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A Chloroplast DNA Helicase II from Pea That Prefers Fork-Like Replication Structures

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A DNA helicase, called chloroplast DNA (ctDNA) helicase II, was purified to apparent homogeneity from pea (*Pisum sativum*). The enzyme contained intrinsic, single-stranded, DNA-dependent ATPase activity and an apparent molecular mass of 78 kD on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The DNA helicase was markedly stimulated by DNA substrates with fork-like replication structures. A 5′-tailed fork was more active than the 3′-tailed fork, which itself was more active than substrates without a fork. The direction of unwinding was 3′ to 5′ along the bound strand, and it failed to unwind blunt-ended duplex DNA. DNA helicase activity required only ATP or dATP hydrolysis. The enzyme also required a divalent cation ($Mg^{2+} > Mn^{2+} > Ca^{2+}$) for its unwinding activity and was inhibited at 200 mM KCl or NaCl. This enzyme could be involved in the replication of ctDNA. The DNA major groove-intercalating ligands nogalamycin and daunorubicin were inhibitory to unwinding (K_i approximately 0.85 μM and 2.2 μM, respectively) and ATPase (K_i approximately 1.3 μM and 3.0 μM, respectively) activities of pea ctDNA helicase II, whereas ellipticine, etoposide (VP-16), and camptothecin had no effect on the enzyme activity. These ligands may be useful in further studies of the mechanisms of chloroplast helicase activities.

A special class of DNA-interacting enzymes known as DNA helicases catalyze the unwinding of energetically stable duplex DNA in an ATP-dependent manner, and thus play an important role in DNA replication, repair, recombination, and transcription (Matson et al., 1994; Lohman and Bjornson, 1996; Tuteja and Tuteja, 1996; Tuteja, 1997). DNA helicases are ubiquitous enzymes now known for their analogy to motor proteins such as myosin, kinesin, and dynein, which use ATP hydrolysis for energy (West, 1996). All helicases contain intrinsic DNA-dependent ATPase activity, which provides energy for the reaction (Kornberg and Baker, 1991). Helicases generally bind in the ssDNA or in the ss-/ds-DNA junctions and translocate unidirectionally along the bound strand, either in the 3′ to 5′ or in the 5′ to 3′ direction. Many DNA helicases have been isolated from bacteria, bacteriophage, virus, and eukaryote systems (Thommes and Hubscher, 1992; Matson et al., 1994; Tuteja and Tuteja, 1996).

Chloroplasts are highly polyploid, semiautonomous, intracellular organelles that contain their own genetically active genomes. In higher plants the ctDNA is a ds, circular molecule that ranges in size from 120 to 160 kb and encodes about 130 genes. The mechanism of DNA replication is well defined in bacteria, viruses, bacteriophage, plasmids, and, to a lesser extent, yeasts (Kornberg and Baker, 1991), but is still not understood in plant systems. DNA replication requires the concerted assembly and activity of many proteins (Hubscher and Spadari, 1994). Most of the studies on replication of DNA in plants have focused on ctDNA because of the relative ease of isolating and handling the intact chloroplast genome (Tewari, 1987; Meeker et al., 1988). A number of enzymes that may be involved in replication, such as DNA polymerases (Sala et al., 1980; McKown and Tewari, 1984; Heinhorst et al., 1990), DNA topoisomerases (Siedlecki et al., 1983; Lam and Chua, 1987; Nielson and Tewari, 1988), and DNA helicase (Tuteja et al., 1996), have been purified from the chloroplasts of higher plants. DNA primase activity has also been reported from pea (*Pisum sativum* L.) chloroplasts (Nielson et al., 1991).

Most organisms encode multiple DNA helicases because of their involvement in numerous biological reactions at different stages of cell metabolism. A set of 13 different DNA helicases have been reported in *Escherichia coli* (Lohman, 1992; Matson et al., 1994). Nine different DNA helicases have been purified to homogeneity from HeLa cells (Tuteja et al., 1990, 1991, 1992, 1993, 1994, 1995, 1996). In plants multiple DNA helicases are also expected to be present. However, to date only one DNA helicase has been purified to homogeneity from pea chloroplasts (Tuteja et al., 1996). Although the existence of two other DNA helicases, one from lily (Hotta and Stern, 1978) and another from soybean (Cannon and Heinhorst, 1990), has been reported, these enzymes were neither purified nor well characterized.

We are studying the detailed molecular mechanism of ctDNA replication with the underlying goals of establishing a well-defined in vitro replication system and establishing the use of organelle DNA for the transformation of plants. In this context we have initiated systematic studies of the DNA helicases present in pea chloroplasts with the objective of isolating and characterizing them and eventually cloning their genes for functional study. Here we report the purification and characterization of pea ctDNA helicase II, which is stimulated by fork-like replication

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Abbreviations: ATPγS, adenosine 5′-O-(3-thiotriphosphate); ds, double-stranded; NTP, nuceloside triphosphate; ss, single-stranded; VP-16, 4′-demethyl-epipodophyllotoxin-β-D-ethylidene glucoside.

Table I. *Purification of pea ctDNA helicase II*

Step	Fraction	Total Volume	Total Protein	DNA Helicase Activity	
				Total	Specific activity
		mL	*mg*	*units*	*units/mg*
	Pea leaves (2.2 kg)				
I	Chloroplast lysate (after dialysis)	660	5152	n.d.[a]	
II	DE-52 cellulose	1200	2060	n.d.	
III	CM-52 cellulose	95	2.85	n.d.	
IV	Heparin-Sepharose	7.2	0.465	3600	7,742
V	dsDNA cellulose	3	0.055	1500	27,273
VI	ssDNA cellulose	1	0.0017	665	391,176

[a] n.d., Not determined, due to presence of nucleases.

structures. The properties of this enzyme make it a candidate for a DNA replicative helicase. We have also tested the effect of different DNA-interacting ligands on the unwinding and ATPase activities of pea ctDNA helicase II.

