Purification and Characterization of RNA Polymerase from *Fremyella diplosiphon*  

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**ABSTRACT**

We have purified and characterized a DNA-dependent RNA polymerase from the blue-green alga *Fremyella diplosiphon*. This enzyme, purified by gel filtration, DEAE-cellulose chromatography, and glycerol gradient centrifugation, is comprised of five polypeptide subunits. Their masses are 161,000, 134,000, 91,000, 72,000, and 41,000 daltons. Preparative electrophoresis of the purified enzyme on nondenaturating gels separates the 41,000-dalton polypeptide from the rest of the enzyme. The enzyme is extremely labile in the presence of a variety of salts of strong acids and bases; the purification procedure was devised to avoid exposure to such compounds.

The blue-green algae represent a type of procaryotic organization distinct from other bacteria. These phototrophs undergo a number of simple developmental processes, such as complementary chromatic adaptation (4), and heterocyst formation (11). They also contain Chl a, evolve O₂, and bear many structural and biochemical similarities to the chloroplasts of higher plants (7).

We have purified and characterized a DNA-dependent RNA polymerase from the blue-green alga *Fremyella diplosiphon*. The subunit composition of the RNA polymerase from *F. diplosiphon* reported here is compared with the corresponding enzymes isolated from another blue-green alga, *Anacystis nidulans* and other bacteria.

**MATERIALS AND METHODS**

**Cell Culture.** *Fremyella diplosiphon* (B. and F.) Drouet (strain 481) was obtained in axenic culture from the collection at Indiana University (25). The alga was grown in modified medium C (15) with Sequestrene (sodium ferric diethylenetriamine pentaacetate, Geigy Agricultural Chemicals, Ardsley, N.Y.) added to a final concentration of 40 mg/l as a substitute for ferric sulfate. Sodium citrate was omitted entirely. The pH of the medium was adjusted to 7.0 prior to autoclaving. The alga was grown at 37 °C in a 14-liter Microferm (New Brunswick Scientific Co., New Brunswick, N.J.) equipped with cool-white fluorescent lights. The cells were harvested in late log phase by permitting them to settle to the bottom of the fermenter vessel at 4 °C and then concentrating by centrifugation. The packed cells were washed once with 0.05 M TGM² and stored in liquid N₂ until needed.

**Assay for Polymerase Activity.** RNA polymerase activity was measured as follows: The reaction mixture (100 μl) contained 10 μg of calf thymus DNA (type I, Sigma Chemical Co.), 8 μmol of Tris-HCl (pH 8.5), 0.2 μmol each of GTP, UTP, and CTP (Calbiochem), 0.02 μmol of [³⁵S]ATP (Schwarz/Mann, 53.2 Ci/mol), 3 μmol of MgCl₂, and 0.20 μl of enzyme solution. The mixture was incubated at 36 °C for 30 min. The reactions were terminated by addition of 2 ml of ice-cold 5% trichloroacetic acid in 20 mM Na-pyrophosphate. Each precipitate was collected on an 0.45-μm Millipore filter (Millipore Corp.) disc by vacuum filtration, then washed with 20 ml of 5% trichloroacetic acid in 20 mM pyrophosphate and soaked in 10% trichloroacetic acid in 20 mM sodium phosphate, 1 M KCl at 25 °C for 1 hr. The filters were dried, placed in vials containing Liquifluor (New England Nuclear), and counted in a Packard liquid scintillation spectrophotometer. A unit of activity is defined as 1 nmol of [³⁵S]AMP (1.58 × 10⁶ cpm) incorporated per 30-min assay.

**Determinations.** Protein was measured by the Lowry procedure (21), using Cyt c as a standard. DNA concentrations were determined by the diphenylamine method (2) using calf thymus DNA as a standard. Gels were scanned in a Gilford 2000 spectrophotometer equipped with a linear transport assembly (Gilford Instruments Co., Oberlin, Ohio). Standards used to calibrate the Sepharose column and glycerol gradients were detected as follows. Phycoerythrin was assayed spectrophotometrically at 560 nm (3). Ribulose bisP carboxylase was assayed using the method of Goldthwaite and Bogorad (10). Catalase was detected by mixing a small aliquot of the fraction to be assayed and a drop of H₂O₂; the evolution of O₂ bubbles was diagnostic for catalase. β-Galactosidase was detected by its A at 280 nm.

