Partial Purification of a Chloroplast DNA Polymerase from *Euglena gracilis*

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A single DNA polymerase has been purified 965 fold from isolated chloroplasts of *Euglena gracilis* with a yield of 53%. The isolation methods include solubilization of the enzyme with 1M NaCl, ammonium sulfate precipitation, DNA affinity and DEAE-cellulose chromatography. The enzyme requires all four deoxynucleotide triphosphates, magnesium and denatured DNA for maximal activity. The chloroplast DNA polymerase is free of contaminating nucleases and phosphatases, has a sharp pH optimum at pH 7.2 and magnesium optimum of 6mM.

Chloroplasts have been considered as semi-autonomous organelles, since they have been shown to synthesize their own DNA independently of the nuclear DNA and incorporate radioactive deoxynucleotide triphosphates *in vitro* (1-4). Recent inhibitor studies, however, suggest that the chloroplast replication enzymes depend upon nuclear transcription and cytoplasmic translation (5,6). As a first step in determining whether the chloroplast contains unique replication proteins or uses the same nuclear enzymes, we have purified DNA polymerase from *Euglena gracilis* chloroplasts and from whole cell extracts. This preliminary communication describes the properties of the chloroplast enzyme. The properties of the other cellular DNA polymerases will be reported in a later communication (7).

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Materials and Methods

Growth: *Euglena gracilis* strain Z was grown autotrophically in 1.2L of media in 2L flasks in a New Brunswick PsychroTherm incubator at 26° C, 200 ft.-candles, and with rigorous shaking (8). Stationary cultures were used for the isolation of chloroplasts, as described by Eisenstadt and Brawerman (9). Whole cell extracts were also prepared from stationary autotrophic cultures (7).

Purification: All steps were performed at 4° C except where otherwise noted. Freshly isolated chloroplasts were washed twice in TME-I buffer (0.05 M Tris, 0.005 M 2-mercaptoethanol, 0.001M EDTA, pH 8.0). They were resuspended in 2 volumes of TME-I and sonicated briefly until breakage was >99%. NaCl (4M) was added to a final molarity of 1M, and the extraction was allowed to continue for 30 minutes. Unbroken chloroplasts, membranes and debris were removed by centrifuging 150,000 x g for 90 minutes in the Spinco 50 Ti rotor. The supernatant was drawn off and brought to 60% saturation with $(NH_4)_2SO_4$ by the dropwise addition of a saturated solution. After 30 minutes, the precipitate was collected by centrifugation (15,000 x g for 15 min.), and redissolved in TMM buffer (0.02M Tris, 0.001M 2-mercaptoethanol, 0.005M Mg-acetate, pH 8.0), containing 50 μg/ml pancreatic DNase. The solution was allowed to warm to room temperature for 30 minutes, and then 2 volumes of buffer (0.02M Tris, 0.001M 2 mercaptoethanol, 0.02M EDTA, 5% glycerol; pH 8) was added to terminate the reaction. The solution was diluted in TME-II (0.02M Tris, 0.001M 2-mercaptoethanol, 0.001M EDTA, 5% glycerol, pH 8.0) to a protein concentration of 1-2 mg/ml, and applied to a 1x2 cm DNA-cellulose column, prepared by the method of Alberts (10). The flow was regulated by gravity feed at a rate of 0.5 ml/min. and collection of 2 ml fractions started

immediately. After completion of the sample charge, the column was eluted stepwise first with 20 ml of 0.15M NaCl in TME-II and second with 20 ml 0.8M NaCl in TME-II. All fractions containing DNA polymerase (0.8M NaCl, eluate) were pooled and rapid dialysed against 3 changed of TME-II (11). This fraction was then chromatographed over a 1x6 cm DEAE-cellulose column, using a 0 to 0.6M NaCl linear gradient in TME-II. Fractions containing DNA polymerase activity were pooled and used for subsequent experiments. The enzyme could be stored at -196°C if 200 µg/ml bovine serum albumin was added with a loss of 25% on freeze-thawing. DNA polymerases from cell extracts were prepared by a similar procedure (7).