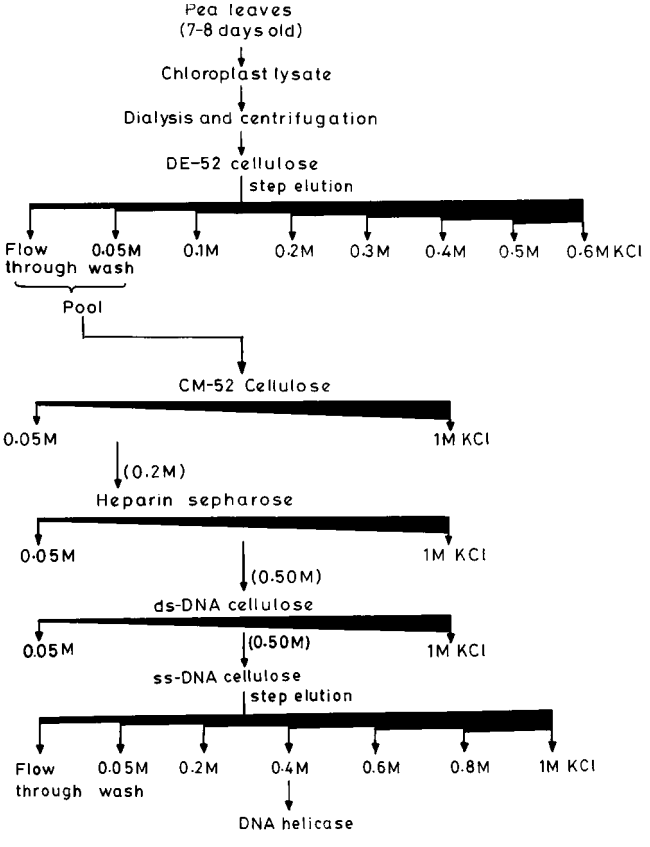

Figure 1. Purification scheme of pea ctDNA helicase II. The strategy used for fractionation of DNA helicase II is shown. The numbers indicate the molarity of the KCl gradient or step elution to elute the enzyme activity.

MATERIALS AND METHODS

DNA, Nucleotides, and Ligands

M13 mp19 ssDNA, dsDNA, and RNA from pea (*Pisum sativum* L.) leaves were prepared as described previously (Sambrook et al., 1989). NTPs and ATPγS were obtained from Boehringer-Mannheim and [γ-^{32}P]ATP (185 Tbq/mmol) and [α-^{32}P]dCTP (approximately 110 Tbq/mmol) were purchased from Amersham. The various oligodeoxyribonucleotides used to construct helicase substrates were synthesized chemically using a DNA synthesizer (model 380 A, Applied Biosystems), and purified electro-

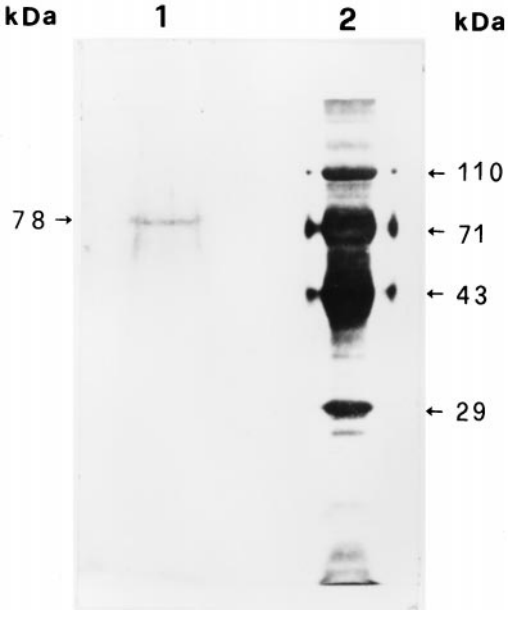

Figure 2. SDS-PAGE of purified pea ctDNA helicase II. Fraction VI (60 ng, lane 1) and the M_r marker (lane 2) were separated on a 12% polyacrylamide gel and visualized by silver staining.

Table II. *Reaction requirements of the purified pea ctDNA helicase II*

Reaction Condition	Unwinding[a]
	%
Complete	50
−Enzyme	<2
+Heated enzyme (56°C for 1 min)	<2
−ATP	<2
−ATP + ATPγS (2 mM)	<2
−ATP + ADP (2 mM) or AMP (2 mM)	<2
$-MgCl_2$	<2
$-MgCl_2 + CaCl_2$ (1.5 mM)	10
$-MgCl_2 + ZnSO_4$ (1.5 mM)	<2
$-MgCl_2 + MnCl_2$ (1.5 mM)	31
$-MgCl_2 + CdCl_2$ (1.5 mM)	<2
$-MgCl_2 + CuCl_2$ (1.5 mM)	<2
$-MgCl_2 + NiCl_2$ (1.5 mM)	<2
$-MgCl_2 + AgNO_3$ (1.5 mM)	<2
$-MgCl_2 + CoCl_2$ (1.5 mM)	<2
+KCl or NaCl (200 mM)	4
$+(NH_4)_2SO_4$ (45 mM)	<2
$+KPO_4$ (pH 8.0, 100 mM)	<2
+EDTA (5 mM)	<2
+M13 ssDNA (30 mM as phosphate)	<2
+M13RFI DNA (30 mM as phosphate)	15
+Pea leaves total RNA (30 mM as phosphate)	20
+*E. coli* tRNA (30 mM as phosphate)	25
+Trypsin (1 unit)	<2

[a] Helicase reaction was carried out with 5 ng of pure protein (fraction VI) and 1 ng of substrate (5′-tailed).

phoretically. The sequences and details of the oligodeoxyribonucleotides were described previously (Tuteja et al., 1994, 1996). The DNA-interacting ligands daunorubicin, ellipticine, camptothecin, and VP-16 were purchased from Topogene (Columbus, Ohio). Nogalamycin was from Sigma.

Preparation of DNA Helicase Substrates

The DNA substrate used in the helicase assay consisted of ^{32}P-labeled complementary oligodeoxyribonucleotides hybridized to M13 mp19 phage ssDNA to create a partial duplex. A substrate with a 5′-hanging tail was used for purification and for most of the characterization unless otherwise stated. The structures of the DNA substrates (circular or linear) were as described previously (Tuteja et al., 1994, 1996). The 3′ to 5′ and 5′ to 3′ direction-specific substrates were constructed as described previously (Tuteja et al., 1994, 1996).

