Identification and Characterization of a 3′ to 5′ Exonuclease Associated with Spinach Chloroplast DNA Polymerase†

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ABSTRACT: Spinach chloroplast DNA polymerase was shown to copurify with a 3′ to 5′ exonuclease activity during DEAE-cellulose, hydroxylapatite, and heparin–agarose column chromatography. In addition, both activities comigrated during nondenaturing polyacrylamide gel electrophoresis and cosedimented through a glycerol gradient with an apparent molecular weight of 105 000. However, two forms of exonuclease activity were detected following velocity sedimentation analysis. Form I constituted ~35% of the exonuclease activity and was associated with the DNA polymerase, whereas the remaining activity (form II) was free of DNA polymerase and exhibited a molecular weight of ~26 500. Resedimentation of form I exonuclease generated both DNA polymerase associated and DNA polymerase unassociated forms of the exonuclease, suggesting that polymerase/exonuclease dissociation occurred. The exonuclease activity (form I) was somewhat resistant to inhibition by *N*-ethylmaleimide, whereas the DNA polymerase activity was extremely sensitive. Using in situ detection following SDS–polyacrylamide activity gel electrophoresis, both form I and II exonucleases were shown to reside in a similar, if not identical, polypeptide of ~20 000 molecular weight. Both form I and II exonucleases were equally inhibited by NaCl and required 7.5 mM $MgCl_2$ for optimal activity. The 3′ to 5′ exonuclease excised deoxyribonucleoside 5′-monophosphates from both 3′-terminally matched and 3′-terminally mismatched primer termini. In general, the exonuclease preferred to hydrolyze mismatched 3′-terminal nucleotides as determined from the V_{max}/K_m ratios for all 16 possible combinations of matched and mismatched terminal base pairs. These results suggest that the 3′ to 5′ exonuclease may be involved in proofreading errors made by chloroplast DNA polymerase.

DNA polymerases are commonly found associated with 3′ to 5′ exonuclease activities which function to proofread base substitution errors made during DNA synthesis [for a review, see Kunkel (1988), Echols and Goodman (1991), and Linn (1991)]. These exonuclease activities may reside either in the same polypeptide as the polymerase or in a separate subunit. For example, *Escherichia coli* DNA polymerase I contains both the polymerase and 3′ to 5′ exonuclease activities in the same polypeptide (Setlow et al., 1972; Derbyshire et al., 1988). Alternatively, *E. coli* DNA polymerase III holoenzyme exhibits polymerase and 3′ to 5′ exonuclease activities as separate proteins designated α- and ϵ-subunits, respectively (Scheuermann et al., 1983; Scheuermann & Echols, 1984). All five classes of eukaryotic DNA polymerases (α, β, γ, δ, ϵ) have also been shown to be associated with 3′ to 5′ exonucleases (Cotterill et al., 1987; Mosbaugh & Meyer, 1980; Kunkel & Soni, 1988; Byrnes et al., 1976; Sabatino & Bambara, 1988). However, the subunit structure and location of the exonuclease active site remain to be determined in most cases.

Plant cells have been less extensively studied regarding DNA polymerase and/or exonuclease activities. Nevertheless, two distinct classes of DNA polymerases have been identified (Amileni et al., 1979; Sala et al., 1980). One class appears to have properties similar to the eukaryotic DNA polymerase α (Amileni et al., 1979; Misumi & Weissbach, 1982). As the most abundant form, the α-like DNA polymerase appears to play an essential role in replicating nuclear DNA (Sala et al., 1981). This plant polymerase has a native molecular weight of 180 000 and is inhibited by aphidicolin, *N*-ethylmaleimide (NEM),[1] and high ionic strength (Amileni et al., 1979; Sala et al., 1981). Partially purified preparations of the *Oryza sativa* α-like DNA polymerase have been reported to contain a 3′ to 5′ exonuclease activity that preferentially removes mismatched 3′-terminal bases (Amileni et al., 1979). The second class of plant polymerase, which is found in the chloroplast, resembles eukaryotic DNA polymerase γ (Sala et al., 1980, 1981). The chloroplast DNA polymerase has been purified from *Euglena gracilis* (Keller et al., 1973), *Chlamydomonas reinhardtii* (Wang et al., 1991), spinach (Spencer & Whitfield, 1969; Sala et al., 1980), and pea (McKown & Tewari, 1984). These chloroplast DNA polymerases have been characterized and exhibit (i) an absolute requirement for a divalent metal cation (Mg^{2+} or Mn^{2+}), (ii) stimulation by NaCl or KCl, (iii) a broad alkaline pH optimum, (iv) inhibition by NEM, (v) insensitivity to aphidicolin, and (vi) a native molecular weight ranging from 87 000 to 110 000 as determined by glycerol gradient sedimentation analysis (Sala et al., 1980; McKown & Tewari, 1984; Wang et al., 1991). Using SDS–polyacrylamide activity gel electrophoresis, two catalytic polypeptides were recently identified with *C. reinhardtii* chloroplast DNA polymerase (Wang et al., 1991). The polypeptides had molecular weights of 80 000 and 116 000 which both possessed DNA polymerase activity. The possible subunit

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[1] Abbreviations: SDS, sodium dodecyl sulfate; LF, large fragment of *Escherichia coli* DNA polymerase I; EDTA, ethylenediaminetetraacetate adjusted to pH 8.0; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

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structure and association of other catalytic activities of this and other chloroplast DNA polymerases remain to be described.

In this paper, we (i) purify the spinach chloroplast DNA polymerase, (ii) identify an associated 3′ to 5′ exonuclease which appears to be dissociable from the polymerase, (iii) characterize the biochemical and physical properties of the exonuclease activity, and (iv) determine the specificity for excising 3′-terminally mismatched substrates.

Experimental Procedures

Materials

Unlabeled 2′-deoxyribonucleoside 5′-triphosphates, prestained molecular weight markers (SDS-7B), dithiothreitol, Tris, and *N*-ethylmaleimide were from Sigma. Triton X-100 was obtained from Pierce, and Pharmacia was the source of 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP). Poly(ethylenimine)–cellulose thin-layer chromatographic plates were from Brinkmann, and [^{3}H]dTTP, [α-^{32}P]dGTP, and [γ-^{32}P]ATP were from New England Nuclear.

Escherichia coli DNA polymerase I large fragment (LF) was purchased from New England Biolabs. T4 polynucleotide kinase was from Bethesda Research Laboratories, and calf intestinal alkaline phosphatase was from Sigma.

Methods

Preparation of DNA Substrates. M13mp2(ΔC106) and M13mp2 (A87, T87, G87, and C87) DNA molecules were isolated as described by Kunkel et al. (1987). Oligodeoxyribonucleotides GGCGATTAAGTTGGG (15-mer), TAACGCCAGGGTTTTCCCN where N represents A, T, G, or C (A19-mer, T19-mer, G19-mer, or C19-mer, respectively), and GTGCTGCAAGGCGATTAAGTTGGN where N signifies T or G (T24-mer or G24-mer, respectively) were prepared using an Applied Biosystems Model 380A DNA synthesizer by the Center for Gene Research and Biotechnology (Oregon State University).

Deblocked/deprotected oligonucleotides were purified and ^{32}P-labeled at the 5′ end [(3.0–3.5) × 10^6 cpm/pmol of 5′ ends] as described previously (Longley & Mosbaugh, 1989, 1991a). Oligonucleotides to be labeled at the 3′ end were similarly 5′-phosphorylated except [γ-^{32}P]ATP was replaced with 1 mM ATP and EDTA was omitted from the termination reaction (Longley et al., 1990). Terminally matched and mismatched 3′-end ^{32}P-labeled G24-mers were prepared as described (Longley & Mosbaugh, 1991b), purified by 20% polyacrylamide/8.3 M urea gel electrophoresis, ethanol-precipitated, and hybridized to M13 DNA, forming G24-mer/M13mp2-matched (G·C)[2] or G24-mer/M13mp2(ΔC106)-mismatched (G·A) DNA substrates. Labeled oligonucleotides were determined to be ≥95% pure prior to hybridization and had a specific activity of (1.8–3.1) × 10^6 cpm/pmol of 3′ ends. Double-stranded DNA substrates were prepared by hybridization to M13mp2 DNA as described by Longley and Mosbaugh (1991b) using 0.2–0.32 pmol of 5′ ends/μg of M13 DNA at a final concentration of 63 μg/mL.