Enzyme Assays: The composition of the DNA polymerase reaction mixtures is given in the legends. Acid and alkaline phosphatase, phosphodiesterase and DNase were determined as previously reported (12). 1 unit of DNA polymerase activity is defined as the incorporation of 1 nM/hr. of ^{3}H-TTP. Radioactivity was determined by the filter paper method as described previously (13).

Chemical Determinations: Protein was measured by the Lowry procedure (14) in crude fractions and by ultraviolet absorption (15) in the partially purified fractions. NaCl was measured by a Radiometer conductivity bridge.

Results

Enzyme Purification: As evidenced in Table 1, chloroplast DNA polymerase specific activity has been increased 965-fold over the intact chloroplasts. This value may be considered as an overestimation, since chloroplasts contain DNases and phosphatases which interfere with the polymerase determination (16,17). We have attempted to optimize the polymerase reaction in crude

Table 1: Purification of Chloroplast DNA Polymerase

Procedure	Total Protein (mg)	Total Activity (units)	SP. Act.	% recovery	Purification
1. Sonicated chloroplasts	127	5.77	0.045	100	1
2. 1M NaCl Extract	103	1.48	0.015	26	0.35
3. Dialysed 150,000 x g sn.	104.2	1.54	0.015	27	0.33
4. 0-60% $(NH_4)_2SO_4$ ppt.	62	13.34	0.215	231	4.74
5. 0.8M eluant, DNA-cellulose	0.42	4.07	11.83	86	263
6. 0.015M eluant, DEAE-cellulose	0.07	3.05	43.50	53	965

25g chloroplasts from Euglena gracilis was used as the starting material. 125 µl assays containing 25 µl enzyme were incubated at 35°C for 30 minutes in a reaction containing: 0.02M Tris pH 8.0, 0.004M EDTA; 0.004M 2-mercaptoethanol; 0.006M Mg-acetate; 0.01mM each of dATP, dCTP, dGTP; 1 µc/ml of ^{3}H-TTP adjusted to 2 x 10^5 cpm/nM; and 110 µg/ml of calf thymus denatured DNA. 2mM ATP was added to fractions 1-4 to counteract non-specific phosphatases. 15 mM NaCl and 80 µg/ml bovine serum albumin were added to fraction 6.

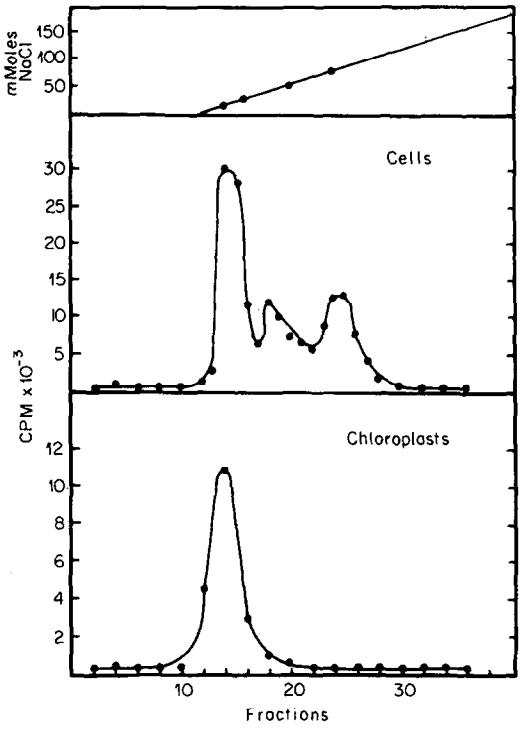

Figure 1: DEAE-cellulose chromatography of chloroplast DNA polymerase and whole cell DNA polymerases. Polymerase activity was eluted at 0.015M NaCl (Pol. I.), 0.03M NaCl (Pol. II), and 0.06M NaCl (Pol. III). Chromatographic conditions are described in the text. DNA polymerase activity was measured as described in Table 1 legend. 9

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extracts by the addition of 2mM ATP and high concentrations of DNA. Considerable activity was lost during DNA-cellulose chromatography, which could not be recovered in the 0.15 M wash or by 2M NaCl-TBE-II elution. Subsequent chromatography over DEAE-cellulose increases the purification, but 25% of the activity is lost relative to the initial charge. Discontinuous elution from DEAE-cellulose by 0.3M NaCl in TME-II allows recovery of >90% of the initial activity and may suggest a cooperative reaction occurs in the 0.8M DNA-cellulose eluate. The chloroplast DNA polymerase chromatographs homogeneously in DEAE-cellulose (Figure 1).