DNA Helicase Assay

The helicase assay measures the unwinding of a labeled oligodeoxyribonucleotide fragment from a partial duplex molecule, catalyzed by ctDNA helicase II. The standard reaction mixture (10 μL) consisted of 20 mM Tris-HCl (pH 8.5), 8 mM DTT, 4% (w/v) Suc, 80 μg/mL BSA, 2 mM ATP, 1.5 mM $MgCl_2$, 100 mM KCl, approximately 1 ng of ^{32}P-labeled DNA substrate (approximately 1000 cpm), and the helicase fraction. The reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 1.5 μL of 75 mM EDTA, 2.25% SDS, 37.5% (by volume) glycerol, and 0.3% bromphenol blue. The products were separated by 12% native PAGE, and the gel was dried and exposed to film for autoradiography. The DNA unwinding was quantitated by excising the radioactive bands from the gel and counting the radioactivity in Beckman liquid-scintillation fluid. One unit of helicase activity is defined as the amount of enzyme that unwinds 30% of the DNA helicase substrate at 37°C in 30 min in the linear range of enzyme concentrations. For examining the effect of DNA-interacting ligands on helicase activity, different types were added to the helicase reaction mixture prior to the addition of the enzyme.

DNA-Dependent ATPase Assay

The hydrolysis of ATP catalyzed by ctDNA helicase II was assayed by measuring the formation of ^{32}P from [γ-^{32}P]ATP. The reaction conditions were identical to those described for the helicase reaction, except that the ^{32}P-labeled helicase substrate was replaced by 1665 Bq [γ-^{32}P] ATP. The reaction was performed both in the presence and absence of 50 ng of M13 mp19 ssDNA, followed by TLC and quantitation as described earlier (Tuteja et al., 1992). For inhibition of pea ctDNA helicase II ATPase activity, the DNA-interacting ligands were included in the reaction prior to the addition of the enzyme, as described earlier (Tuteja et al., 1997).

Other Methods

DNA topoisomerase, polymerase, ligase, nicking, and nuclease activities were performed as described earlier (Tuteja et al., 1990, 1991, 1992, 1993, 1994, 1995, 1996). Protein concentration was determined using the Bio-Rad protein assay kit. SDS-PAGE was performed by the method of Laemmli (1970), followed by silver staining of the gel with the Bio-Rad kit.

RESULTS

Purification of ctDNA Helicase II

DNA helicase activity was biochemically assayed as described previously (Tuteja, 1997). The DNA substrate used for the purification procedure and for most of the characterization consisted of a ^{32}P-labeled 32-base oligonucleotide annealed through its last 17 nucleotides (position 16–32) to M13 mp19 ssDNA. This partial duplex DNA substrate contained a 5′-end protruding tail of 15 nucleotides. The displacement of a DNA fragment was measured by native gel electrophoresis. The results of purification are summarized in Table I, and the purification scheme is outlined in Figure 1. All of the purification steps were

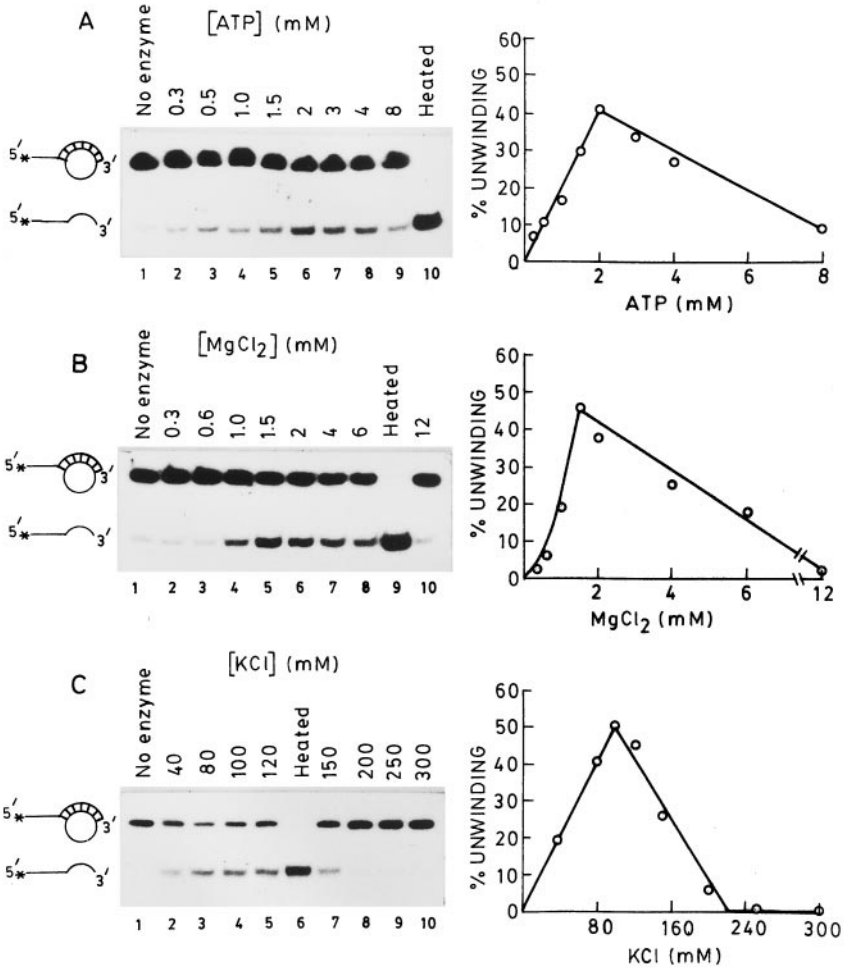

Figure 3. Effect of ATP (A), $MgCl_2$ (B), and KCl (C) on pea ctDNA helicase II activity. In each reaction 5 ng of fraction VI with 1 ng of the 5′-tailed substrate was used with varying concentrations of ATP, $MgCl_2$, or KCl. Quantitative data are displayed on the right side of each autoradiogram. The structure of the substrate is shown on the left side of each gel. Asterisks denote the ^{32}P-labeled end. Lanes marked "No enzyme" and "Heated" are the reactions without the enzyme and with heat-denatured substrates, respectively. The activity is shown as percent unwinding.

performed at 4°C. Triton X-100-disrupted chloroplast lysate (fraction I, 660 mL) was prepared from 2.2 kg of pea leaves (7- to 8-d-old plants) and dialyzed against buffer A (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 1 mM sodium-metabisulfite, 1 μM pepstatin, 1 mg/mL benzamidine, and 1 μM leupeptin) as described earlier (Tuteja et al., 1996). Fraction I was loaded onto a 240-mL DEAE-cellulose column (DE-52, Whatman) equilibrated with buffer A as described previously (Tuteja et al., 1996). The flow-through and wash fractions were collected (fraction II, 1200 mL) and loaded onto an 85-mL cellulose column (CM-52, Whatman) equilibrated with buffer A. After a thorough washing, bound proteins were eluted with an 850-mL linear gradient of 0.05 to 1 M KCl in buffer A. Fractions eluted at around 0.2 M KCl contained helicase activity. The active fractions were pooled and dialyzed against buffer A (fraction III, 95 mL). Up to this step the activity was not quantitated due to contamination with nuclease activity.

