RNA Polymerase from Sporulating Bacillus subtilis

PURIFICATION AND PROPERTIES OF A MODIFIED FORM OF THE ENZYME CONTAINING TWO SPORULATION POLYPEPTIDES*

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A new form of DNA-dependent RNA polymerase termed enzyme III has been purified from sporulating cells of Bacillus subtilis. In addition to the subunits of core RNA polymerase (β', β, α, and ω), enzyme III contains sporulation-specific polypeptides of 85,000 (P86) and 27,000 (PZ) daltons. P86 corresponds to an RNA polymerase binding protein previously identified by precipitation of RNA polymerase from crude extracts of sporulating cells with antibody directed against core enzyme. Both P86 and PZ are purified with RNA polymerase highly purified by gel filtration, DEAE-cellulose chromatography, phosphocellulose chromatography, and glycerol gradient centrifugation. Enzyme III bound more tightly to phosphocellulose and sedimented more rapidly during zone centrifugation than did RNA polymerase lacking the sporulation polypeptides. RNA polymerase containing P86 and PZ transcribed Bacillus subtilis DNA about 4.5 times more actively than did core RNA polymerase, although both enzymes exhibited similar activities with poly(dA-dT) and phage φ2 DNA as templates. Enzyme III and core RNA polymerase also differed in their response to increasing concentrations of MgCl2 and KCl.

The DNA-dependent RNA polymerase of Bacillus subtilis is thought to undergo modifications during the process of sporulation (for a review, see Ref. 1). The onset of sporulation, for instance, is associated with a marked decrease in the activity of the σ subunit of RNA polymerase (2, 3). The inhibition of σ is a specific event in the process of sporulation since it is prevented in mutants blocked at an early stage of sporulation, including certain mutants of RNA polymerase isolated by resistance to rifampicin (4, 5). Extracts of sporulating cells appear to contain a specific component that inhibits σ activity probably by interfering with the binding of σ to core RNA polymerase (5-7).

While lacking σ, RNA polymerase from sporulating cells can be isolated in association with a large sporulation-specific polypeptide (8). This species was first identified by precipitation of RNA polymerase from crude extracts of sporulating cells with antibody directed against core enzyme from vegetative bacteria (8). A reconstitution experiment confirmed that this sporulation polypeptide binds specifically to RNA polymerase. The binding protein first appears early during sporulation and is either absent or present in greatly reduced amounts in enzyme from stationary phase cells of mutants blocked early in sporulation (9). Here we report on the purification and properties of a form of RNA polymerase containing this previously described sporulation protein and an additional sporulation-specific polypeptide.

MATERIALS AND METHODS

Buffers—Buffer G, 0.05 M Tris-HCl, pH 7.5/0.01 M MgCl2/0.2 M KCl/0.3 mM dithiothreitol/0.1 mM EDTA/10% (v/v) glycerol; Buffer I, 0.05 M Tris-HCl, pH 7.9/0.3 M KCl/0.3 mM dithiothreitol/0.01 M EDTA/10% (v/v) glycerol; Buffer II, 0.05 M Tris-HCl, pH 7.9/0.3 mM dithiothreitol/0.01 M EDTA/20% (v/v) glycerol. All buffers also contained 5% (v/v) of a freshly prepared solution of phenylmethylsulfonylfluoride (6 mg/ml of 95% ethanol).

Cells—Bacillus subtilis SMY, a sporulating Marburg strain, was used in all experiments unless noted otherwise. Cells were grown in Difco sporulation medium (9) in a 20-liter New Brunswick fermentor and harvested with a refrigerated Sharples continuous flow centrifuge. To prevent proteolysis of RNA polymerase during purification (2), cells were washed immediately after harvesting with 800 ml of an ice-cold buffer containing 0.05 M Tris-HCl (pH 7.5), 1.0 M KCl, 0.01 M EDTA, and 5% (v/v) phenylmethylsulfonylfluoride solution. Cells were frozen over a bath of dry ice in acetone and stored at -80°C.

