1% dithiothreitol, 0.0013% bromphenol blue, heated 5 min in boiling H$_2$O, and applied to the gel. The gel was run for 30 min at 15 mA, then for about 21/2 h at 25 mA. It was then stained for 60 min in 50% methanol, 7.5% acetic acid, 0.5% Coomassie brilliant blue R-250; and destained in 45% methanol, 9% acetic acid.

The system of Laemmli (28) was used essentially as published, except for alterations in the Temed and persulfate concentrations to allow slower polymerization; and in the concentrations of acrylamide and N,N'-methylenebisacrylamide in the stacking gel to allow proper formation of sample wells. Also, the concentration of acrylamide in the running gel was reduced to 5% to allow maximal separation of the β and β′ subunits. The system is as follows.

**Running gel:** 5% acrylamide, 0.13% N,N'-methylenebisacrylamide, 0.375 M Tris, 0.1% SDS, 0.0167% Temed, 0.035% persulfate, pH 8.8. **Stacking gel:** 4.5% acrylamide, 0.15% N,N'-methylenebisacrylamide, 0.125 M Tris, 0.1% SDS, 0.1% Temed, 0.03% persulfate, pH 6.8. Upper and lower reservoir buffers: 0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3.

The gel was pre-run for 30 min at 25 mA. Samples were dialyzed against 0.0625 M Tris, 10% glycerol, 5% mercaptoethanol, 2% SDS, pH 6.8. They were then diluted 1:1 with this same buffer including 0.002% bromphenol blue, heated for 5 min in boiling H$_2$O and applied to the gel. It was then run between 15 and 25 mA for about 2½ h. It was stained and destained as discussed above.

The third system used was the anionic glycine system of Wu and Bruening (14). It was used as published, except that the acrylamide and N,N'-methylenebisacrylamide concentrations were altered as discussed in the above systems. Also, the buffer listed as upper reservoir buffer by Wu and Bruening was used as both upper and lower reservoir buffer. The system is as follows.

**Running gel:** 5% acrylamide, 0.133% N,N'-methylenebisacrylamide, 0.109 M ethanalamine, 0.06 M HCl, 8 M urea, 0.073% persulfate, pH 9.4. **Stacking gel:** 4.5% acrylamide, 0.15% N,N'-methylenebisacrylamide, 0.0875 M triethanolamine, 0.06 M HCl, 8 M urea, 0.091% persulfate, pH 7.5. Upper and lower reservoir buffer: 0.0735 M ethanalamine, 0.06 M glycine, 0.1% SDS, pH 6.7.

Samples were mixed with equal volumes of 0.0875 M triethanolamine, 0.06 M HCl, 8 M urea, 12.5% sucrose, 0.5% SDS, 10 mM dithiothreitol, 0.0036% bromphenol blue, pH 7.5. They were then heated for 5 min in boiling H$_2$O and applied to the gel. The gels were run at 15 mA for 30 min, then at 25 mA for about 2½ h. They were then stained and destained as described above.

**Materials** — All reagents were purest available commercial products which were used without further purification except where noted, and their sources were: polyethylene glycol (6,000 to 7,500); cyanogen bromide; ammonium persulfate; ethanalamine; and boric acid were from Matheson, Coleman and Bell; urea (super pure); Tris (super pure), and [I]HUTP were from Schwarz/Mann; spectrophotometer, and mercaptoethanol were from Eastman Organic Chemicals; unlabeled nucleoside triphosphates were from Boehringer Mannheim Biochemicals (type II); Coomassie brilliant blue G, and glycine were from Sigma Chemical Co.; Sepharose 4B and 6B, dextran T500, and blue dextran 2000 were from Pharmacia Fine Chemicals; poly[d(A-T)] was from P-L Biochemicals, Inc.; calf thymus DNA was from Worthington Biochemical Corp.; dithiothreitol and phenylmethylsulfonyl fluoride were from Calbiochem; acrylamide, sodium dodecyl sulfate, N,N',N'-methylenebisacrylamide, and N,N',N',N'-tetramethylethylenediamine were from Bio-Rad Laboratories; diisopropylfluorophosphate and triethanolamine were from Aldrich Chemical Co., Inc.