Fraction III was applied to a 3-mL heparin Sepharose column equilibrated with buffer A. Following washing with buffer A, the bound proteins were eluted with a 36-mL linear gradient from 0.05 to 1 M KCl in buffer A. The active fractions eluted at about 0.5 M KCl, and were then pooled and dialyzed against buffer B (fraction IV, 7.2 mL. 3600 units). Buffer B was buffer A plus 1 mM ATP and 1 mM $MgCl_2$. Fraction IV was loaded onto a 1.2-mL dsDNA-cellulose column equilibrated in buffer B. The column was washed thoroughly, and bound proteins were eluted with an 18-mL linear gradient of KCl (0.05 to 1 M) in buffer B. The activity eluted from the column at about 0.5 M KCl (fraction V, 3 mL, 1500 units). Fraction V was first diluted with buffer B to adjust the KCl concentration to 0.05 M and was then loaded onto a 0.5-mL ssDNA-cellulose column equilibrated with buffer B. After washing the column with 10 mL of buffer B the bound proteins were eluted in steps with 0.2, 0.4, 0.6, 0.8, and 1 M KCl in buffer B. The helicase activity was detected only in the 0.4 M KCl fraction (fraction VI, 1 mL, 665 units).

SDS-PAGE analysis followed by silver staining revealed the presence of only one polypeptide of 78 kD in fraction VI (Fig. 2, lane 1), which showed that ctDNA helicase II was purified to apparent homogeneity with a specific activity of 3.9×10^5 units/mg (Table I). The enzyme preparation did

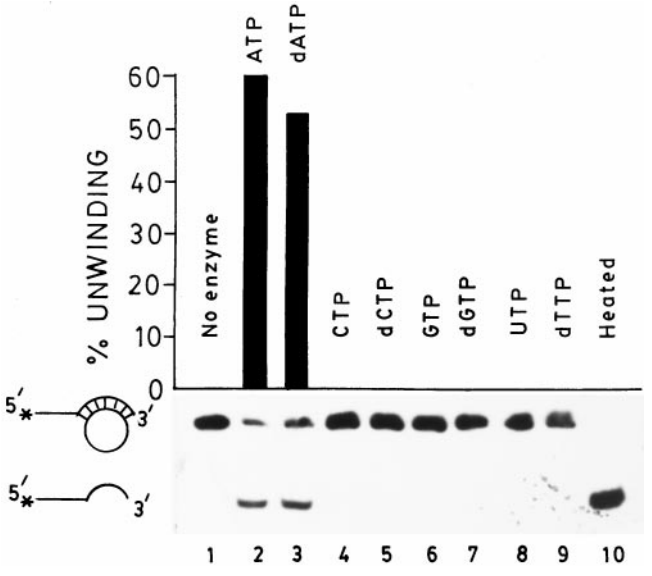

Figure 4. Preference of nucleotides for pea ctDNA helicase II activity. The standard helicase reactions were performed with 5 ng of fraction VI, 1 ng of 5′-tailed substrate, and 2 mM NTP or deoxyribonucleoside triphosphate. The amount of unwound DNA was quantitated and plotted as a histogram above the autoradiogram of the gel. Lanes 1 and 10 are the reactions without the enzyme and with the heat-denatured substrate, respectively. Lanes 2 to 9 are reactions in the presence of ATP, dATP, CTP, dCTP, GTP, dGTP, UTP, and dTTP, respectively. The structure of the substrate is shown on the left side of the autoradiogram. The asterisk denotes the ^{32}P-labeled end.

not contain any detectable DNA polymerase, ligase, topoisomerase, nicking, or nuclease activities. ssDNA-dependent ATPase activity was present at a level of 400 pmol of ATP hydrolyzed in 30 min by 5 ng of pure enzyme (fraction VI).

Cofactor and Reaction Requirements

The reaction requirements of pea ctDNA helicase II are shown in Table II. The enzyme is heat labile and loses its activity upon heating at 56°C for 1 min. Significant unwinding activity was observed in a broad pH range (pH 7.5–9.5) with an optimum near pH 8.5 (data not shown). The activity was completely inhibited by trypsin (1 unit), EDTA (5 mM), potassium phosphate (100 mM), ammonium sulfate (45 mM), and ssDNA (30 mM as phosphate). However, dsDNA, pea leaf total RNA, and *Escherichia coli* tRNA inhibited the helicase activity to 70%, 60%, and 50%, respectively (Table II). The enzyme showed an absolute requirement for divalent cations. Mg^{2+} (1.5 mM) optimally fulfilled this requirement (Fig. 3B), whereas Mn^{2+} and Ca^{2+} at equivalent concentrations supported 62% and 20% of the activity, respectively. However, at 12 mM $MgCl_2$ the activity was totally inhibited (Fig. 3B). Other divalent cations, such as Zn^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Ag^{2+}, and Co^{2+}, were unable to support the reaction (Table II). The optimum concentration of KCl required for the helicase reaction was 100 mM. At a greater concentration (200 mM) of KCl the activity was inhibited (Fig. 3C).

DNA helicase activity was totally dependent upon ATP with an optimum concentration requirement of 2 mM (Fig. 3A). At greater than 8 mM ATP the enzyme was inhibited. dATP also supported 90% of the activity, whereas other