Assay—RNA polymerase activity was assayed as previously described (10, 11) with 0.15 M [3H]ATP (2 mCi/mmol) except that no KCl was added unless indicated otherwise. Template were either 10 μg of poly(dA-dT), 7.5 μg of φ2 DNA, or 18 μg of B. subtilis DNA. One unit of RNA polymerase activity incorporates 1 nmol of [3H]AMP in 10 min at 37°C.

Antibody Precipitation—Methods for the precipitation of RNA polymerase from crude extracts of sporulating cells by anti-core RNA polymerase antibody and for the analysis of the precipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis have been described (2, 8).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (12). The following proteins served as stand-

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ard: β-galactosidase (135,000 daltons), phosphorylase a (93,000 daltons), fructose-6-phosphate kinase (78,000 daltons), transferrin (78,500 daltons), bovine serum albumin (68,000 daltons), catalase (59,000 daltons), fumarase (48,500 daltons), aldolase (40,000 daltons), and carbonic anhydrase (29,000 daltons). Sodium dodecyl sulfate slab gel electrophoresis in Tris-glycine was as described by Studier (13) and was employed for the subunit structure analyses illustrated in the figures. The "separating" gels were 14 x 24 cm and contained a 5 to 12.5% gradient of acrylamide. Electrophoresis was at a constant current of 10 mA (with the voltage limited to 250 volts) for 3 hours. Gels were stained for 1 hour at 37°C with the stain described by Vesterberg (14) and destained in a solution of 5% (v/v) methanol and 7.6% (v/v) acetic acid.

Purification of RNA Polymerase—Forty-five grams of B. subtilis harvested either during the 3rd hour of sporulation or during vegetative growth were disrupted in 260 ml of Buffer G by sonication for a total of 1 hour at 0-5°C. The extract was centrifuged at 120,000 x g for 90 min and the resultant supernatant fluid was adjusted to 60% saturation with solid ammonium sulfate. After centrifugation at 100,000 x g for 30 min, the precipitate was resuspended in 8 ml of Buffer I. Pancreatic RNase (20 mg) was added and the solution was dialyzed for 2 hours against 1 liter of Buffer I. MgCl₂ (to a concentration of 0.02 M) and DNAse I (9 mg) were then added. After 3 hours at 0°C the enzyme solution was centrifuged for 1 hour at 100,000 x g and the supernatant fluid was applied to a Bio-Gel A-1.5m agarose column (4 x 80 cm) internally supported by glass beads (15). RNA polymerase activity was eluted as a single peak with Buffer I. Next, the effluent fractions containing the bulk of the enzyme were pooled, diluted to a final concentration of 0.1 M KCl by the addition of Buffer II, and applied to a DEAE-cellulose column (100 ml). The column was washed with Buffer II containing 0.1 M KCl and the enzyme then eluted step-wise with Buffer II containing 0.24 M KCl. The enzyme was then diluted to a final concentration of 0.1 M KCl and slowly applied to a phosphocellulose column (20 ml). After the column was washed with Buffer II containing 0.1 M KCl, enzyme I was eluted step-wise with Buffer II containing 0.24 M KCl. Next, enzymes II and III were eluted from the phosphocellulose column with a linear gradient of KC1 as described in the legend to Fig. 1 and these forms of RNA polymerase were further purified by zone centrifugation as described in the legend to Fig. 6.