The enzyme was deionized three passages through a desalting column (consisting of AG501-X8 (D) mixed bed resin; Bio-Rad Laboratories).

**RESULTS**

**Purification of Core and Holoenzyme** — In order to obtain purified subunits of RNA polymerase for reconstitution studies it was necessary to develop procedures which would prevent proteolysis during purification of the enzyme and during dissociation of the enzyme into subunits; furthermore, relatively rapid methods for obtaining dissociated subunits in...
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FIG. 2. The SDS-polyacrylamide gel electrophoresis pattern of RNA polymerase core and holoenzyme obtained from the DNA-cellulose column. The urea-SDS-polyacrylamide gel electrophoresis system of Wu and Bruening (14) was used. The core pattern (a) was observed in Fractions 31 to 42 and 35 to 39 of Fig. 1, a and b, respectively. The holoenzyme pattern (b) was observed in Fractions 44 to 56 and 41 to 53 of Fig. 1, a and b, respectively. Pooled holoenzyme is shown in c. In a and b the polyacrylamide gel concentration was 5% and in c, 10%.

Fig. 3. Rechromatography of core on DNA-cellulose. a, pooled fractions of core from an initial DNA-cellulose column were chromatographed on a second DNA-cellulose column with a shallow linear gradient from 0.3 to 0.8 M KCl in Buffer D. b, urea-SDS-polyacrylamide gel electrophoresis of Fractions 16, 19, 22, 25, 28, 31, 34, and 37 from this column. Dye fronts were marked with India ink.

good yields were required to optimally study reassociation of the subunits. The use of hemoglobin-Sepharose for the batch and column removal of proteases and peptidases which abound in extracts from B. subtilis has been described in detail under "Experimental Procedures" and by Nakayama et al. (18). The purification procedure was essentially that as described by Fukuda and Doi (22) except that the concentration of glycerol and KCl were increased in the buffer used in chromatography on DNA-cellulose from 10% and 0.02 M to 20% and 0.1 M, respectively, and the column was washed with 0.3 M KCl before gradient elution of the enzyme. While the yield of polymerase did not increase significantly, the modification of the buffer resulted in a much higher yield of holoenzyme relative to the core enzyme. This is shown in Fig. 1. When Fukuda and Doi (22) used 10% glycerol in their preparations about twice as much core (Fig. 1a, Fractions 32 to 42) as holoenzyme (Fig. 1a, Fractions 42 to 52) was obtained. The
inclusion of 0.1 M KCl and 20% glycerol in the column equilibration buffer increased the yield of holoenzyme (Fig. 1b, Fractions 40 to 54) from about 30% to about 80%. Since holoenzyme had a higher specific activity than core enzyme, these results explain the loss of activity they reported when the enzyme obtained from the DEAE-cellulose was applied to DNA-cellulose. It was also important during chromatography on DNA-cellulose to elute the column with a salt gradient to separate the core (Fig. 2a) and holoenzyme (Fig. 2b). The SDS-urea-gel electrophoresis analysis of the fractions containing the core and holoenzyme revealed additional subunit differences. In addition to the subunits, αβ′β′, the core fractions contained ω and δ (Fig. 3). These two subunits had been reported previously (29, 30). The holoenzyme fractions contained αβ′β′α and ω′; no δ was present (Fig. 2c).

Another small polypeptide, probably ω′ (51), was found loosely associated with both core and holoenzyme. While the polypeptide was found to be associated with the polymerase after sedimentation through a glycerol gradient (5), it was not associated in stoichiometric quantities with core after rechromatography on DNA-cellulose (Fig. 3).