Isolation of Chloroplasts. Spinach chloroplasts were isolated by a procedure similar to that described by Tewari (1986). Fresh spinach leaves (5000 g) obtained from a local market were washed with cold water, and 250-g batches were suspended in homogenization buffer [500 mL of 50 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 500 mM sucrose, and 0.2 mM PMSF]. Each batch was homogenized in a Waring blender for 10 s. The crude homogenate was filtered through two layers of cheesecloth and then through six layers of Miracloth (Calbiochem). Following filtration, the solution was centrifuged in a Sorvall GSA rotor at 2000*g* for 5 min at 4 °C. The pellets were resuspended in 1250 mL of buffer A [50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 50 mM NaCl, 10% (w/v) glycerol, and 0.2 mM PMSF]. After centrifugation at 1000*g* for 5 min at 4 °C in a Sorvall SA600 rotor, the chloroplast pellet was resuspended and the process repeated with final resuspension in 666 mL of buffer A.

Enzyme Assays. Standard chloroplast DNA polymerase reaction mixtures (100 μL) contained 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 7.5 mM $MgCl_2$, 0.5 mM EDTA, 75 mM NaCl, 120 mM KCl, 15 μM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (375 cpm/pmol), 100 μg/mL activated calf thymus DNA, and 25 μL (0.005–0.5 unit) of DNA polymerase. After incubation at 37 °C for 60 min, 200 μL of 1 mg/mL BSA in 0.1 M sodium pyrophosphate was added on ice. The DNA was precipitated with 1 mL of 10% (saturation) trichloroacetic acid and collected on Schleicher & Schuell No. 30 glass fiber filters as previously described (Mosbaugh, 1988). Acid-insoluble radioactivity was measured using a Beckman LS-6800 liquid scintillation spectrometer with 0.4% 2,5-bis(5-*tert*-butyl-2-benzoxazolyl)thiophene in toluene as the scintillator. One unit of chloroplast DNA polymerase activity catalyzed the incorporation of 1 nmol of total dNMP into DNA in 60 min at 37 °C.

Standard 3′ to 5′ exonuclease reaction mixtures (15 μL) contained 50 mM Tris-HCl (pH 8.0), 2 mM DTT, 10 mM $MgCl_2$, 50 mM NaCl, 12.6 μg/mL [5′-^{32}P]T24-mer/M13mp2 DNA (3′-terminal T·C mispair), and 3 μL (0.001–0.09 unit) of enzyme. When necessary, exonuclease was diluted in 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, 100 mM NaCl, 100 μg/mL BSA, and 10% (w/v) glycerol. Reactions were incubated at 37 °C for the times indicated (0–75 min), then transferred to ice, and terminated by the addition (15 μL) of 95% deionized formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. Samples (3 μL) were analyzed by electrophoresis using a 12% polyacrylamide (29:1)/8.3 M urea DNA sequencing gel at 1000 V (45 mA/gel) for 3 h as described by Kunkel and Mosbaugh (1989). The gels were dried, and autoradiography was performed at room temperature using Kodak XAR-5 film. To quantitate the radioactivity in various DNA products, the gel bands were excised, placed into 5 mL of Ready Protein$^+$ (Beckman), and counted using a liquid scintillation spectrometer. One unit of 3′ to 5′ exonuclease activity digested 1 pmol of [5′-^{32}P]24-mer hybridized to M13mp2 DNA in 60 min at 37 °C.

Calf intestinal alkaline phosphatase was assayed in a reaction mixture (525 μL) containing 100 mM Tris-HCl (pH 8.0), 1 mM *p*-nitrophenyl phosphate, and 25 μL of enzyme. Following incubation at 37 °C to allow color development, the reactions were placed on ice and quenched by adding 500 μL of 0.1 M NaOH (Mosbaugh et al., 1977). The reaction product was detected by measuring the absorbance at 410 nm using a Beckman DU-40 spectrophotometer.

Glycerol Gradient Centrifugation. The native molecular weight of chloroplast DNA polymerase/exonuclease was determined by centrifugation through a linear 10–30% (w/v) glycerol gradient (11.2 mL) containing 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, 100 mM NaCl, and 100 μg/mL BSA. Centrifugation was performed in a

[2] Base pairs and mispairs are indicated in the order of primer·template (i.e., G·C contains a G in the primer and a C in the template).

Beckman SW41 rotor at 39000 rpm for 40 h at 4 °C. Fractions (7 or 14 drops) were collected from the bottom of the tube. The location of calf intestinal alkaline phosphatase (MW = 100000; 6.2 S) was detected as described above. Molecular weights were determined by the method of Martin and Ames (1961).

In Situ Activity Polyacrylamide Gels. In situ detection of chloroplast DNA polymerase and 3′ to 5′ exonuclease activities following nondenaturing polyacrylamide gel electrophoresis was performed essentially as described by Longley and Mosbaugh (1991a). Briefly, a 5-cm resolving gel contained 10% acrylamide, 0.37% *N,N′*-methylenebis(acrylamide), 375 mM Tris-HCl (pH 8.8), 50 μg/mL bovine fibrinogen, 2 mM EDTA, 0.95 μg/mL each of 5′-end ^{32}P-labeled T24-mer/M13mp2 DNA and 5′-end ^{32}P-labeled 15-mer/M13mp2 DNA, 0.07% ammonium persulfate, and 0.03% TEMED. A stacking gel was overlaid containing 2.5% acrylamide, 0.625% *N,N′*-methylenebis(acrylamide), 62.5 mM Tris-HCl (pH 6.8), 20% sucrose, 2 mM EDTA, 5 μg/mL riboflavin, and 0.1% TEMED. Samples were adjusted to 30% (w/v) glycerol and 0.007% bromophenol blue and loaded into a 0.5-cm-wide well. Electrophoresis was performed at 4 °C using an analytical gel format (Longley & Mosbaugh, 1991a) and with the running buffer containing 25 mM Tris base and 192 mM glycine (pH 8.3). Migration through the stacking gel occurred at 100 V and then continued at 200 V until the dye approached the bottom of the resolving gel.

SDS–polyacrylamide activity gels were performed similar to that previously described by Longley and Mosbaugh (1991a). Chloroplast DNA polymerase was resolved through a denaturing 10% polyacrylamide resolving gel [acrylamide: *N,N′*-methylenebis(acrylamide) ratio 37.5:1] containing the components described above, except that 0.1% SDS was included. The stacking gel was composed of 4.5% acrylamide, 0.2% *N,N′*-methylenebis(acrylamide), 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 2 mM EDTA, 0.07% ammonium persulfate, and 0.06% TEMED. Samples (25 μL) were prepared by heating at 37 °C for 3 min in 54 mM Tris-HCl (pH 6.8), 1.7 mM EDTA, 120 mM 2-mercaptoethanol, 6.25% (w/v) glycerol, 0.8% SDS, and 0.03% bromophenol blue. Electrophoresis was performed at 4 °C as described for the nondenaturing gels except that the running buffer contained 0.1% SDS. Following electrophoresis, SDS was removed from the resolving gel by immersion in 100 mL of SDS extraction buffer [10 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, and 25% (v/v) 2-propanol] for 30 min at 25 °C with gentle agitation. SDS extraction buffer was then replaced (100 mL) and SDS removal allowed to continue for an additional 30 min. After removal of the SDS extraction buffer, the gel was briefly rinsed with 20 mL of renaturation buffer [50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 75 mM NaCl, 120 mM KCl, 400 μg/mL BSA, and 10% (w/v) glycerol] at 4 °C for 3 min. The rinse buffer was removed, renaturation buffer (80 mL) was added, and the gel was incubated at 4 °C for 17 h with gentle agitation to allow enzyme renaturation.