Attempts to find additional DNA polymerase from isolated chloroplasts by direct DEAE-cellulose chromatography of the crude supernatant or the ammonium sulfate precipitate did not yield an elution profile different from Figure 1. Monitoring the purification using native DNA in the reaction mixture did not reveal other polymerases or significantly alter the results in Table 1.

Cellular DNA Polymerases: Extracts prepared from entire cells revealed three peaks of DNA polymerase activity (Figure 1). Each polymerase fraction has been shown to contain a number of distinct properties as well as to be chromatographically reproducible (7). The chloroplast enzyme was recovered as the most active enzyme from whole cell extracts, despite variations in physiology or the method of extraction.

Properties of Chloroplast DNA Polymerase: As other DNA polymerases, the enzyme requires Mg^{++}, DNA primer, and the four deoxynucleotide triphosphates (Table 2). NaCl stimulates the reaction in the range of 10 to 15mM by 48%. No requirement has been demonstrated for EDTA or 2-mercaptoethanol. One unit of enzyme was saturated by 50 µg of DNA and concentrations of denatured DNA above 500 g/unit enzyme proved inhibitory. ^{3}H-TTP diluted with non-radioactive TTP saturated the reaction at 50pM/unit enzyme. The pH curve in phosphate or Tris buffer yielded a single sharp optimum at pH 7.2. The curve fell off sharply having 10% enzyme activity at pH 6.0 and 30% enzyme activity at pH 9.5. The magnesium optimum was reached at 6-8mM in magnesium acetate and no appreciable reaction could be measured by substituting Zn^{++}, Co^{++}, Mn^{++}, or Cu^{++}. The reaction product from the chloroplast enzyme incubation mixture was DNase digestible and was banded in isopycnic CsCl gradients at the same density as the

Table 2: Characterization of the Chloroplast DNA Polymerase Reaction

COMPLETE	100		
$-Mg^{++}$	0	+ATP (2mm)	100
-2-mercaptoethanol	86	+NaCl	148
-EDTA	99	+Serum albumin	135
-dATP, dCTP, dGTP	0	+Acriflavin (10 µM)	50
-dATP, dGTP	11	+Ethidium bromide (10 µM)	50
-dGTP, dCTP	17.5	+DNase (50 µg/ml)	0
-dATP, dCTP	0	+RNase (50 µg/ml)	100
		+Pronase (50 µg/ml)	100

0.5 µg chloroplast DNA polymerase was added to 125 µl reaction mixtures described in Table 1. Appropriate deletions or additions were made and duplicate assays run at 35°C for 30 minutes. Enzymes were added after the initial reaction and reincubated for an additional 30 minutes at 35°C. The control reaction incorporated 1115 cpm. (all values expressed as % control).

primer DNA. The chloroplast DNA polymerase was tested for its sensitivity to ethidium bromide and acriflavin, since these dyes have been useful in plasmid elimination (13), mitochondrial mutagenesis (19), and oncogenic tumor virus studies (20). 10 µM concentrations of either ethidium bromide or acriflavin inhibited the chloroplast DNA polymerase 50%, so that its sensitivity to comparable to calf thymus DNA polymerase and several RNA dependent DNA polymerases (20).

Discussion

Our results confirm many earlier experiments which suggest that the chloroplast contain DNA polymerase (1-3). Only one DNA polymerase could be recovered from intact chloroplasts. The partially purified enzyme is similar to other eukaryotic DNA polymerases having a neutral pH optimum, low magnesium optimum, a

preference for denatured DNA, and a slight reaction (20%) in the presence of 3 deoxynucleotide-triphosphates (21-23). DNA repair has been observed in Euglena chloroplasts (24), so that our enzyme could be involved in this function rather than in replication. However, no detectable DNase activity (25) could be associated with the chloroplast-DNA polymerase fraction. In summary, chloroplasts of Euglena gracilis contain an enzyme which has the potential for replication/repair. Whether this enzyme is restricted to the chloroplast, and is encoded in the chloroplast genome will be the subject for later communications.

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