Core enzyme was rechromatographed on DNA-cellulose (Fig. 3b). The fractions containing the core enzyme were analyzed by urea-SDS-polyacrylamide gel electrophoresis (Fig. 3b). The stoichiometry of the α′ remained unchanged in the core fractions. One ω′ was found per α′. One δ subunit was present per core in only the early fractions (Nos. 12 to

Fig. 5. The separation of the α and β subunits from the β′ subunit by chromatography on blue dextran-Sepharose. α, core enzyme (12 mg) was dissociated in Buffer G containing 6.5 M urea and applied to a column (1.8 × 15 cm) of blue dextran-Sepharose at 17 ml/h as described under "Experimental Procedures." The column was washed with Buffer G until protein was no longer eluted. The column was then step-eluted with Buffer G containing 1 M NH₄Cl (Fraction 30). Fractions of 1.7 ml were collected. Protein was determined by measuring A₅₉₅ of 25 µl of each fraction in 3 ml of Bradford's reagent (25). β, pooled Fractions 8 to 18 and 39 to 43 were analyzed by urea-SDS-polyacrylamide gel electrophoresis. The pooled fractions are labeled a and b, respectively.

Fig. 6. The separation of α and β subunits by phosphocellulose chromatography. α, the α′ and β subunits (Fractions 8 to 18, Fig. 5) obtained by chromatography of urea-dissociated core on blue dextran-Sepharose were pooled and applied to a phosphocellulose column (1.8 × 16 cm) equilibrated with Buffer G. The protein concentration of the eluant was determined immediately by a modification of Bradford's method (see Fig. 5). The column was washed with Buffer G until the A₅₉₅ due to the α′ and β′ subunits had fallen to 0.06 (i.e. one-half of the peak value). The β′ subunit was then eluted from the column with Buffer G + 1 M NH₄Cl. β, Fractions 15, 19, 23, and 30 from the phosphocellulose column were analyzed by urea-SDS-polyacrylamide gel electrophoresis.
complex, p, p', o, and α subunits, respectively. The gels were 8.5 cm long, 17 cm wide, and 0.1 cm thick.

SDS-polyacrylamide gel system of Neville (27). c, SDS-polyacrylamide gel system of Laemmli (28). Panels 1, 2, 3, 4, and 5 represent the core enzyme which was then dialyzed against Buffer E containing 10% glycerol and 0.02 M KCl prior to chromatography. These conditions destabilized the holoenzyme and caused the release of σ and δ factors. Under these conditions, the σ and δ factors were found in the flow-through fractions from phosphocellulose while the core was eluted with Buffer E + 0.5 M KCl. The core obtained from the phosphocellulose column had a subunit composition of αββ′α′.

The core was dissociated and fractionated into its subunits by two different methods. The first method consisted of dissociating the core into subunits by treatment with urea followed by passage through a phosphocellulose column (Fig. 4). The order of elution of the subunits was α, β, and β′. Although the separation of the subunits was very effective, the recovery of the β and β′ subunits was poor. The β subunit of polymerases from other bacterial species has been found to elute at a lower ionic strength than the β′ subunit (8).

A more suitable method was developed which provided a rapid and effective purification of the subunits. Urea was added to core enzyme which was then dialyzed against Buffer G containing 6.5 M urea and applied to a blue dextran-Sepharose column (see “Experimental Procedures”). The α, α′, and β subunits did not bind to the column and were found in the flow-through fractions (Fig. 5). The β′ subunit did bind to the column and was eluted by 1 M NH₄Cl. Halling et al. (10) demonstrated that the α′ subunit obtained by this rapid method contained the 2 zinc atoms associated with the enzyme. To separate the α′ from the β subunit, this mixture was passed through a phosphocellulose column (Fig. 6a) as described under “Experimental Procedures.” The α′ subunit was present in the flow-through fractions while the β subunit was eluted with 1 M NH₄Cl (Fig. 6b). The yield of the β subunit was 70 to 90% by this method. A critical factor in obtaining good recovery of the β subunit was the immediate elution of the β subunit after the α′ subunit had eluted from the column. Recovery of the β subunit was very poor, if the α and β subunits were applied slowly or allowed to remain on the column. While the fractions containing the β subunit were not contaminated with α′, a small amount of β was sometimes found in the later α fractions (Fig. 6b, Fraction 19).