Following nondenaturing polyacrylamide gel electrophoresis or after enzyme renaturation for SDS–polyacrylamide activity gels, the resolving gel was cut vertically into several ~0.2 × 5 cm slices through the sample lane. The slices were each immersed in 5 mL of DNA polymerase/exonuclease reaction buffer [50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 7.5 mM $MgCl_2$, 100 mM NaCl, 120 mM KCl, 400 μg/mL BSA, 15% (w/v) glycerol, and 100 μM ddTTP]. In situ reactions were performed at 25 °C for 0–60 min with gentle mixing. Each reaction was terminated by adjusting to 10 mM EDTA and placing the gel on ice. Reaction buffer was then removed, replaced with 5 mL of ice-cold stop buffer [50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 10 mM EDTA, 50 mM NaCl, 400 μg/mL BSA, and 15% (w/v) glycerol], and incubated at 4 °C for 30 min. [^{32}P]DNA reaction products within the gel were analyzed by electrophoresis through a second-dimensional 20% polyacrylamide/8.3 M urea DNA sequencing gel as described by Longley and Mosbaugh (1991a). Electrophoresis was performed at 1200 V, the gel dried, and autoradiography conducted at room temperature using Kodak XAR-5 film.

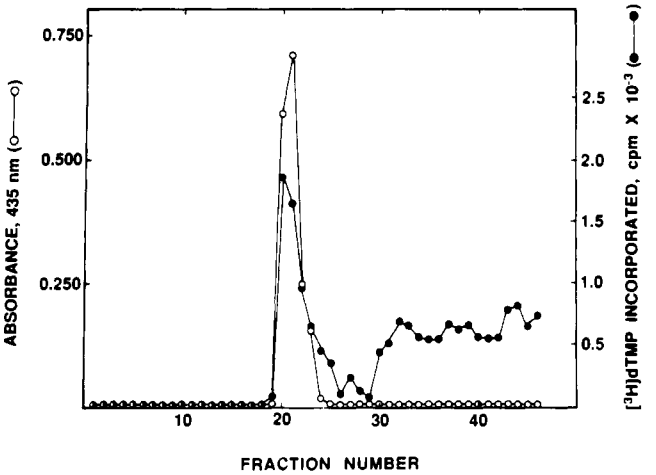

FIGURE 1: Cosedimentation of DNA polymerase with spinach chloroplasts. Chloroplasts were isolated from spinach leaves (500 g) through the Miracloth filtration step as described under Experimental Procedures. After centrifugation at 1000*g* for 3 min, the pellet was resuspended using a soft-bristled paint brush in 125 mL of homogenization buffer. The suspension was then centrifuged at 1000*g* for 10 min, and the pellet was resuspended in buffer (74-mL final volume) containing 50 mM Tris-HCl (pH 8.0), 25 mM EDTA, 0.35 M sorbitol, and 0.2 mM PMSF. A sample (12 mL) of resuspended chloroplasts was layered onto a 30%/52% discontinuous sucrose gradient and centrifuged in a Beckman SW28 rotor at 25000 rpm for 30 min at 4 °C. The step gradient was prepared with a 18-mL cushion containing 52% sucrose, 50 mM Tris-HCl (pH 8.0), and 25 mM EDTA which was overlayed with 7 mL of the same buffer but containing 30% sucrose. Fractions (~800 μL) were collected from the bottom of the tube and assayed for DNA polymerase activity (●) as described under Experimental Procedures except unlabeled dTTP was omitted. Chlorophyll was detected by measuring the absorbance at 435 nm in samples that were diluted 200-fold with distilled water (○). Sedimentation was from right to left.

Results

Association of DNA Polymerase Activity with Chloroplasts. To assess the possible association of DNA polymerase activity with the chloroplast fraction, we examined the initial organelle preparation by sucrose gradient sedimentation (Figure 1). Both chlorophyll and DNA polymerase activity were observed to cosediment as a sharp peak at the expected position of intact chloroplasts. In addition, polymerase activity was also detected as a broad peak at the top of the gradient. We suspect this activity may represent chloroplast DNA polymerase which escaped through leaching or disruption of this organelle. This interpretation was based on the observations that subsequent purification revealed only a single chromatographic species of polymerase and the activity was detected in reactions containing 75 mM NaCl plus 120 mM KCl. Both polymerase peaks differed from the plant α-like DNA polymerase isolated from spinach, which has been reported to be completely inhibited by high salt concentrations (Misumi & Weissbach, 1982).

Purification of DNA Polymerase. (*A*) *Preparation of Chloroplast Extract.* Chloroplasts were isolated from 5 kg of spinach leaves by a technique similar to that described by Tewari (1986). All procedures were performed at 0–4 °C unless otherwise indicated. Isolated chloroplasts were lysed by adjustment to 2% (v/v) Triton X-100 using a 10% (v/v) detergent stock and designated fraction I.

(*B*) *Ammonium Sulfate Precipitation.* Fraction I was brought to 30% saturation with powdered ammonium sulfate by gradual addition over a 30-min period while stirring on ice. After an additional 15 min of mixing, the precipitate was collected by centrifugation at 10000*g* for 15 min. With care to avoid the floating coagulated material, the supernatant fraction was removed and brought to 70% saturation with ammonium sulfate as described above. The protein precipitate was again collected by centrifugation, and the pellet was resuspended in 60 mL of buffer A. Following extensive dialysis against buffer A, the precipitate that formed was removed by centrifugation at 10000*g* for 10 min. The supernatant fraction was designated fraction II.

(*C*) *DEAE-Cellulose Chromatography.* Two identical DEAE-cellulose columns were equilibrated with buffer A. Half the volume (~40 mL) of fraction II was loaded onto each column and then washed with 90 mL of buffer A at a flow rate of ~33 mL/h. Chloroplast DNA polymerase was eluted with buffer A containing 250 mM NaCl (Figure 2A). This procedure provided rapid removal of residual Triton X-100 (wash fractions) from the enzyme preparation. Fractions containing DNA polymerase activity from each column were pooled as fraction III.

(*D*) *Hydroxylapatite Chromatography.* After dialysis against 20 mM potassium phosphate (pH 7.5), 5 mM 2-mercaptoethanol, 200 mM NaCl, and 10% (w/v) glycerol, fraction III was applied to a hydroxylapatite column equilibrated in the same buffer. After the column was washed with 80 mL of equilibration buffer, the enzyme was eluted with a 160-mL linear gradient from 0 to 400 mM potassium phosphate in the equilibration buffer at a flow rate of ~27 mL/h. DNA polymerase activity eluted as a symmetrical peak at 120 mM potassium phosphate (Figure 2B). Fractions containing polymerase activity were pooled and designated fraction IV.

(*E*) *Heparin–Agarose Chromatography.* Fraction IV was dialyzed against 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, 100 mM NaCl, and 10% (w/v) glycerol and loaded onto a heparin–agarose column equilibrated in the same buffer. The column was then washed with 60 mL of equilibration buffer containing 100 μg/mL BSA and eluted with a 100-mL linear gradient from 0 to 1.5 M NaCl in equilibration buffer containing BSA at ~22 mL/h. DNA polymerase activity eluted (Figure 2C) as a sharp peak at ~480 mM NaCl, and those fractions containing >20% of the polymerase activity were pooled as fraction V. At this stage in the purification, the addition of 100 μg/mL BSA was essential for retaining enzyme activity. Without added BSA, only 6% of the polymerase activity applied to the column was recovered, and that activity was rapidly lost upon storage.