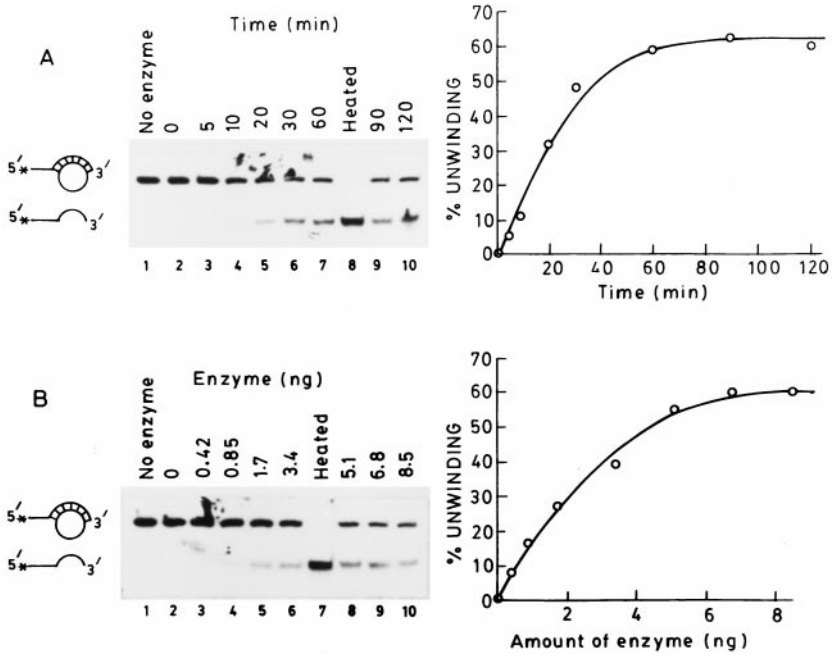

Figure 5. Kinetics and concentration dependence of pea ctDNA helicase II. The enzyme activity data from the autoradiograms (left) were quantitated and are shown on the right. The structure of the substrate used is shown on the extreme left. Asterisks denote the ^{32}P-labeled end. A, The standard reaction was carried out with 5 ng of fraction VI at the times indicated. B, An increasing amount of fraction VI was used in the standard helicase assay. The concentrations are indicated at the top of each lane. Lanes labeled "No enzyme" and "Heated" are the reactions without the enzyme and with the heat-denatured substrate, respectively.

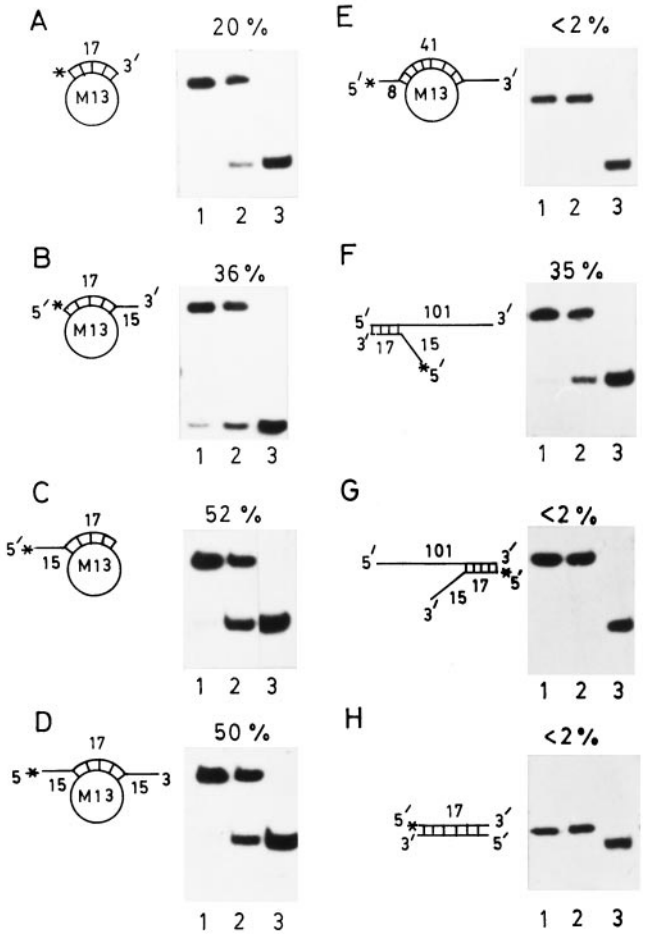

Figure 6. Preference of forked DNA structures for unwinding activity of pea ctDNA helicase II. The DNA helicase reactions were performed under standard conditions using different DNA substrates that contained either no tail (A), a 3′ tail (B), a 5′ tail (C), or both 3′ and 5′ tails (D and E). Small linear substrates with forked structures, which also represent 3′ to 5′ (F) or 5′ to 3′ (G) direction specificity, and a blunt-ended DNA substrate (H) were also used. The schematic structure of each substrate is shown on the left side of the autoradiogram of the gel. Asterisks denote the ^{32}P-labeled end. The percent unwinding is shown at the top of each panel. In each panel, lane 1 is the reaction without the enzyme, lane 2 is the reaction with the enzyme (5 ng), and lane 3 is the heat-denatured substrate.

NTPs or deoxyribonucleoside triphosphates did not support the unwinding activity (Fig. 4). ADP, AMP, and the poorly hydrolyzable ATP analog ATPγS were inactive (Table II).

Kinetics and Titration of Helicase Activity

The kinetics of the helicase reaction under the standard assay conditions with 5 ng of purified enzyme (fraction VI) showed a linear rate of up to 30 min (Fig. 5A). After further incubation it deviated from the linearity and became saturated at 60 min. Titration of helicase activity with increasing amounts of the pure enzyme showed an approximate linear response; up to 55% unwinding with 5 ng of the protein and approximately 1 ng of the substrate (Fig. 5B).

Helicase Activity Is Stimulated by Fork Structures

The influence of fork structures in the DNA substrate on the unwinding activity of pea ctDNA helicase II was examined by using four different substrates in standard assay conditions. All four substrates had the same duplex length (17 bp) with an identical sequence but differed in the presence of noncomplementary tails at the 3′ end (Fig. 6B), 5′ end (Fig. 6C), both of the ends (Fig. 6D), or without a tail (Fig. 6A). The substrate without the tail supported helicase activity poorly (20% unwinding, Fig. 6A). In the presence of a 3′ tail, the helicase activity was stimulated and showed more unwinding (36% unwinding) compared with the no-tail substrate (Fig. 6B). However, substrates containing either a 5′ tail alone or both 5′ and 3′ tails were the most efficiently displaced. At the same concentration of enzyme (fraction VI, 5 ng) the 5′-tailed and 5′- and 3′-tailed fork structures showed unwinding of 52% and 50% of the duplex, respectively (Fig. 6, C and D). The enzyme was unable to unwind the longer duplex even if it contained both tails (Fig. 6E). The enzyme also failed to unwind blunt-ended duplex DNA (Fig. 6H).