The relative mobility of β and β′ was compared by the use of three SDS-polyacrylamide gel electrophoresis systems (Fig. 7). The results with all three systems indicated that the subunit responsible for rifampin resistance (see below) had the slowest mobility indicating that it was also the largest subunit. For B. subtilis RNA polymerase subunits the urea-SDS-polyacrylamide gel electrophoresis system of Wu and Bruening (14) was the most effective in separating the β and β′ subunits.

Two types of reconstitution experiments were carried out. In the first set of experiments the reconstitution was carried out between the αβ and β′ fractions obtained by blue dextran-Sepharose chromatography. The results are shown in Table I.

Reassociation of Subunits to Form RNA Polymerase - Experiments were performed to see whether enzymatic activity could be obtained by reassociation of the subunits and to determine which of the subunits was responsible for rifampicin resistance of the enzyme. For this purpose subunits were purified from the enzyme obtained from the wild type strain and a rifampicin resistant mutant of B. subtilis.

Two types of reconstitution experiments were carried out. The αβ and β′ subunits were obtained by blue dextran-Sepharose (24) column chromatography in the presence of urea. The reconstitution conditions and assay system are described under “Experimental Procedures.” The subunit mixtures were kept at the temperatures for the time indicated under “Conditions” and then 5-μl aliquots were added to 250 μl of the reaction mixture and incubated for 20 min at 37°. When rifampicin (Rif) (1 μg) was included in the reaction mixture, it was introduced before the enzyme was added. All activities (cpm) are the average of triplicate assays.

| Conditions | αβ + β′ | αβ + β′ | αβ + β′ | αβ + β′ |
|------------|---------|---------|---------|---------|
| 30°, 0 min + Rif | 102 | 53 | 1,381 | 638 |
| 30°, 20 min + Rif | 1,485 | 826 | 9,806 | 2,898 |
| 30°, 60 min + Rif | 1,522 | 946 | 9,824 | 4,327 |
| 30°, 60 min + Rif | 135 | 61 | 10,091 | 5,759 |

Reconstitution of RNA polymerase core from subunits of wild type and a rifampicin-resistant mutant

The circled subunits are from the rifampicin-resistant mutant. The αβ and β′ subunits were obtained by blue dextran-Sepharose column chromatography in the presence of urea. The reconstitution conditions and assay system are described under “Experimental Procedures.” The subunit mixtures were kept at the temperatures for the time indicated under “Conditions” and then 5-μl aliquots were added to 250 μl of the reaction mixture and incubated for 20 min at 37°. When rifampicin (Rif) (1 μg) was included in the reaction mixture, it was introduced before the enzyme was added. All activities (cpm) are the average of triplicate assays.

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Identification of the subunit responsible for rifampicin resistance

The circled subunits are from the rifampicin-resistant mutant. The subunits were isolated by chromatography of core on blue dextran-Sepharose (24) and phosphocellulose columns in the presence of urea as described under Experimental Procedures. Core enzymes were reconstituted by mixing α, β, and β′, in that order, in Buffer K containing 35% glycerol and 0.3 M KCl (6) and assayed as described under Experimental Procedures. For holoenzyme activity, 2.1 μg of φ29 DNA and 0.12 μg of σ factor were used. All activities are the average of triplicate assays. Rifampicin (Rif) (1 μg) was added to each reaction mixture before the enzyme. All combinations of only two different subunits did not incorporate significant counts.

| Subunit combinations | RNA polymerase activity | φ29 DNA | poly(dAT) | -Rif | +Rif | -σ | +σ |
|----------------------|-------------------------|---------|-----------|------|------|----|----|
| α + β + β′           | 1571                    | 121     | 447       | 2269 |
| α + β + β′           | 1380                    | 71      | 1516      |      |
| α + β′ + β′          | 1065                    | 0       |          |      |
| α + β + β            | 1010                    | 1082    |          |      |
| α + β′ + β′          | 812                     | 0       |          |      |
| α + β′ + β′          | 619                     | 745     |          |      |
| α + β′ + β′          | 798                     | 1050    | 368       | 1269 |