(*F*) *Glycerol Gradient Centrifugation.* Fraction V was concentrated 25–50-fold before samples (200 μL) were layered onto 10–30% linear glycerol gradients and sedimentation performed as described in Figure 2D. DNA polymerase activity was detected as a symmetrical peak with a molecular weight of 105 000 ± 3000, relative to the external protein marker calf intestinal alkaline phosphatase. Fractions 17–20 were pooled and designated fraction VI, and this polymerase fraction was used for all subsequent experiments. Further

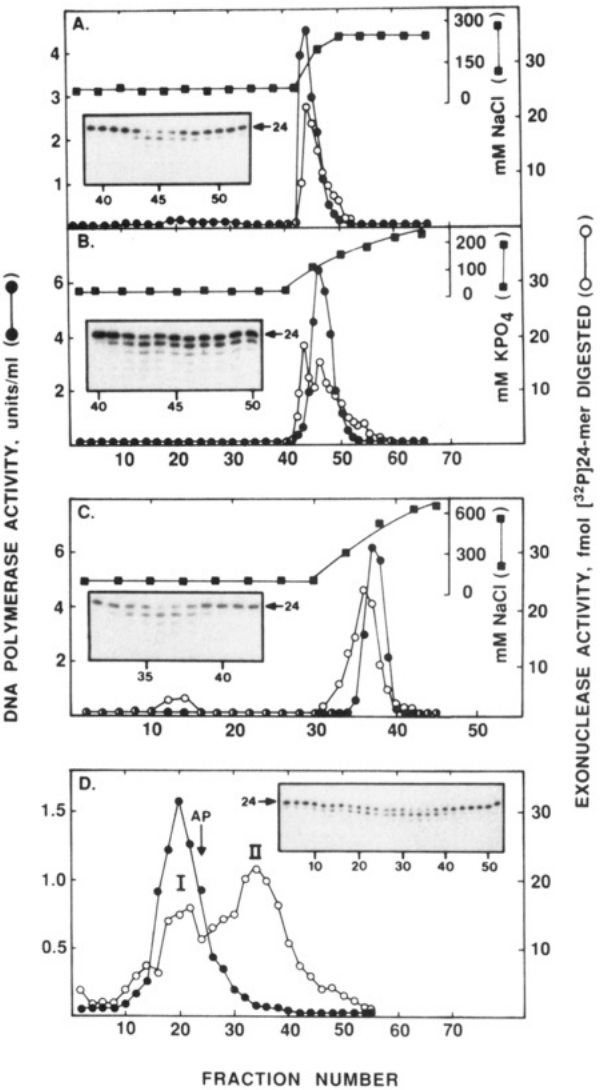

FIGURE 2: Purification of chloroplast DNA polymerase/3′ to 5′ exonuclease. (A) DEAE-cellulose chromatography (DE-52, Whatman) of fraction II chloroplast DNA polymerase (93 units loaded on each of the two identical 6.2 cm² × 20 cm columns). (B) Fraction III DNA polymerase (130 units) from both DEAE-cellulose columns was dialyzed into equilibration buffer and subjected to hydroxylapatite chromatography (Bio-Gel HTP, Bio-Rad) using a 6.2 cm² × 18 cm column. (C) Fraction IV DNA polymerase (100 units) was dialyzed, applied to a heparin–agarose column (2.5 cm² × 16 cm), and eluted in buffer containing 100 μg/mL BSA. (D) Glycerol gradient centrifugation of fraction V DNA polymerase. After the enzyme was concentrated 25–50-fold by centrifugation using a Centriprep 10 (Amicon) ultrafiltration unit in a Sorvall GSA rotor at 2000*g* for 6–8 h, a 200-μL sample (33 units of polymerase) was layered onto each of two 10–30% linear glycerol gradients. In the case shown, calf intestinal alkaline phosphatase (2.6 units) was added as an internal molecular weight standard which sedimented as indicated (AP, arrow). Centrifugation was performed in a Beckman SW41 rotor at 39 000 rpm for 40 h at 4 °C. Details of column elutions and gradient centrifugation are described under Results. Fractions were assayed for DNA polymerase (●) and 3′ to 5′ exonuclease (○) activity as described under Experimental Procedures. Standard exonuclease assays contained samples (3 μL) that had been diluted 25-fold and incubated 20 min (A and B), 50-fold and incubated 60 min (C), and undiluted and incubated 75 min (D), all at 37 °C. The insets show autoradiograms detecting 3′ to 5′ exonuclease activity across peak fractions of chloroplast DNA polymerase.

attempts to purify the DNA polymerase resulted in extremely poor recovery of activity. Due to the necessity of including BSA after fraction IV for enzyme stability, we were unable to accurately quantify this purification scheme beyond the hydroxylapatite chromatographic step.[3] However, the overall

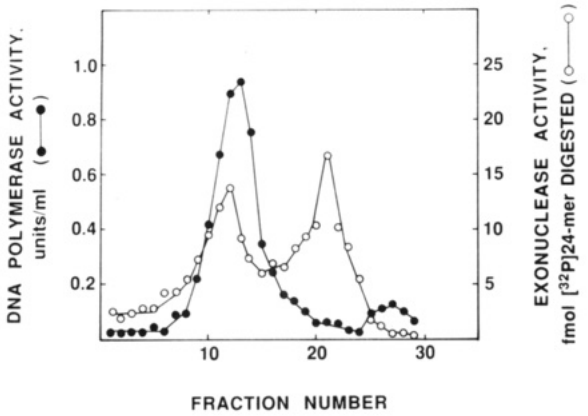

FIGURE 3: Recentrifugation of DNA polymerase/exonuclease (fraction VI) on a glycerol gradient. Chloroplast DNA polymerase from fractions 17–20 (Figure 2D) was pooled and concentrated 10-fold by a Centricon 10 (Amicon) in a Sorvall SA600 rotor at 2500*g* for 3 h at 4 °C. A sample (200 μL) was layered onto a linear 10–30% glycerol gradient and centrifugation performed in a Beckman SW41 rotor at 39000 rpm for 40 h at 4 °C as described under Experimental Procedures. Fractions (~400 μL) were collected from the bottom of the tube and assayed for DNA polymerase activity (●). Exonuclease activity (○) was detected using 6 μL of each fraction after incubation at 37 °C for 30 min as described under Experimental Procedures. Sedimentation was from right to left.

yield of chloroplast DNA polymerase activity from fractions II–VI was 30%, and the final enzyme preparation was relatively stable for up to 4 weeks when stored on ice.

Copurification of DNA Polymerase and 3′–5′ Exonuclease Activity. Since many cellular DNA polymerases including those isolated from mitochondria contain an associated 3′ to 5′ exonuclease activity (Kunkel & Soni, 1988; Kunkel & Mosbaugh, 1989; Kaguni & Olson, 1989; Insdorf & Bogenhagen, 1989), we assayed for both activities throughout enzyme purification. DNA polymerase and 3′ to 5′ exonuclease activities were observed to coelute from the DEAE-cellulose column as a single peak (Figure 2A). Further chromatography on hydroxylapatite and heparin–agarose columns resulted in partial but not complete resolution of these two activities (Figure 2B,C). Analysis by glycerol gradient centrifugation clearly revealed one species of DNA polymerase and two species of exonuclease activity (Figure 2D). Exonuclease form I constituted approximately 35% of the total activity and cosedimented with the chloroplast DNA polymerase at 6.4 S (MW = 105000). On the other hand, form II exonuclease represented ~65% of the recovered activity, lacked detectable DNA polymerase activity, and exhibited a significantly lower molecular weight of 26500. The existence of the form I exonuclease with polymerase activity led us to suspect that these two enzymatic activities were associated. To examine this point, the leading side of the polymerase/exonuclease peak was pooled (fractions 17–20) and resedimented in an identical glycerol gradient as shown in Figure 3. Interestingly, two peaks of exonuclease were again detected corresponding to form I (53% total activity) and form II (47% total activity) exonuclease. As before, only form I exonuclease was associated with polymerase activity. Thus, it appeared that the polymerase and exonuclease activities were physically associated.

[3] Chloroplast DNA polymerase (fraction IV) was purified about 200-fold on the basis of the increase in specific activity from fraction II. The presence of Triton X-100 in fraction I DNA polymerase interfered with accurate activity measurements. We estimated that fraction VI DNA polymerase was purified >6900-fold. This value was based on the specific activity calculated from an activity measurement obtained using a preparation prepared with BSA and a protein determination obtained with a preparation lacking BSA.