Small, linear, partial-duplex substrates with 5′- or 3′-tailed fork-like structures (Fig. 6, F and G) were also examined. The results show that pea ctDNA helicase II was able to unwind only a 5′-tailed substrate (Fig. 6F) and not the 3′-tailed substrate (Fig. 6G). It should be noted that the length of the tails (15 nucleotides) was significantly shorter, so the enzyme loaded and translocated unidirectionally on the other free ssDNA strand. Since these are direction-specific substrates and only the 5′-tailed substrate was

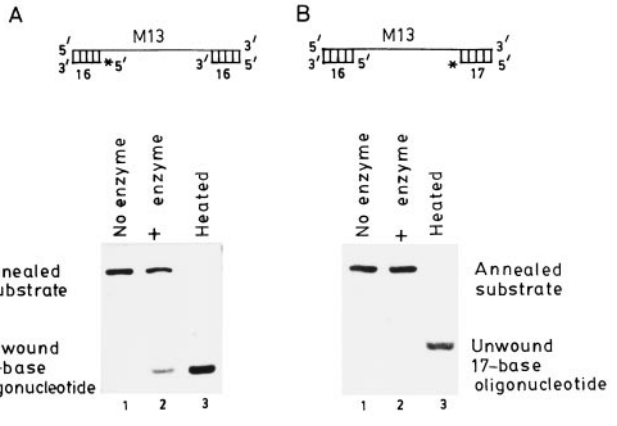

Figure 7. Direction of unwinding by pea ctDNA helicase II. The structure of the linear substrate for the 3′ to 5′ direction (A) and 5′ to 3′ direction (B) is shown on top of the autoradiogram. In each gel, lane 1 is the reaction without enzyme; lane 2 is the reaction with 8.5 ng of fraction VI; and lane 3 is the heat-denatured substrate. Asterisks denote ^{32}P-labeled ends.

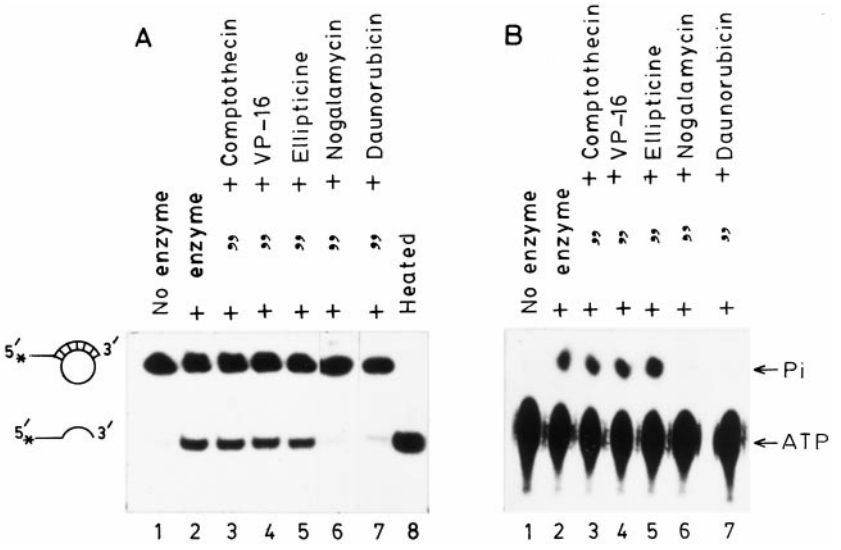

Figure 8. Effect of DNA-interacting ligands on DNA unwinding (A) and ATPase (B) activities of pea ctDNA helicase II. The standard helicase reaction was performed with 5 ng of fraction VI, 1 ng of the 5′-tailed substrate, and 50 μM of the compound. The name of each compound is located on top of each autoradiogram.

unwound (Fig. 5F), the pea ctDNA helicase II must translocate in the 3′ to 5′ direction, as is illustrated below.

Direction of Unwinding by Pea ctDNA Helicase II

The direction of unwinding by helicase is defined by the strand to which the enzyme binds and moves. The results in Figure 6, F and G, show that the enzyme moves in the 3′ to 5′ direction (Fig. 6F) and not in the 5′ to 3′ direction (Fig. 6G). This finding was further confirmed by constructing two other direction-specific substrates. The construction of the substrates was described earlier (Tuteja et al., 1996), and these structures are shown in Figure 7. These substrates consisted of a longer, linear M13 ssDNA with short stretches of duplex DNA at both ends (Fig. 7, A and B). The results show that the pea ctDNA helicase moved unidirectionally from 3′ to 5′ along the DNA strand to which it bound (Fig. 7A). The enzyme did not show unwinding in the 5′ to 3′ direction (Fig. 7B).

Influence of DNA-Interacting Ligands on DNA Unwinding and ATPase Activities of the Enzyme

A set of five different DNA-interacting ligands, including both nonintercalative (camptothecin, VP-16) and intercalative (ellipticine, daunorubicin, and nogalamycin), were included separately in the standard helicase and ATPase reactions to determine their effect on these enzyme activities. The chemical structures of the ligands used are described in Tuteja et al. (1997). Initially, each ligand was used at a final concentration of 50 μM. The results are shown in Figure 8. Camptothecin, VP-16, and ellipticine did not show any effect on helicase (Fig. 8A) or DNA-dependent ATPase (Fig. 8B) activities of the enzyme. However, anthracycline antibiotics, daunorubicin, and nogalamycin were inhibitory to both of the enzyme activities (Fig. 8, A and B).

The kinetics of inhibition of helicase and ATPase activities were also tested by using different concentrations of daunorubicin and nogalamycin, and the results are shown in Figures 9 and 10. The apparent K_i values for inhibition of both the unwinding and ATPase activities of pea ctDNA helicase II by daunorubicin were 2.2 μM (Fig. 9A) and 3.0 μM (Fig. 10A), respectively. However, the apparent K_i values for nogalamycin as an inhibitor of the unwinding and ATPase activities of the enzyme were 0.85 μM (Fig. 9B) and 1.3 μM (Fig. 10B), respectively.

DISCUSSION

Green plant cells contain three separate genomes (nuclear, mitochondrial, and chloroplast), which replicate, transcribe, and express their genetic information independently of each other (Tuteja, 1997). At least one helicase activity is required for the unwinding of duplex DNA ahead of an advancing fork, which is an absolute requirement for DNA replication (Kornberg and Baker, 1991). A reasonable attribution of functions has been obtained for many of the *E. coli*, yeast, and viral DNA helicases. On the contrary, very little is known about the plant DNA helicases and the in vivo role of most of the eukaryotic DNA helicases. In this study we have described the purification and properties of a DNA helicase II from pea chloroplast, which requires fork structures for maximum unwinding activity and is inhibited by major DNA groove-binding ligands.

ctDNA helicase II was fractionated on the basis of its behavior on a DEAE-cellulose column, to which it did not bind. In this property, as well as several others, it differs from our previously described DNA helicase I from the same source (Tuteja et al., 1996) as shown in Table III.