**DNA Isolation.** Suspensions of cells of *F. diplosiphon* or *Tolyphothrix temus* disrupted by five cycles of freezing and thawing were diluted with 2 ml/g fresh weight of 1.5 mM Na-citrate, 15 mM NaCl (1/10 SSC; standard saline citrate is 15 mM Na-citrate, 150 mM NaCl) and extracted six times with equal volumes of 1/10 SSC-saturated phenol. The DNA was precipitated from the aqueous phase with 10 volumes of ice-cold absolute ethanol at −20 °C. The precipitate was washed once with cold absolute ethanol, twice with cold absolute ethyl ether, and dried under a stream of N₂. The DNA was further purified by centrifugation on two CsCl gradients.

The first centrifugation was in a block gradient consisting of two layers of CsCl. The bottom layer (2 ml) was adjusted to a density of 1.75 g/cm³ and the top layer (2 ml) to 1.50 g/cm³. About 500 μg of DNA was loaded onto each gradient and centrifuged at 200,000g for 12 hr in a Beckman Spincos SW 56 rotor. The DNA sediments through the top layer and remains at the interface between the layers. (The contaminating polysaccharides remain at the top and the RNA forms a pellet at the bottom of the tube.) The gradient interface was mixed with 4 ml of CsCl adjusted to a density of 1.701 g/cm³ and centrifuged in the same rotor at 200,000g for 72 hr. Fractions of 0.2 ml were collected and scanned at 260 nm. The DNA-containing fractions were pooled, dialyzed exhaustively against 1/10 SSC, and stored at −20 °C.
RESULTS

Purification of RNA Polymerase. The RNA polymerase activity extracted from *F. diplosiphon* is, like other RNA polymerases, sensitive *in vitro* to the ionic strength of the assay medium (20, 23). Figure 1 shows the inhibition by KCl. The activity of the fraction V enzyme is 95% inhibited by 0.25 M KCl. Other salts of strong acids such as NaCl, (NH₄)₂SO₄, and NH₄Cl inhibit to the same degree. Unlike other well characterized polymerases, this salt impairment of activity seems not to be reversed by removal of the salt.

We have conducted numerous experiments in attempts to reverse lability to salts. Samples of the fraction II enzyme were dialyzed for various amounts of time in dialysis tubing which would retain molecules larger than 300, 1,000, or 10,000 daltons. The controls were held at 4°C without dialysis for the same amount of time. The original activity was 7,100 cpm/20 μl of fraction II extract. The activity after the addition of KCl to 0.5 M was about 800 cpm. This activity further decreased to 550 cpm after 6 hr of dialysis. Dialysis of fraction II enzyme without the addition of salt results in a 3 to 5% loss in activity/hr relative to the controls. The undialyzed controls maintained their activities over a time period of 12 hr.

We also attempted to dialyze rapidly the enzyme solutions to which KCl had been added using various devices and columns designed for rapid dialysis in an attempt to free the enzyme from salt rapidly. The Dow hollow fiber filter (B/HFU-1) was able to reduce a 2 M KCl solution to 0.2 M in 20 min and to 0.05 M in 40 min. The fraction II enzyme, made 0.5 M with KCl, was dialyzed in this filter for 40 min. Samples were taken every 5 min and tested for polymerase activity and salt concentration. These results can be summarized by saying that during the 40-min dialysis, the KCl concentration dropped from 0.5 M to about 0.01 M KCl and the activity originally 8,300 cpm/20 μl and 630 cpm after the addition of the KCl dropped to 590 cpm. Also tried were Amicon filtration devices and a Sephadex G-50 column to desalt the extract in a few min. All of these devices gave the same results as simple dialysis. Recombining the dialyze or filtrate with the dialyzed polymerase extract did not lead to recovery of the activity.