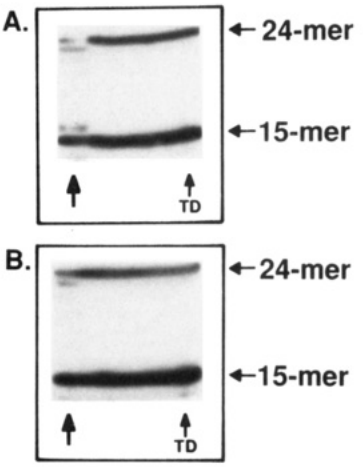

FIGURE 4: In situ detection of chloroplast DNA polymerase and 3′ to 5′ exonuclease following nondenaturing polyacrylamide gel electrophoresis. A sample (30 μL) of DNA polymerase/exonuclease (fraction VI; 0.09 unit) was loaded onto a nondenaturing 10% polyacrylamide activity gel containing [^{32}P]15-mer/M13mp2 DNA and [^{32}P]T24-mer/M13mp2 DNA as described under Experimental Procedures. Following electrophoresis, the protein gel was sliced vertically into segments (~0.2 cm × 5 cm) and placed into test tubes (A) with buffer (5 mL) containing 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 100 mM NaCl, 120 mM KCl, 400 μg/mL BSA, and 15% (w/v) glycerol or (B) with the same buffer but also containing 10 mM NEM. Preincubation with NEM was performed for 5 min at 4 °C; then 7.5 mM $MgCl_2$ and 100 μM ddTTP were added, and each reaction was incubated for 60 min at 25 °C. The [^{32}P]DNA products were then resolved from the gel by electrophoresis and detected by autoradiography as described under Experimental Procedures. The location of the polymerase and/or exonuclease activities is indicated by the vertical arrows, and the position of the tracking dye (TD) is located by an arrow near the right end of the gel.

This result also suggested that form II exonuclease may have disassociated from the polymerase/exonuclease species. The relationship between these activities consequently became the next focus of this investigation.

Detection of DNA Polymerase and 3′ to 5′ Exonuclease by Activity Gel Electrophoresis. To determine whether the chloroplast DNA polymerase and 3′ to 5′ exonuclease activities resided in the same or separate polypeptides, we analyzed the preparation using activity gel electrophoresis. As our first approach, a sample of DNA polymerase/exonuclease (fraction VI) was resolved through a native polyacrylamide gel containing two 5′-end ^{32}P-labeled oligonucleotides annealed to M13mp2 DNA. One oligonucleotide (15-mer) was fully complementary to positions 106–120 within the *lacZ*$_\alpha$ gene and served as a primer for DNA synthesis. The other oligonucleotide (T24-mer) was separately hybridized to M13mp2 DNA at a similar location (positions 106–129). However, this DNA contained a 3′-terminal T·C mispair at position 106 and served as a substrate for detecting 3′ to 5′ exonuclease activity. Following electrophoresis and in situ enzymatic reactions, [^{32}P]DNA products that resulted from polymerase or exonuclease reactions were resolved according to size by a second dimension of electrophoresis through a denaturing DNA sequencing gel. Detection of degraded and extended [^{32}P]DNA products identified the location of exonuclease and DNA polymerase activities, respectively. A 3′ to 5′ exonuclease activity would be expected to degrade the 15-mer and/or 24-mer to smaller products depending on the catalytic preference for matched and mismatched 3′-termini. On the other hand, DNA polymerase activity would be anticipated to incorporate only the next complementary nucleotide (ddTMP) onto the primer (15-mer), forming a [^{32}P]16-mer. Extension of the 24-mer was not expected since dTMP was not the next

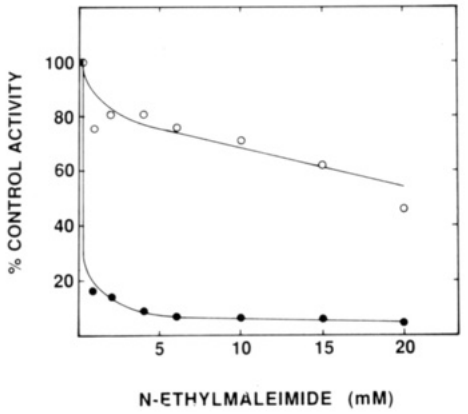

FIGURE 5: *N*-Ethylmaleimide inhibition of DNA polymerase and 3′ to 5′ exonuclease activities. Chloroplast DNA polymerase/exonuclease (fraction VI) was dialyzed against 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, and 10% (w/v) glycerol to remove 2-mercaptoethanol from the sample. Standard DNA polymerase reactions (●) but lacking 2-mercaptoethanol were prepared containing 0.06 unit each of polymerase and were preincubated for 5 min at 4 °C with various amounts of *N*-ethylmaleimide as indicated in the figure. After incubation for 60 min at 37 °C, the reactions were terminated, and acid-insoluble radioactivity was analyzed as described under Experimental Procedures. For 3′ to 5′ exonuclease assays (○), the enzyme samples were diluted 20-fold with buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 100 μg/mL BSA, and 10% (w/v) glycerol. Exonuclease reactions (25 μL) were performed under standard conditions except that DTT was omitted and preincubation with NEM was as described above. After incubation for 30 min at 37 °C, the reaction products were analyzed by polyacrylamide gel electrophoresis as described under Experimental Procedures. Control activity (100%) represented 0.02 unit of exonuclease and produced ~25% digestion of the [^{32}P]T24-mer/M13mp2 DNA.

complementary nucleotide for incorporation on this primer/template. From the result in Figure 4A, both the chloroplast DNA polymerase and 3′ to 5′ exonuclease activities were observed to have comigrated during electrophoresis (R_f = 0.15). This observation taken together with the copurification (Figure 2) and cosedimentation (Figure 3) results confirmed that the two activities were indeed physically associated.

To explore the relationship between the polymerase and exonuclease catalytic activities, we examined their sensitivities to *N*-ethylmaleimide (Figure 5). Both activities were inhibited; however, the polymerase was significantly more sensitive (>80% inhibition at 1 mM NEM) than the exonuclease, which retained >50% activity at 20 mM NEM. We again observed differential sensitivity of the two activities upon reexamining the native polyacrylamide active gels but in the presence of NEM (Figure 4B). Together these results indicated that the catalytic sites for polymerase and exonuclease activities were not identical.

In an attempt to determine the identity of the polymerase and exonuclease polypeptides, in situ activity gel electrophoresis was repeated using SDS-denaturing conditions. Following polyacrylamide gel electrophoresis, the SDS was removed by 2-propanol extraction, and the enzymes were allowed to renature. DNA polymerase and exonuclease reactions were performed, and [^{32}P]DNA products were resolved by DNA sequencing gel electrophoresis, as before. A single band of exonuclease activity was detected with a polypeptide molecular weight of ~20 000 (Figure 6A). Unfortunately, no DNA polymerase activity was recovered. Since this exonuclease activity was originally associated with the DNA polymerase and cosedimented at 105 000 molecular weight, we suspect that it represented a separate polypeptide which was complexed to the polymerase.

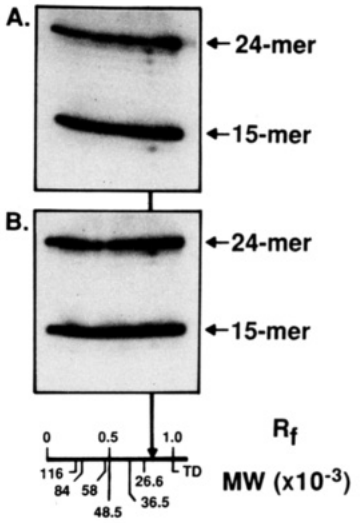

FIGURE 6: In situ activity analysis of chloroplast DNA polymerase and exonuclease following denaturing polyacrylamide gel electrophoresis. (A) A sample (25 μL) of chloroplast DNA polymerase/exonuclease (fraction VI) containing 1.6 units of polymerase activity was denatured and applied to a 13-mm-wide lane of a 10% polyacrylamide/0.1% SDS activity gel containing 0.95 μg/mL each of [5′-^{32}P]15-mer/M13mp2 DNA and [5′-^{32}P]24-mer/M13mp2 DNA. Electrophoresis, SDS extraction, enzyme renaturation, in situ enzyme reactions (25 °C for 50 min), and [^{32}P]DNA product analysis were performed as described under Experimental Procedures. (B) A sample (25 μL) of form II exonuclease was treated identically and loaded into another well; electrophoresis and enzyme detection were carried out as described in panel A. The horizontal arrows indicate the positions of the [^{32}P]24-mer and [^{32}P]15-mer substrates. Prestained polypeptide molecular weight markers, α_2-macroglobulin (180 000), β-galactosidase (116 000), fructose-6-phosphate kinase (84 000), pyruvate kinase (58 000), fumarase (48 500), lactate dehydrogenase (36 500), and triosephosphate isomerase (26 600), were resolved in an adjacent lane and used to determine polypeptide molecular weights. The vertical arrow locates the position of both exonuclease activities. The position of the tracking dye (TD) is indicated on the scale with the molecular weight markers. Migration of the enzyme in the first dimension of electrophoresis was from left to right.