RESULTS

RNA Polymerase from Sporulating Bacillus subtilis—RNA polymerase was purified from B. subtilis harvested at the 3rd hour of sporulation as described under "Materials and Methods" and as summarized in Table I. Chromatography on phosphocellulose resolved three different forms of RNA polymerase that are referred to as enzymes I, II, and III. Enzyme I was eluted stepwise between 0.1 and 0.24 m KCl.

| Fraction | Total protein | Total activity | Specific activity | Yield |
|----------|---------------|----------------|------------------|-------|
|          | mg            | units          | units/mg         | %     |
| 1. Ammonium sulfate enzyme | 1190 | 14,100 | 12 | 100 |
| 2. Pooled Agarose enzyme | 270 | 6,670 | 25 | 47 |
| 3. DEAE-cellulose 0.24 M eluate | 141 | 4,100 | 29 | 29 |
| 4. Phosphocellulose 0.24 M eluate | 2.8 | 305 | 109 | 109 |
| 0.30 M eluate | 1.43 | 1,040 | 730 | 109 |
| 0.36 M eluate | 1.12 | 1,140 | 1012 | 1012 |

* Protein was determined as described (10).

* RNA polymerase activity is expressed as nanomoles of AMP incorporated in 10 min at 37°C with poly(dA-dT) as template at 0.15 M KCl.

while enzymes II and III were eluted with a linear gradient of KC1 at 0.30 and 0.36 m KCl, respectively (Fig. 1). Enzymes II and III accounted for the bulk of the RNA polymerase and were therefore studied in greatest detail. Measurements of the template specificity of enzyme in fractions from the gradient elution indicated that enzyme III transcribed B.
B. subtilis DNA about 4.5 times more actively than did enzyme II, although both forms of RNA polymerase exhibited similar activities with either poly(dA-dT) or phage ϕe DNA as templates (Figs. 1 and 2). This difference in template preference was confirmed under conditions of linear dependence of enzyme activity on protein concentration (Fig. 2). Enzymes II and III also differed in their response to Mg²⁺. Transcription of the bacterial DNA by enzyme III was stimulated more markedly by increasing concentrations of Mg²⁺ than was RNA synthesis by enzyme II (Fig. 3).

Another difference between enzymes II and III was in their sensitivity to KCl. Although transcription of B. subtilis DNA by both enzymes was inhibited by KCl (Fig. 4a), increasing ionic strength stimulated transcription of phage ϕe DNA by enzyme III but partially inhibited RNA synthesis by enzyme II (Fig. 4b). In contrast, KCl slightly stimulated transcription of poly(dA-dT) by enzyme II while having little effect on the transcription of the synthetic template by enzyme III (Fig. 4c). For comparison, the effect of KCl on enzyme II was similar to that observed for core RNA polymerase from vegetative bacteria. For example, the addition of 0.15 M KCl decreased activity with ϕe DNA as template by about 50% and stimulated transcription of poly(dA-dT) by about 40%. This finding is consistent with the gel analyses described below that indicate that enzyme II corresponded to core RNA polymerase. (A more detailed account of the effect of KCl on RNA synthesis by vegetative core polymerase and holoenzyme has been presented previously (10).)

Slab gel electrophoresis of RNA polymerase in fractions from the phosphocellulose gradient elution revealed polypeptides of 85,000 and 27,000 daltons (and small amounts of a 65,000-dalton species) that co-eluted with the subunits of core RNA polymerase (β', β, α, and ω) in enzyme III (Fractions 20 to 22; Fig. 5). Gel analysis also revealed a contaminating polypeptide of 60,000 daltons that was eluted in fractions throughout the gradient and a 90,000-dalton contaminant that largely eluted after enzyme III. The stoichiometries of the 85,000- and 27,000-dalton species in enzyme III (hereafter referred to as P85 and P27, respectively) were between 0.5 and 1.0 per core RNA polymerase. Both of these species were absent in enzyme II (fractions 14 to 16; Fig. 5). Phosphocellulose-purified enzyme I (not shown) also contained, in addition to the subunits of core enzyme, small amounts of P85 and P27, and a protein with the mobility of a newly described subunit of holoenzyme in vegetative bacteria called δ (16). (It is interesting to note that a form of RNA polymerase from phage SP01-infected B. subtilis also elutes from phosphocellulose between 0.1 and 0.24 M KCl and contains this δ polypeptide in addition to phage-induced subunits. Conceivably, δ lowers the affinity of RNA polymerase for phosphocellulose.)