Cell Disruption. Twenty to 30 g of frozen cells were disrupted by two rapid consecutive phase changes in a Raper-Hyatt press (23). The frozen pellet of broken cells was thawed in the presence of 2 ml of 0.05 M TGM/g fresh weight. The broken cell suspension (fraction I) was centrifuged at 100,000 x g for 1 hr. More than 95% of the polymerase activity remains in the supernatant fluid after centrifugation (fraction II).

Sepharose 4B Chromatography. Sepharose 4B was purchased from Pharmacia Fine Chemicals (Piscataway, N.J.) and prepared as recommended by the manufacturer. Seven to 10 ml of fraction II (200 mg of protein) were applied to a column (2.5 x 20 cm) (void volume 45 ml) equilibrated with 0.05 M TGM. The Sepharose column was washed with 0.05 M TGM at a flow rate of 30 ml/hr and 48 ml of 6 ml each were collected. Fractions comprising the activity peak were pooled as fraction III (Fig. 2). Fraction III enzyme activity is stable for at least 6 months when stored in liquid N₂.

DEAE-Cellulose Chromatography. DEAE-cellulose (DE52, Whatman Chemical Co., Clifton, N.J.) was prepared for chromatography as described by Burgess (5). The DEAE-elution buffers consisted of TGM containing various concentrations of Tris/HCl as noted; all were at pH 8.5. Fraction III activity would bind to the DEAE-cellulose only after centrifugation at 150,000 x g for 1 hr to remove the remaining Chl-containing membrane fragments. About 200 ml (50 mg of protein) of clarified fraction III were applied to a DEAE-cellulose column (1 x 7 cm). The column was then washed with 100 ml of TGM and 150 ml of 0.2 M Tris/HCl TGM. The RNA polymerase activity was eluted with a 50-ml linear gradient of 0.2 to 0.6 M Tris-HCl TGM. The enzymatic activity eluted at 0.28 M Tris-HCl as a single peak (Fig. 3). The active fractions were pooled as fraction IV. Fraction IV has a t₁/₂ of several weeks when stored in liquid N₂.

Glycerol Gradient Centrifugation. For purification by density gradient centrifugation, 1 ml of fraction IV enzyme (250 μg of protein) was loaded onto a 12-ml linear gradient consisting of 12 to 30% glycerol TGM and centrifuged at 200,000 x g for 2 hr in a Beckman Spinco SW 40 rotor. After centrifugation, the activity appeared in the middle of the gradient and was distributed in a symmetrical peak which corresponds with the protein peak (Fig. 4). The gradients were collected in 1-ml fractions and the peak fraction from each gradient was designated fraction V. The fraction V activity is stable for at least 9 months.

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![Figure 1](https://example.com/figure1.png)  
**Fig. 1.** Effect of KCl on activity of enzyme. Standard assay conditions were employed except for inclusion of KCl at concentrations noted.

![Figure 2](https://example.com/figure2.png)  
**Fig. 2.** Elution profile of Sepharose 4B column. Eluate was collected in 6-ml fractions. (---) O.D. at 280 nm; (-----) RNA polymerase activity. Ribulose bisP carboxylase and phycoerythrin were chromatographed with the fraction II extract. β-Galactosidase was run with phycoerythrin on the same column at a different time. Mol wt of standards are: ribulose bisP carboxylase, 560,000; β-galactosidase, 520,000; phycoerythrin, 280,000.
Gel Electrophoresis. Occasionally the enzyme was purified further to step VI by electrophoresis on 5% acrylamide gels under nondenaturating conditions. Polyacrylamide gels were prepared as described by Laemmli (16) and run in the apparatus devised by Ames (1). Electrophoresis under nondenaturing conditions used the same buffers and electrode solutions but with SDS omitted. The gels were stained and destained using the method of Fairbanks (9).