Because the polypeptide molecular weight of form I exonuclease was similar to the native molecular weight of form II exonuclease determined by glycerol gradient sedimentation analysis (MW = 26 500), we also examined the form II exonuclease using the SDS–polyacrylamide activity gel technique (Figure 6B). The results indicated that both form I and II polypeptides exhibited the same molecular weight of ~20 000, suggesting they were very similar if not the same polypeptides.

Characterization of the Exonuclease and DNA Polymerase Reaction Properties. Since the evidence above suggested that form I and II exonucleases might be related, we examined their catalytic properties. Both exonuclease activities were inhibited in an identical manner by addition of NaCl (Figure 7A). At 100 mM NaCl, both exonuclease activities were approximately 50% inactivated. Similarly, both form I and II exonucleases showed an absolute requirement for a divalent metal cation, and 7.5 mM $MgCl_2$ was the optimal concentration (Figure 7B). In addition, each exonuclease shared a similar pH optimum with maximal activity detected in 50 mM Tris-HCl (pH 8.0) though significant activity existed between pH 7.0 and 9.0 (data not shown).

The chloroplast DNA polymerase activity was also examined and shown to be stimulated by up to 125 mM NaCl (Figure 7A). Activity detected at 250 and 500 mM was about 70% and 2% of the optimum, respectively. Like the exonuclease, the polymerase required a divalent metal cation but was optimally satisfied by 15 mM $MgCl_2$ (Figure 7B).

Exonucleolytic Excision of 3′-Terminally Mismatched

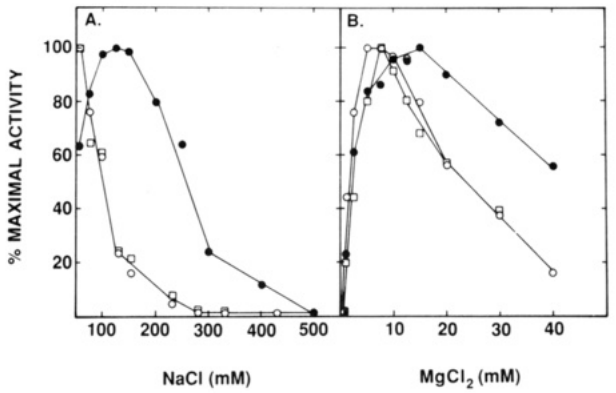

FIGURE 7: Effect of NaCl and $MgCl_2$ on DNA polymerase and 3′ to 5′ exonuclease activities. Standard DNA polymerase and 3′ to 5′ exonuclease reaction conditions were as described under Experimental Procedures except NaCl (A) or $MgCl_2$ (B) was added as indicated in the figure. Reactions contained 0.03 unit of DNA polymerase (●), 0.005 unit of form I exonuclease (○), or 0.007 unit of form II exonuclease (□). The total concentration of NaCl in each assay is indicated and accounts for the contribution by both the enzyme and DNA substrate.

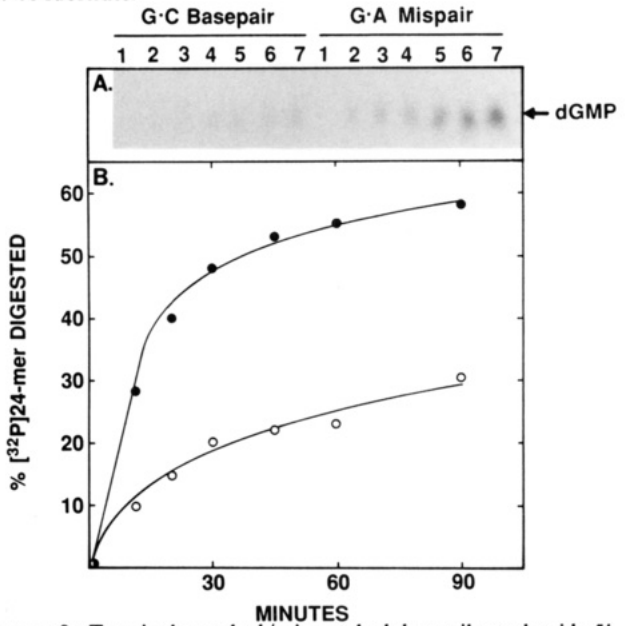

FIGURE 8: Terminal matched/mismatched deoxyribonucleoside 5′-monophosphate excision activity of chloroplast DNA polymerase/exonuclease. A standard 3′ to 5′ exonuclease reaction mixture (200 μL) was prepared with 0.16 unit of fraction VI DNA polymerase/exonuclease as described under Experimental Procedures except 100 μg/mL BSA and 12.6 μg/mL of either 3′-end labeled [^{32}P]G24-mer/M13mp2 DNA or [^{32}P]G24-mer/M13mp2(ΔC106) DNA were included. After incubation at 37 °C for 0, 10, 20, 30, 45, 60, and 90 min (lanes 1–7, respectively), samples (25 μL) were removed and reactions terminated on ice by addition of 2.5 μL of 0.1 M EDTA. (A) Samples (10 μL) were spotted on a PEI thin-layer chromatography sheet along with dGMP and dGTP external standards. Ascending chromatography was performed until the 1.2 M LiCl solvent had migrated 17 cm. Standards were located under 254-nm light (Mineralight Model UVGL-25) and reaction products detected by autoradiography. Only that portion of the PEI TLC plate (R_f 0.43–0.57) containing dGMP is shown. (B) [^{32}P]DNA substrate and [^{32}P]dGMP product were excised from the plate, placed into 5 mL of Ready Protein$^+$, and counted for radioactivity. Open circles (○) represent the percent of dGMP formed per reaction for the matched G·C primer/template, and closed circles (●) reflect the same for the mismatched G·A substrate.

Nucleotides by the DNA Polymerase/Exonuclease Complex. To examine substrate specificity and to identify the reaction product of the form I exonuclease activity, two similar DNA substrates were constructed. A 3′-end ^{32}P-labeled G24-mer was separately hybridized to M13mp2 DNA and M13mp2-(ΔC106) DNA, forming 3′-terminally matched (G·C) and mismatched (G·A) molecules, respectively. Each substrate was digested with form I exonuclease, and the products formed with time were analyzed by thin-layer chromatography using poly(ethylenimine)–cellulose plates. In all cases, deoxyguanosine 5′-monophosphate was the only product detected from the 3′ end of the matched or mismatched DNA substrates (Figure 8A). A comparison of the initial velocities of hydrolysis on each substrate indicated that the 3′-terminally mismatched DNA was preferred by ~2.8-fold (Figure 8B).

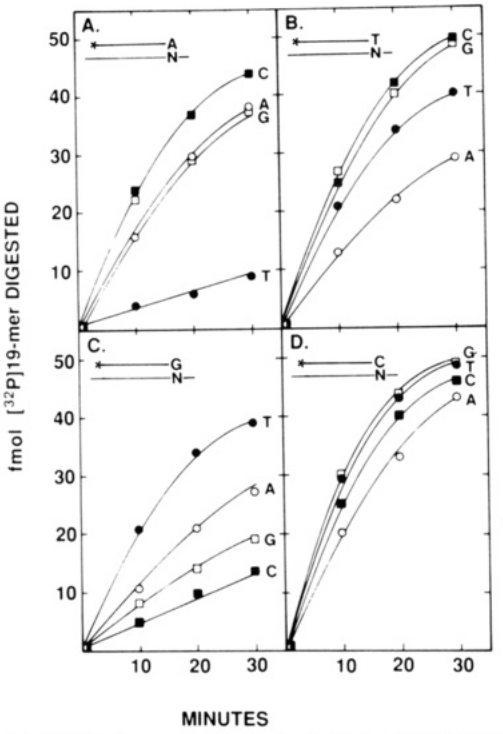

FIGURE 9: Substrate specificity for hydrolysis of matched and 3′-mismatched termini by the 3′ to 5′ exonuclease associated with chloroplast DNA polymerase. Sixteen individual 3′ to 5′ exonuclease reactions (50 μL) containing 50 mM Tris-HCl (pH 8.0), 2 mM DTT, 10 mM $MgCl_2$, 85 mM NaCl, 100 μg/mL BSA, 0.06 unit of DNA polymerase (fraction VI), and 6.3 μg/mL [^{32}P]19-mer/M13mp2 DNA. In separate annealing reactions, the 4 (A, T, G, C)19-mers were hybridized to 4 different M13mp2 (A87, T87, G87, or C87) DNA molecules, and 1 of the 16 substrates was used for each exonuclease reaction. After incubation at 37 °C for the times indicated, samples (10 μL) were removed and added to 10 μL of 95% deionized formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. The 5′-end ^{32}P-labeled reaction products were analyzed on 12% acrylamide/urea DNA sequencing gels, and the amount of 19-mer digested was determined as described under Experimental Procedures. Oligonucleotides A19-mer (A), T19-mer (B), G19-mer (C), and C19-mer (D) were annealed to M13mp2 DNA containing nucleotides A87 (○), T87 (●), G87 (□), or C87 (■) as indicated at the position designated by N in the template.