When the αβ and the β′ subunits were mixed in equimolar concentrations and dialyzed at 0°C for 8 h, very little or no activity was obtained with wild type enzyme. If the subunit mixture was incubated at 30°C for 20 min prior to assay, the activity was significantly greater. The activity was sensitive to rifampicin when the wild type αβ subunits were present in the reassociated enzyme, whereas it was resistant to rifampicin if the αβ subunits were obtained from the enzyme isolated from the rifampicin-resistant mutant. Furthermore, the reassociation of the mutant subunits occurred more efficiently at the lower temperature since the activity prior to heat activation at 30°C was higher for the mutant enzyme than the wild type enzyme. Another interesting observation was the stimulation of the mutant enzyme by rifampicin. Rifampicin resistance was expected and the stimulation of the reconstituted enzyme with rifampicin confirmed the results reported by Sumida-Yasumoto and Doi (15) with crude extracts from rifampicin-resistant mutants.

The reconstitution experiments were also carried out under conditions whereby all core subunits had been separated, since it was still possible that the α subunit was responsible for the rifampicin resistance in these organisms (6). Table II illustrates the results obtained with the various combinations of subunits obtained from the wild type and rifampicin-resistant mutant. All combinations containing fully reconstituted core (αββ′) were able to synthesize RNA. When the reconstituted enzymes were tested in the presence of rifampicin, only those enzymes containing β subunit from the rifampicin-resistant mutant were resistant to the antibiotic. The last column of Table II shows that the reconstituted core activity can be stimulated on φ29 DNA by the presence of σ factor. Thus, the largest subunit in B. subtilis RNA polymerase is the β subunit, whereas, in E. coli the second largest subunit is responsible for rifampicin resistance.

Discussion

Purification methods have been developed to obtain B. subtilis RNA polymerase enzyme and its subunits rapidly and in good yield. This has been facilitated in part by the use of hemoglobin-Sepharose (18) as an affinity substrate for intracellular proteases and peptidases which are present in crude extracts of B. subtilis and which are extremely difficult to inhibit completely by use of chemical inhibitors. The use of the urea-SDS-polyacrylamide gel electrophoresis system of Wu and Bruening (14) has permitted a much greater separation of the β and β′ subunits of B. subtilis than the methods of Laemmli (28) and Neville (27). The urea-SDS-gel system is useful in identifying all the B. subtilis RNA polymerase subunits without the necessity of extended electrophoresis. The blue dextran-Sepharose column has been exploited already by Halling et al. (10) to show that the β′ subunit of B. subtilis RNA polymerase contains zinc. The advantages of this rapid isolation procedure are that zinc is not lost from the β′ subunit even in the presence of 6.5 M urea, the duration of exposure of the subunit to urea is minimized, and milligram quantities of the β′ subunit can be obtained.

The proportion of holoenzyme to core enzyme isolated was dependent on the purification procedure used. When the KCl and glycerol concentrations in the buffer used to dialyze the enzyme and equilibrate the DNA-cellulose column were 0.92 M and 10%, respectively, the σ factor was released from holoenzyme during chromatography on DNA-cellulose. This may be similar to the results of Halling et al. in E. coli, and thus could be useful for quickly determining the loci of ts mutations that map in the σ factor. These studies would not necessitate the separation of the α and the β subunits from each other. However, when the reconstitution of the polymerase from all subunits is necessary, the α and β subunits can be separated from each other by chromatography on phosphocellulose as described in the second method. Reconstitution was achieved in buffer containing 35% glycerol and 0.3 M KCl (6). The data indicate that an immature core is formed which can be activated by heat as found with E. coli (6). The σ stimulation of heat-activated core suggests that a proper conformation has been attained. Optimal conditions and kinetics of reconstitution are now being studied. The development of this reconstitution method should allow the identification of mutations in core subunits and the elucidation of the function of core-associated polypeptides such as α′, δ, δ′, and β′.