As further evidence that P85 and P27 were bound to RNA polymerase, enzymes II and III were subjected to glycerol gradient centrifugation in buffer containing 1.0 M KCl. For comparison, the effect of KCl on enzyme II was similar to that observed for core RNA polymerase from vegetative bacteria. For example, the addition of 0.15 M KCl decreased activity with ϕe DNA as template by about 50% and stimulated transcription of poly(dA-dT) by about 40%. This finding is consistent with the gel analyses described below that indicate that enzyme II corresponded to core RNA polymerase. (A more detailed account of the effect of KCl on RNA synthesis by vegetative core polymerase and holoenzyme has been presented previously (10).)

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Fraction Number

FIG. 6. Zone centrifugation of enzymes II and III. Phosphocellulose-purified enzymes II and III (130 units of each) were concentrated against Ficoll to 0.6 ml and separately sedimented through 12-ml linear gradients of 10 to 30% (v/v) glycerol in Buffer II containing 1.0 M KCl for 28 hours at 2°C and at 200,000 x g. Fractions (0.54 ml) were collected, and 20-μl samples were assayed with poly(dA-dT) as template. Enzyme II, •••; enzyme III, △△△. The positions of β-galactosidase (βgal) and hemoglobin (Hb), sedimented in a parallel gradient, are indicated by the arrows.

more rapidly (Fig. 6). (This small difference in sedimentation coefficient was reproducibly observed in several independent experiments.) As evidence of high purity, enzyme activity at this stage of purification was coincident with a peak of protein (17). Fig. 7 compares the subunit structures of glycerol gradient-purified enzymes II and III in the fractions of peak activity with enzyme III in Fraction 21 (Fig. 5) of the phosphocellulose gradient elution. Enzyme II contained β′, β, α, and ω and appeared to correspond to core RNA polymerase. Glycerol gradient-purified enzyme III contained P85 and P27 (Fig. 7) and slab gel analysis of enzyme in fractions across the glycerol gradient (not shown) revealed that these species had co-sedimented with the core subunits of enzyme III. However, the stoichiometries of these sporulation polypeptides had diminished somewhat during sedimentation of enzyme III through the glycerol gradient of high salt concentration. Glycerol gradient-purified enzyme III transcribed B. subtilis DNA more actively than did the purified enzyme II.

To determine whether P85 corresponded to the RNA polymerase-binding protein previously identified by antibody precipitation (8), RNA polymerase was precipitated from a crude extract of radioactively labeled sporulating cells by antibody directed against vegetative core enzyme. The antibody precipitate contained, in addition to core RNA polymerase, a polypeptide with the mobility of P85 (Fig. 8). In a previous study, we (8) showed that this species is sporulation-specific; also, a reconstitution experiment indicated that this antibody-precipitated polypeptide binds to RNA polymerase. Although we had earlier estimated a molecular weight of 70,000, we now calculate a higher molecular weight using the molecular weight markers listed under "Materials and Methods." Since the antibody-precipitated polypeptide co-migrated with P85, we assume that these polypeptides are the same species.

Finally, we have noted that the relative amounts of enzymes II and III are apparently characteristic of the stage of sporulation. Phosphocellulose chromatography of RNA polymerase from cells harvested at the 5th hour of spore formation, for instance, yielded only enzymes I and III. This finding could indicate that P85 and P27 are most abundant at late times during the sporulation process.

Fig. 7. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of enzymes II and III. Sample A, enzyme III in Fraction 21 of the phosphocellulose gradient elution (Fig. 1); Sample B, glycerol gradient-purified enzyme II; Sample C, glycerol gradient-purified enzyme III.