Since the exonuclease preferred a 3′-terminally mismatched substrate, we examined the specificity of hydrolyzing each of the 16 possible matched and mismatched combinations of 3′-termini. Four similar 5′-end ^{32}P-labeled oligonucleotides (A19-mer, C19-mer, G19-mer, and T19-mer) each with an identical DNA sequence but containing a different 3′-terminal nucleotide were individually hybridized to four different M13mp2 DNA molecules.[4] The M13mp2 DNA molecules

[4] The 19-mers were hybridized to nucleotide positions 87–105 within the transcript DNA sequence of the $lacZ_\alpha$ gene contained in M13mp2 DNA. The 3′-terminal residue of the oligonucleotides (A19-mer, T19-mer, G19-mer, C19-mer) was located at position 87 of each M13mp2 DNA molecule.

Table I: Efficiency of Matched and Mismatched 3′-Terminal Nucleotide Excision by the 3′ to 5′ Exonuclease Associated with Chloroplast DNA Polymerase[a]

substrate[b] primer/ template	initial velocity[c] [DNA$_1$] (1.6 nM)	[DNA$_2$] (4.8 nM)	DNA$_2$/DNA$_1$[d]	V_{max}/K_m[e]
mispairs				
C/A	1.2	3.3	2.8	45.9
A/C	1.0	3.3	3.3	44.8
T/C	1.2	3.5	2.9	44.4
A/A	1.0	2.5	2.5	43.1
T/G	1.1	3.6	3.3	40.7
T/T	1.0	2.9	2.9	38.6
G/A	0.7	1.7	2.4	36.5
C/C	1.1	3.7	3.4	36.0
A/G	0.7	3.1	4.4	29.9
C/T	1.0	3.1	3.1	29.2
G/T	0.8	2.5	3.1	26.2
G/G	0.5	1.1	2.2	21.0
matched pairs				
C/G	1.0	3.2	3.2	30.8
T/A	0.8	1.9	2.4	21.9
A/T	0.3	1.6	5.3	21.7
G/C	0.4	1.0	2.5	13.4

[a] Reactions (50 μL) were performed to determine the 3′ to 5′ exonuclease substrate specificity as described in Figure 9. Five sets of 16 reactions (all matched and mismatched primer/template combinations) were prepared, each containing 0.06 unit of DNA polymerase (fraction VI) and either 4.3, 6.3, 8.4, 12.6, or 18.9 μg/mL [^{32}P]19-mer/M13mp2 DNA. Reactions were incubated at 37 °C for 0, 10, 20, and 30 min; samples (10 μL) were removed, terminated with formamide/EDTA/dye mixture (10 μL), and analyzed on 12% acrylamide/urea DNA sequencing gels as described under Experimental Procedures. [b] Substrates were (A, T, G, C)19-mer hybridized to all combinations of M12mp2 (A87, T87, G87, or C87) DNA. Base pairs at position 87 of the *lacZ*$_\alpha$ sequence of M13mp2 are indicated as primer/template nucleotides. [c] Slopes of the 3′ to 5′ exonuclease activity (femtomoles of [^{32}P]19-mer digested per 50-μL reaction) versus time (minutes) curves were determined for the five sets of reactions. Only initial velocities for the 16 reactions containing 6.3 μg/mL (1.6 nM 3′ ends) and 18.9 μg/mL (4.8 nM 3′ ends) 19-mer/M13mp2 DNA are indicated as [DNA$_1$] and [DNA$_2$], respectively. [d] If the velocity of exonuclease activity was directly proportional to substrate concentration then initial velocity ratios of DNA$_2$ to DNA$_1$ would equal 3.0. [e] The V_{max} (femtomoles of 19-mer digested per hour) to K_m (nanomolar) ratios were obtained using the procedure described by Boosalis et al. (1989).

consisted of the wild-type M13mp2 (T87) and three M13mp2 (A87, G87, C87) point mutants. Thus, hybridization of the four [5′-^{32}P]19-mer oligonucleotides with the four M13mp2 DNAs generated the 16 unique 3′-terminally matched and mismatched substrates with each 3′-terminal residue located in an identical DNA sequence context. The initial velocity of terminal excision of each substrate was determined as observed in Figure 9. For three of the four sets of primer/templates (Figure 9A–C), the mismatched substrates were preferred by 1.4–6.2-fold over the corresponding 3′-terminally matched substrate (A·T, T·A, or G·C). In contrast, the substrate containing a 3′-terminal C·G pair was hydrolyzed with equal or slightly better efficiency than the three cognate mispairs (C·T, C·A, and C·C) as observed in Figure 9D. The initial velocity of hydrolyzing a C residue from any 3′-terminus was also found to be relatively high when comparing all 16 DNA substrates.

The kinetic parameters of these 16 reactions were also examined to determine the substrate specificity constant defined by V_{max}/K_m. In an attempt to obtain individual K_m and V_{max} values, time course experiments were repeated, and initial velocities were measured for each substrate at five different primer/template concentrations (Table I). In all cases, the initial velocity was roughly proportional to the substrate concentration even at the highest primer/template concentration tested. Thus, the maximal velocity had not been reached. It is important to emphasize that individual measurements of K_m and V_{max} constants need not be obtained to determine the V_{max}/K_m ratio. Accurate estimates of V_{max}/K_m can be determined at relatively low primer/template concentrations from the slope of the velocity versus substrates curves (Boosalis et al., 1989). The V_{max}/K_m ratios for each substrate were therefore obtained by this method (Table I). Those DNA substrates that showed larger V_{max}/K_m values were recognized by the exonuclease with greater efficiency. After the constants were ranked from highest to lowest, it becomes evident that the mismatched substrates were preferred over the matched substrates with the exception of the C·G pair. Thus, the 3′ to 5′ exonuclease appears to preferentially hydrolyze 3′-terminal mispairs.

Discussion

We have demonstrated that spinach chloroplast DNA polymerase preparations contained a 3′ to 5′ exonuclease activity. This exonuclease copurified with the chloroplast DNA polymerase through six steps that resolved proteins on the basis of different separation principles. In addition, both activities cosedimented and coelectrophoresed under nondenaturing conditions. These results represent the first demonstration of a chloroplast DNA polymerase physically associated with a 3′ to 5′ exonuclease. Association between polymerase and 3′ to 5′ exonuclease activities has been reported for DNA polymerase γ isolated from porcine liver mitochondria (Kunkel & Mosbaugh, 1989). In fact, association of these two activities is a common feature among various biological sources of DNA polymerase γ (Kunkel & Soni, 1988; Kaguni & Olson, 1989; Insdorf & Bogenhagen, 1989). Whether this pattern extends to chloroplast DNA polymerases from other biological sources needs to be examined. Other studies have reported the presence of exonuclease activity throughout a substantial portion of the chloroplast DNA polymerase purification (McKown & Tewari, 1984; Wang et al., 1991). However, in those cases, the exonuclease activity was eventually removed from the polymerase preparation. This would not be inconsistent with our finding that the 3′ to 5′ exonuclease activity was dissociable from the spinach chloroplast DNA polymerase.