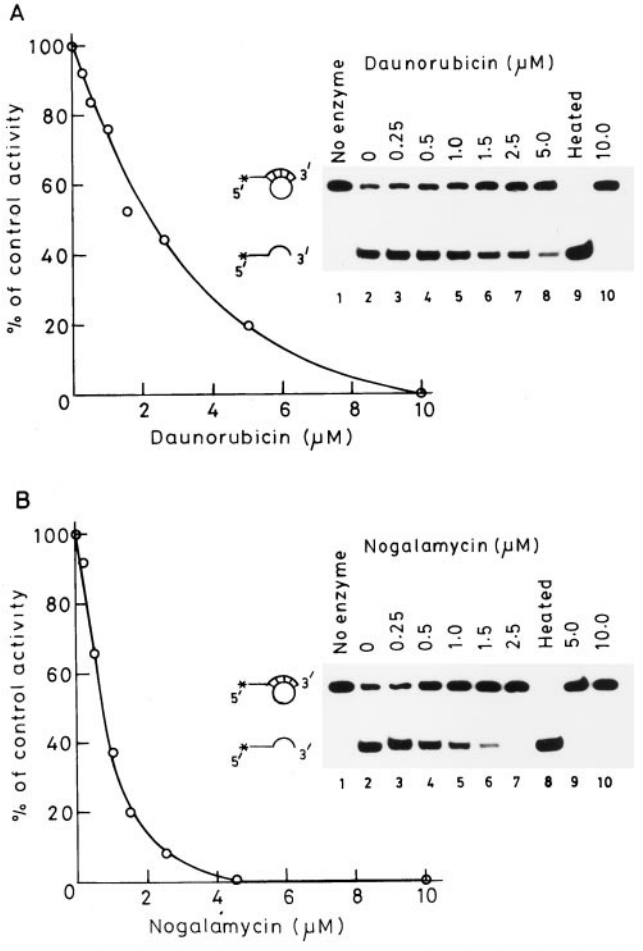

Figure 9. Titration of inhibition of unwinding activity of pea ctDNA helicase II by daunorubicin (A) and nogalamycin (B). The DNA helicase reactions were performed in the presence of increasing concentrations of the ligand using 1 ng of ^{32}P-labeled substrate and 5 ng of the pure enzyme. The quantitative curve is shown on the left side of each autoradiogram. Various concentrations of each ligand used are located at the top of each lane.

However, similar activity was observed by Cannon and Heinhorst (1990) in soybean chloroplasts, which did not adsorb to DEAE cellulose, but the enzyme was not further studied.

The pea ctDNA helicase II translocates in the 3′ to 5′ direction along the bound strand in a manner similar to that of the previously described ctDNA helicase I (Tuteja et al., 1996), human DNA helicase I, II, III, V, VI, and α (Seo and Hurwitz, 1993; Tuteja and Tuteja, 1996), simian virus-40 large tumor antigen (Stahl and Knippers, 1987), and nDNA helicase I and II from calf thymus (Zhang and Grosse, 1991).

The most striking feature of the enzyme was its preference for fork-like structures of the substrate, unlike ctDNA helicase I (Tuteja et al., 1996). A 5′-tailed fork structure was more stimulatory than the 3′-tailed structure. Similar results were reported for human DNA helicase α (Seo and Hurwitz, 1993). The observation that the 3′-tailed DNA was a relatively poor substrate compared with the 5′- and 5′-and 3′-tailed substrate suggested that the pea ctDNA helicase II translocated in the 3′ to 5′ direction, since the displacement efficiency probably depends on the availability of ssDNA for binding and subsequent translocation. The results reported in Figure 6, F and G, indicate that helicase II needs more than 15 and less than 84 nucleotides of the free-loading zone of ssDNA for binding and moving along it. The increased activity with the 3′-tailed substrate (Fig. 6B) compared with the nontailed substrate (Fig. 6A) indicates that the enzyme needs a fork-like situation for effective binding. The failure to unwind the 41 bp (Fig. 6E), even though it contains both the tails, was due to the longer length of the base-paired region. It probably needs some additional supporting protein(s) to unwind longer du-

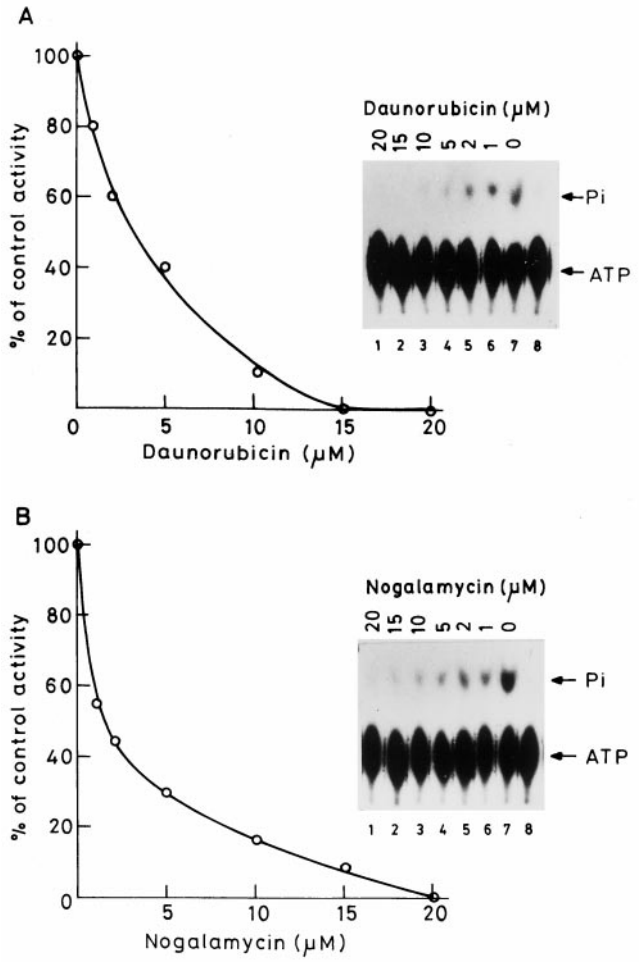

Figure 10. Titration of inhibition of DNA-dependent ATPase activity of pea ctDNA helicase II by daunorubicin (A) and nogalamycin (B). The standard ATPase reactions were performed in the presence of increasing concentrations of the ligand using 5 ng of the pure enzyme. The quantitative curve is shown on left side of each autoradiogram of the TLC plate. The positions of the Pi and ATP spots are indicated by arrows. Various concentrations of each ligand used are indicated at the top of each lane.