RNA Polymerase from Vegetative Bacteria and From an Asporogenous Mutant—The following lines of evidence indicate that P85 and probably also P27 are absent in vegetative bacteria. First, gradient elution from phosphocellulose of RNA polymerase from vegetative bacteria yielded enzyme that eluted only at 0.30 M KCl and that lacked the sporulation polypeptides (17). Second, high resolution two-dimensional
Thus, although this RNA polymerase-binding protein is not electrophoresis (not shown) of crude extracts from these during stationary phase is severely restricted. Asporogenous bacteria indicated that SpoOa-5NA produced enzyme III activity (17). Furthermore, this protein is not encoded in the genome of B. subtilis, as it was not detected by conventional purification procedures (16, 22, 23). One of these purification procedures was to use the antibody directed against core RNA polymerase from vegetative bacteria. This antibody precipitate was soluble and subjected to gel electrophoresis in a slot adjacent to glycerol gradient-purified enzyme III. After staining and destaining, the gel was dried and the gel slice was extracted in a 3% solution of Protosol (New England Nuclear) and the radioactivity was measured in a scintillation counter. A photograph of the slot containing the glycerol gradient-purified enzyme III is aligned below the radioactive profile.

**DISCUSSION**

This paper describes the isolation of a form of RNA polymerase-binding protein identified by antibody precipitation. RNA polymerase in an extract of [3H]tryptophan-labeled Bacillus subtilis harvested at the 4th hour of sporulation was precipitated with antibody directed against core RNA polymerase from vegetative bacteria (8). This antibody precipitate was solubilized and subjected to slab gel electrophoresis in a slot adjacent to glycerol gradient-purified enzyme III. After staining and destaining, the gel was dried and the slot containing the radioactive sample cut into 3-mm slices. The slices were extracted in a 3% solution of Protosol (New England Nuclear) and the radioactivity was measured in a scintillation counter. A photograph of the slot containing the glycerol gradient-purified enzyme III is aligned below the radioactive profile.

Electrophoresis by the procedure of O’Farrell (18) indicated that P** is absent in crude extracts of vegetative bacteria. P** from crude extracts of sporulating bacteria streaked to some extent in the isoelectric focusing dimension of the two-dimensional electrophoresis but was nevertheless resolved from other bacterial proteins (data not shown). We have not yet searched for P** in crude extracts. Third, an earlier “double label” experiment in which RNA polymerase was isolated by antibody precipitation from a mixture of vegetative and sporulating cells separately labeled with two different radioisotopes had indicated that the large sporulation subunit of RNA polymerase is absent in enzyme from vegetative bacteria (8). An analogous double label experiment (not shown) confirms that P** is also missing from vegetative RNA polymerase.

Phosphocellulose chromatography of RNA polymerase from stationary-phase cells of a mutant blocked at stage 0 of spore formation (SpoOa-5NA; Ref. 19) yielded only a small peak of enzyme III activity (17). Furthermore, two-dimensional electrophoresis (not shown) of crude extracts from these asporogenous bacteria indicated that SpoOa-5NA produced considerably less of enzyme III than did wild type sporulating cells. Enzyme III thus appears to be tightly associated with enzyme III since they co-purified with enzyme purified to apparent homogeneity by conventional purification procedures. RNA polymerase containing the sporulation polypeptides differed from core enzyme (enzyme II) in both purification and transcriptional properties. Enzyme III eluted at a higher salt concentration during phosphocellulose chromatography and sedimented more rapidly during zone centrifugation than did enzyme lacking the sporulation polypeptides. Enzyme III also transcribed B. subtilis DNA more actively than did enzyme II and RNA synthesis by each of these enzymes was affected differently by Mg** and by KCl.

Subsequent to our isolation of an RNA polymerase-binding protein from sporulating cells (8), Nishimoto and Takahashi (20) reported the isolation of a form of B. subtilis RNA polymerase containing a sporulation-specific polypeptide of 95,000 daltons. The purification properties of this form of RNA polymerase are similar to those described here for enzyme III and, despite the discrepancy in molecular weight, their sporulation-specific subunit of RNA polymerase probably corresponds to the species described in this laboratory. Recently, Fukuda et al. (21) have reported small polypeptides of 27,000 and about 20,000 daltons associated with RNA polymerase from sporing B. subtilis.

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