Linn et al. (31) had reported that the second largest polypeptide of B. subtilis RNA polymerase was β based on the isolation of a rifampicin-resistant mutant of B. subtilis, LS3, S. M. Halling, K. C. Burtis, R. H. Doi, unpublished observations.

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which simultaneously was asporogenous and had an altered mobility of the second largest RNA polymerase subunit in sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies. However, the nature of the mutation has not been established; some of the revertants to sporogeneity retained rifampcin-resistant and had varied sporulation properties (32). Some of these revertants had core enzymes with subunits of wild type mobility. Thus from these studies alone it was not clear whether the second largest B. subtilis RNA polymerase subunit could unequivocally be labeled as $\beta$ or $\beta'$. Although our studies have shown that the electrophoretic properties were not a function of the specific method used, it is possible that bacterial strain differences could account for the faster mobility of the $\beta$ polypeptide in the studies of Linn et al. (31).

A number of observations, summarized in Fig. 8, support labeling the largest polypeptide of B. subtilis RNA polymerase as $\beta$. The $\beta$ subunit from each species is eluted at a lower ionic strength from phosphocellulose than the $\beta'$ subunit (Fig. 6) (12). Zinc has been reported to be associated with the $\beta'$ subunit of E. coli (11) and B. subtilis (10). The $\beta'$ subunit from each species aggregates readily (5). The limited proteolysis of the $\beta'$ subunit of E. coli resulted in the appearance of a polypeptide with a molecular weight of 110,000 (34), suggesting the presence of $\alpha$ factor in fractions containing core but not holoenzyme. The $\beta'$ subunit of B. subtilis results in the appearance of a polypeptide with a molecular weight of 110,000 (34). The $\beta'$ subunit is the more basic subunit of the polymerase in all species of prokaryotes studied thus far; furthermore, the second largest polypeptide of B. cereus RNA polymerase is the most basic subunit and has been designated as $\beta'$ by Zillig (8).

The presence of $\delta$ factor in fractions containing core but not holoenzyme is interesting since recent studies by Pero et al. (29) and Tjian et al. (35) have suggested that $\delta$ plays an important role in promoter recognition during phase infection. The presence of $\delta$ decreases the affinity of core in Buffer D for DNA-cellulose. The $\delta$ factor in vegetative cells appears to be different from the $\delta_1$ and $\delta_2$ factors first reported in sporulating cells by Fukuda et al. (36) since the $\delta_1$ and $\delta_2$ enzymes from sporulating cells had a much higher affinity for DNA-cellulose and had a high specific activity on several DNA templates in the absence of $\sigma$ factor. The $\delta$-containing core from vegetative cells eluted at a lower ionic strength from DNA-cellulose than holoenzyme (37) and had an extremely low specific activity on the poly[d(AT)] template. Although we and Plevani et al. (37) did not find $\delta$ and $\sigma$ in the same core fractions eluted from the DNA-cellulose column, it is possible that $\sigma$ was dissociated from the $\delta$-containing core during DNA-cellulose chromatography. Whether $\sigma'$ is an integral part of the polymerase or not is unclear as we found the association of this polypeptide to be variable. We are currently determining the relationship between the $\delta$ factors which have been reported and the relationship between $\delta$ and $\sigma$ factor in vegetative holoenzyme.

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Fig. 8. Summary of the properties of the large polypeptides of E. coli and B. subtilis RNA polymerase when the polypeptides in the urea-SDS-polyacrylamide gels are labeled as shown.

| Property             | E. coli | B. subtilis |
|----------------------|---------|------------|
| RIFAMPICIN SENSITIVE | $\beta$  | $\beta'$    |
| ELUTION FROM PC     | $\beta$  | $\beta'$    |
| CONTAIN ZINC        | $\beta$  | $\beta'$    |
| LOW SALT AGGREGATION| $\beta'$ | $\beta'$    |
| PROTEOLYSED EASILY  | $\beta'$ | $\beta'$    |

* S. M. Halling and R. H. Doi, unpublished observations.
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