A slab gel (160 × 200 × 1.5 mm) was prepared with a single sample well across the top. Two ml (100 μg of protein) of the fraction V enzyme were applied to this gel and electrophoresed at 100 v (constant voltage) for 5 hr. After electrophoresis a 0.5-cm strip was cut from one edge of the slab and stained. After destaining, the gel strip was realigned with the slab and the polyacrylamide strip corresponding to the stained enzyme band was cut out and sliced into 0.5-mm cubes with a microtome blade. The enzyme was eluted from the gel cubes in 0.5 μTGM for 18 hr at 4 C. Only 10 to 15% of the applied activity was recovered and the specific activity was greatly reduced (Table I).

Purification Data. The specific activity of the step V purified enzyme is comparable to that reported for highly purified plant polymerases (13, 17, 26) but it is lower than those purified from yeasts (27) and bacteria (6, 14, 19). Table I shows that the yield until fraction III is greater than 100%. This could be due to the loss of contaminating nucleases during the purification. In fact, if the fraction II enzyme is frozen in liquid N₂ for 2 or 3 months, the same increase in activity can be demonstrated. On the basis of specific activity, this method gives an over-all purification of about 760-fold.

CHARACTERISTICS OF RNA POLYMERASE

Divalent Cation Requirement. The enzyme requires divalent cations for activity. The maximum activity of the fraction V enzyme is exhibited in the presence of 30 mm MgCl₂ while optimum stimulation with MnCl₂ occurred at 5 mm (Fig. 5).

Sensitivity to Inhibitors. In vivo, 10 μg of rifamycin SV is enough to inhibit the growth rate 95% (data not shown). In vitro, 0.1 μg/ml of rifamycin SV inhibits the RNA polymerase 95% at all stages of purification. Rifamycin AF/01-3 which is capable of inhibiting eucaryotic nuclear II and III RNA polymerases (22) gives the same inhibition. α-Amaritin, a potent inhibitor of eucaryotic RNA polymerase type II as well as type III at higher concentrations (8), has no effect on the F. diplosiphon enzymic activity at concentrations up to 10 μg/ml.

Template Dependence and Specificity. At all stages of purification, the enzyme is more active with native than with denatured DNA (Table II) and requires all four ribonucleoside triphosphates for its activity (Table III). The DNA dependence of the enzyme increased from 60% in fraction II to more than 90% in fraction V. F. diplosiphon DNA and the DNA from T. tenuis, a related blue-
green alga, are relatively poor templates as compared with calf thymus DNA. The enzyme was most active with DNA from the Bacillus subtilis phage φe and the synthetic template poly d(AT) (Table III).

Structure of Purified Enzyme. The fraction V enzyme includes polypeptides of 161,000, 134,000, 91,000, 72,000, and 41,000 daltons (values ± 5%). The fraction VI enzyme purified by preparative electrophoresis lacks the 41,000-dalton polypeptide (Fig. 6). The relative amounts of each polypeptide were estimated by scanning the Coomassie blue-stained polypeptide bands and, after subtracting background, comparing the areas under the peaks. The ratio of the A in each band from the highest to the lowest in mol wt is: 1.13: 0.83: 1.00: 2.14: 5.69. These values were normalized to the 91,000-dalton polypeptide (Fig. 7). The amount of the 134,000-dalton polypeptide varied somewhat from preparation to preparation (lowest, 0.63; highest, 0.95). This could be due to specific proteolysis. Proteolysis of the second largest subunit has been previously observed in A. nidulans (12) and B. subtilis (18).

FIG. 5. Plot of Mg$^{2+}$ and Mn$^{2+}$ concentrations versus RNA polymerase activity. (––): MnCl$_2$; (––): MgCl$_2$.