Table III. *Poperties of pea ctDNA helicase I and II*

Property	DNA Helicase I	DNA Helicase II
Apparent abundance[a] (μg/kg of leaves)	3.73	0.77
Size (kD, SDS-PAGE)	68	78
Size (kD, Native)	136	nd[b]
Behavior on column chromatography		
DE-52 cellulose	Eluted at 0.2–0.3 M salt	Eluted at 0.05 M salt (flow through)
Heparin Sepharose	Eluted at 0.4 M salt	Eluted at 0.5 M salt
dsDNA-cellulose	Eluted at 0.6 M salt	Eluted at 0.5 M salt
ssDNA-cellulose	Eluted at 0.6 M salt	Eluted at 0.4 M salt
Optimum concentration		
ATP (mM)	4	2
$MgCl_2$ (mM)	1	1.5
KCl (mM)	25–250	100
Divalent cation requirements	$Mg^{2+} = Mn^{2+}$	$Mg^{2+} > Mn^{2+} > Ca^{2+}$
Nucleotide requirements	ATP > dATP, CTP, UTP, GTP > dCTP, dTTP, dGTP	ATP = dATP
Sensitivity to salt (200 mM KCl or NaCl)	Inhibition	No inhibition
Preference of fork-like structure of substrate	No	Yes
DNA-dependent ATPase activity (pmol ATP hydrolyzed/ng 30 min^{-1})	35	80

[a] Values are calculated from the final recovery of pure protein. [b] nd, Not determined.

plexes. The enzyme could not unwind the blunt-ended DNA duplex because of the lack of a free ssDNA-loading zone. Chloroplast helicase II was inhibited by dsDNA and RNA, which shows that this enzyme has the affinity to bind them, as was also reported for human DNA helicase IV (Tuteja et al., 1991) and ctDNA helicase I (Tuteja et al., 1996).

The DNA-unwinding activity and the intrinsic ATPase activity of the ctDNA helicase II can be inhibited by DNA-interacting ligands that bind DNA. In *E. coli* and human helicases, this inhibition has been shown to be highly specific with respect to the ligand used (George et al., 1992; Tuteja et al., 1997). The topoisomerase inhibitor etoposide (VP-16) and the cytotoxic alkaloid camptothecin, which are nonintercalating ligands and do not bind DNA directly, showed no inhibition of the helicase or ATPase activities of ctDNA helicase II. Camptothecin and VP-16 were also reported to not inhibit *E. coli* (George et al., 1992) or human DNA helicase II (Tuteja et al., 1997). The ellipticine was reported to inhibit *E. coli* DNA helicase II (George et al., 1992) and yeast topoisomerase II by directly interacting with the protein (Froelich-Ammon et al., 1995).

The two most potent inhibitors of unwinding and ATPase activities were daunorubicin and nogalamycin, similar to *E. coli* DNA helicases (George et al., 1992), human DNA helicase II (Tuteja et al., 1997), and partially purified DNA helicase from HeLa cells (Bachur et al., 1992). Recently, a ctDNA helicase I has also been shown to be inhibited by daunorubicin and nogalamycin (Tuteja and Phan, 1998). These ligands probably inhibited the helicase reaction by intercalating into the major groove of the DNA. This presumably provides a physical block to continued translocation by the ctDNA helicase II, causing the unwinding reaction to be inhibited, as was also suggested by George et al. (1992). Since these ligands were also inhibitory to the ATPase reaction, we can conclude that ATPase requires translocation on the DNA.

The possibility that the helicase reaction could also be inhibited by direct binding of these ligands to the ctDNA helicase II protein was ruled out by preincubating the helicase with inhibitory concentrations of daunorubicin and nogalamycin prior to dilution in an unwinding reaction. Under these conditions the unwinding activity was not inhibited (data not shown). This further confirmed that the inhibition was due to the formation of a ligand-DNA complex that impeded the translocation of the protein. Overall, the data suggest the possibility that inhibitory intercalators must place a functionality in the major groove when bound to DNA. The exact mechanism of DNA unwinding is not yet defined. These results may be important for understanding both the mechanism by which the duplex DNA is unwound by a helicase and also the mechanism by which these ligands inhibit cellular function.

The biological roles of only a few eukaryotic helicases have been determined. Recently, a gene encoding a putative helicase was reported in Arabidopsis by activation of a promoter trap (Wei et al., 1997). In plants the *MCM* proteins/gene products from Arabidopsis (Springer et al., 1995), maize (Sabelli et al., 1996), and *Dactylis glomexata* (Ivanova et al., 1994) were also shown to contain helicase motifs. The MCM proteins, first discovered in minichromosome maintenance mutants of yeast, were reported to be involved in activating the origins of replication. If these MCM proteins from plants are shown to be ATP-dependent helicases by virtue of helicase activity (which has not been shown yet), this will prove a role for a helicase in DNA replication. However, the simian virus-40 large tumor antigen helicase has been shown to play a role in DNA replication (Stahl and Knippers, 1987; Goetz et al., 1988). The E1 protein, a DNA helicase from bovine papilloma virus, is also involved in DNA replication (Seo et al., 1993). Recently, the Werner's syndrome gene product was shown to contain DNA helicase activity (Suzuki et al., 1997). Werner's syndrome is a rare, autosomal recessive genetic disorder causing premature aging accompanied by rare cancer. Recently, the crystal structures of complexes of *E. coli* Rep helicase bound to ssDNA and ADP (Korolev et al., 1997) and a C-terminal fragment of the bacteriophage T7 gene 4 helicase (Bird et al., 1997) have been determined.

The replication of ctDNA was studied by analyzing the structure of replicative intermediates in the electron microscope. In pea, ctDNA replication was initiated by introducing two displacement loops (OriA and OriB D-loops), which expand toward each other and initiate the formation of Cairns replicative forked structures (Tuteja, 1997; Tuteja

and Tewari, 1998). Since ctDNA helicase II prefers thefork structures, it seems likely that the enzyme unwinds the ctDNA through Cairns replicative fork and is thereby involved in ctDNA replication. However, the possibility of its role in other DNA transactions cannot be ruled out.

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