During glycerol gradient centrifugation of the chloroplast DNA polymerase/exonuclease (fraction V), two forms of the exonuclease were resolved. One species, designated exonuclease form I, cosedimented with DNA polymerase activity at a molecular weight of 105000. This was in exact agreement with the molecular weight previously reported for spinach chloroplast DNA polymerase (Sala et al., 1980). The other species, termed exonuclease form II, was not associated with polymerase activity and sedimented with a lower molecular weight of 26500. Several lines of evidence suggested that form I and II exonucleases are related and that form II was derived from a polymerase/exonuclease form I complex. When DNA polymerase/exonuclease (fraction VI) was pooled from the glycerol gradient and analyzed by nondenaturing polyacrylamide activity gel electrophoresis, only the polymerase with its associated exonuclease activity was observed. However, when the same sample was resedimented in an identical glycerol gradient, both form I and II exonucleases appeared in approximately an equal ratio (Figure 3), suggesting that form II dissociated from a polymerase/exonuclease complex. If this interpretation was correct, one might also have expected to observe a second peak of the DNA polymerase that was free of exonuclease activity. However, such a peak was not observed. We propose either that the free form of the polymerase was unstable or that it was contained in the fractions between

the two exonuclease peaks. It would not be surprising to find that the polymerase was unstable when liberated from the exonuclease. Dissociation of the α-subunit from *E. coli* DNA polymerase III core enzyme has been previously reported to yield a less stable form of polymerase (Maki & Kornberg, 1985, 1987). In addition, support for the second possibility is evident from the unequal ratio of polymerase to exonuclease (form I) activity across the resedimented polymerase peak (Figure 3). This suggested that more than one form of the polymerase might exist within the lower molecular weight fractions of this peak. Additional evidence indicated that form I and II exonucleases are related if not identical enzymes. Both exonucleases required the same reaction conditions for optimal activity. These exonucleases (i) possessed an absolute requirement for a divalent metal cation, each being satisfied with 7.5 mM $MgCl_2$ as the optimum, (ii) were identically inhibited by NaCl, and (iii) preferred an alkaline pH 8.0 for maximal activity. Finally, the most convincing evidence that form I and II exonucleases are similar proteins was obtained by in situ detection of exonuclease activity following SDS–polyacrylamide gel electrophoresis. Both activities showed identical polypeptide molecular weights of ~20000. From these results, we concluded that form II exonuclease was derived through dissociation of the DNA polymerase/exonuclease form I complex.

Since form I exonuclease cosedimented with the DNA polymerase at 105000 molecular weight but exhibited a polypeptide molecular weight of ~20000, we suspect that these two activities reside in separate, associated polypeptides. Our finding that the exonuclease was resistant to NEM inhibition, whereas the polymerase was extremely sensitive, strengthens the argument that the catalytic sites for these two activities are distinct. Whether these two activities reside in separate subunits or in polypeptides derived from proteolytic processing remains to be determined. Recently, the chloroplast DNA polymerase isolated from *C. reinhardtii* was shown by SDS–polyacrylamide activity gel analysis to be associated with polymerase bands of 80000 and 116000 molecular weight (Wang et al., 1991). The larger polypeptide had a molecular weight similar to the spinach chloroplast DNA polymerase/exonuclease that we observed by sedimentation analysis. Unfortunately, the gel used to analyze the *C. reinhardtii* DNA polymerase was not assayed for exonuclease activity. It is interesting to speculate that the form II exonuclease (MW = 20000) might associate with an 80000 molecular weight form of DNA polymerase to produce a larger complex (MW ~ 105000) similar to the one we observed in the glycerol gradient.

The function of the 3′ to 5′ exonuclease activity associated with chloroplast DNA polymerase was not determined. However, the observation that this exonuclease generally preferred a 3′-terminally mismatched DNA substrate suggested that the exonuclease might play a role in proofreading base substitution errors made during chloroplast DNA synthesis. It was not particularly surprising to find that the discrimination between matched and mismatched base pairs was relatively low (~1–6.2-fold). Recently, several DNA polymerases including *E. coli* DNA polymerase I (Bebenek et al., 1990), polymerase III core enzyme (Brenowitz et al., 1991), and porcine liver DNA polymerase γ (Kunkel & Mosbaugh, 1989; Longley & Mosbaugh, 1991b) have been examined to determine the influence of proofreading exonucleases on in vitro base substitution fidelity. In all cases, the magnitude of this discrimination in vitro was less than 10-fold. Since the overall contribution of exonucleolytic proofreading toward the fidelity of in vivo DNA synthesis has been estimated at 10^2–10^3-fold (Schaaper, 1988; Echols & Goodman, 1991), we suggest that other factors may influence the in vivo substrate specificity of proofreading exonucleases. Additional studies will be required to determine the exact function of this 3′ to 5′ exonuclease in chloroplast DNA metabolism.

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Registry No. DNA polymerase, 9012-90-2; 3′,5′-exonuclease, 79393-91-2.

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Cloning and Sequencing of the Gene for Rubrerythrin from *Desulfovibrio vulgaris* (Hildenborough)†

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ABSTRACT: The gene coding for rubrerythrin from the sulfate-reducing bacterium *Desulfovibrio vulgaris* (Hildenborough) has been cloned and sequenced. Rubrerythrin is known to contain two types of iron sites: one rubredoxin-like FeS_4 center in each of the two identical subunits and one hemerythrin-like diiron site per dimer [LeGall, J., et al. (1988) *Biochemistry 27*, 1636-1642]. The gene encodes a polypeptide of 191 amino acids, and a normal ribosome binding site is located 11-6 base pairs upstream from the translational start of the gene. There is no evidence for the presence of a leader sequence, suggesting a cytoplasmic location for the protein. The rubrerythrin gene is not part of any other known transcriptional unit in the *D. vulgaris* genome. The nucleotide sequence encodes four Cys residues, the minimum required for ligation to iron in rubredoxin. The pairs of Cys residues occur in Cys-X-X-Cys sequences as they do in rubredoxin, but the 12-residue spacing between the Cys pairs in rubrerythrin is less than half that in rubredoxins. A pair of Arg residues flanking one Cys residue may contribute to the much more positive reduction potential of the rubredoxin-like site in rubrerythrin compared to that of rubredoxin. While the amino acid sequence of rubrerythrin shows no significant overall homology with that of any known protein, the C-terminal region does share some homology with rubredoxin sequences. If folding of the rubredoxin-like amino acid sequence domain in rubrerythrin is similar to that in rubredoxins, then three His residues are brought into proximity. These His residues, which have no counterpart in any rubredoxin, could, therefore, furnish ligands to the hemerythrin-like irons. This proposal is consistent with the absorption spectrum and the positive reduction potential qualitatively observed for the hemerythrin-like diiron site. Assuming that the hemerythrin-like diiron site bridges the two subunits, preliminary X-ray diffraction results on rubrerythrin [Sieker et al. (1988) *Proteins: Struct., Funct., Genet. 3*, 184-186] are consistent with this proposal.

A large number of redox-active metalloproteins have been isolated and characterized from the strictly anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris* (Hildenborough) (LeGall & Fauque, 1988). Among these proteins, rubrerythrin (Rr)[1] is unique in terms of its complement of high reduction potential non-heme iron sites (LeGall et al., 1988). Rr is a homodimer (native M_r 43 000) containing two types of iron sites: two rubredoxin-like (Rd-like) FeS_4 centers (one per subunit) with spectroscopic properties very similar to the tetrahedrally coordinated iron centers found in Rds (Watenpaugh et al., 1979; Adman et al., 1991) and one diiron site with spectroscopic similarities to those in a class of proteins for which Hr is a prototype (Holmes et al., 1991; Sanders-Loehr, 1989). This diiron site is, therefore, referred to as the Hr-like site, although it shows no ability to bind O_2. Despite

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[1] Abbreviations: Rr, rubrerythrin; Rd, rubredoxin; Hr, hemerythrin; SDS, sodium dodecyl sulfate; IPTG, isopropyl β-D-thiogalactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; TE, 10 mM Tris-HCl/0.1 mM EDTA, pH 8; SSC, 0.015 M sodium citrate/0.15 M NaCl, pH 7.2.

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