Table II

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Specific Activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage φe</td>
<td>236</td>
</tr>
<tr>
<td>Native calf thymus</td>
<td>150</td>
</tr>
<tr>
<td>Corn nucleus</td>
<td>109</td>
</tr>
<tr>
<td>T4</td>
<td>89</td>
</tr>
<tr>
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<td>63</td>
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<tr>
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<td>52</td>
</tr>
<tr>
<td>Polynophrith x tenuis</td>
<td>51</td>
</tr>
<tr>
<td>Fremyella diplosifon</td>
<td>46</td>
</tr>
<tr>
<td>Denatured calf thymus</td>
<td>35</td>
</tr>
<tr>
<td>Poly d(AT) (Synthetic)</td>
<td>241</td>
</tr>
</tbody>
</table>

Activity of a constant concentration of RNA polymerase was measured over a range of concentrations of each template. The specific activities given are for the most active template:enzyme ratio. All of these ratios were in the range of between 1-2 μg DNA/0.160 units of polymerase.

DISCUSSION

The purified (fraction V) DNA-dependent RNA polymerase from F. diplosifon is comprised of at least four polypeptides, of 161,000, 134,000, 91,000, and 72,000 daltons, plus a 41,000-dalton protein which is present in a ratio of about 5:1 of the 91,000-
Table III
Requirements for Activity

<table>
<thead>
<tr>
<th>Assay</th>
<th>Counts Incorporated in 30 min</th>
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</thead>
<tbody>
<tr>
<td>All components</td>
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</tr>
<tr>
<td>- Template</td>
<td>153</td>
</tr>
<tr>
<td>- UTP</td>
<td>73</td>
</tr>
<tr>
<td>- CTP</td>
<td>63</td>
</tr>
<tr>
<td>- GTP</td>
<td>51</td>
</tr>
<tr>
<td>- ATP*</td>
<td>26</td>
</tr>
<tr>
<td>- Polymerase</td>
<td>25</td>
</tr>
</tbody>
</table>

* 14C UTP used as label

Fig. 7. Densitometric scan of fraction V enzyme at 520 nm after gel electrophoresis on a 7.5% acrylamide SDS gel and staining with Coomassie blue.

dalton proteins. SDS gel electrophoresis through the last two steps of the purification indicates that the 41,000-dalton polypeptide is consistently associated with the enzymic activity. Most of this polypeptide is purified away during the electrophoresis step but trace amounts remain. Indeed, the loss of this polypeptide may be responsible for the drop in specific activity of the electrophoretically pure enzyme. It is also possible that this polypeptide, although closely associated with the enzyme, is not essential for the type of in vitro activity we are measuring.

The RNA polymerase from *F. diplophon* is sensitive to rifampycin but insensitive to α-amanitin. The RNA polymerase isolated for the unicellular blue-green alga, *A. nidulans* (12), has the same antibiotic sensitivity, but a different polypeptide composition: 190,000, 145,000, 92,000, and 38,000 daltons. Its largest subunit is almost the same size as the 180,000-dalton polypeptide of DNA-dependent RNA polymerases isolated from plant nuclei (13, 17, 26) and chloroplasts (24).

A major structural difference between the *F. diplophon* and the *Escherichia coli* enzymes is the relatively high mol wt of the smallest blue-green algal subunit associated with the enzyme through electrophoresis. Considering the stoichiometric data, this gives a total mol wt of about 550,000 daltons. This enzyme co-migrates and co-chromatographs on Sephadex with the enzyme ribulose bisP carboxylase (mol wt 560,000).

Another major difference between the *F. diplophon* and the bacterial enzymes is in sensitivity to salt. Low concentrations of salt (less than 0.1 m KCl) stimulate the *E. coli* enzyme while concentrations above 0.05 m KCl irreversibly inhibit the *F. diplophon* enzyme. This salt lability is unique with respect to all of the other RNA polymerases that have been purified. While the RNA polymerase II purified from maize exhibits a salt inhibition curve similar to that of the polymerase from *F. diplophon*, the salt inhibition of the maize enzyme can be reversed by dialysis (26). Lability to salt is not dependent on the purity of the *F. diplophon* enzyme. Fraction V polymerase is as sensitive to salt as the fraction I enzyme. This implies that loss of activity results from interaction of the salt directly with the enzyme rather than with some other component of the extract. The fraction V enzyme was electrophoresed on SDS gels before and after salt treatment. The resultant polypeptide pattern on these gels was identical. We were unable to detect any difference in the size of the subunits after salt treatment.

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