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Purification and characterization of a DNA helicase from pea chloroplast that translocates in the 3′-to-5′ direction

Narendra TUTEJA[1], Tuan-Nghia PHAN[1] and Krishna K. TEWARI[1,2]

[1] International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi, India
[2] Department of Molecular Biology and Biochemistry, University of California, Irvine, USA

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An ATP-dependent DNA helicase has been purified to near homogeneity from pea chloroplasts. The enzyme is a homodimer of 68-kDa subunits. The purified enzyme shows DNA-dependent ATPase activity and is devoid of DNA polymerase, DNA topoisomerase, DNA ligase or nuclease activities. The enzyme requires Mg^{2+} or Mn^{2+} for its maximum activity. ATP is the most favoured cofactor for this enzyme while other NTP or dNTP are poorly utilized. Pea chloroplast DNA helicase can unwind a 17-bp duplex whether it has unpaired single-stranded tails at both the 5′ end and 3′ end, at the 5′ end or at the 3′ end only, or at neither end. However, it fails to act on a blunt-ended 17-bp duplex DNA. The enzyme moves unidirectionally from 3′ to 5′ along the bound strand. The unwinding activity is inhibited by the intercalating drugs nogalamycin and daunorubicine.

Keywords: unwinding enzyme; DNA helicase; DNA-dependent adenosine triphosphatase; chloroplast; replication.

The mechanism of DNA replication has been well defined in plasmids, bacteriophages, bacteria, viruses and, to a lesser extent, in yeast [1]. Most studies of the replication of DNA in plants have investigated chloroplast DNA [2]. The replicative intermediates of chloroplast DNA molecules of 120–160 kbp from higher plants are well characterized and the origins of replication have been mapped for pea chloroplast DNA and *Chlamydomonas* chloroplast DNA [3–6]. Of the enzymes involved in DNA replication, only DNA polymerase [7] and DNA topoisomerase [8, 9] have been purified from chloroplasts. The presence of DNA primase has also been reported in pea chloroplasts [10]. DNA helicases are an important class of enzymes that are involved in replication, recombination and repair of DNA [1, 11, 12]. These enzymes unwind duplex DNA and provide single-stranded DNA template. Many DNA helicases have been isolated from bacteriophage, bacterial, viral and eukaryotic systems [11, 12]. Multiple DNA helicases are present in a cell because of the different structural requirements of the substrates at various stages of repair, replication and recombination. In *Escherichia coli* functions for most of the DNA helicases have been attributed [11]. Six DNA helicases have been isolated from human cells by Tuteja et al. [13–18]. In plants, there are two reports of DNA helicases, one in lily [19] and the other in soybean [20]. In both of these studies, the enzymes were not purified to homogeneity or extensively characterized.

We now report the purification and detailed characterization of a DNA helicase from pea chloroplast, which is a homodimer of 68-kDa subunits and requires divalent cations for activity.

Correspondence to N. Tuteja, Plant Molecular Biology, ICGEB, Aruna Asaf Ali Marg, New Delhi, India 110 067

Abbreviations. ATP[S], adenosine 5′-[γ-thio]-triphosphate; SV, simian virus.

Enzymes. DNA-dependent ATPase (EC 3.6.1.–); DNA-directed DNA polymerase (EC 2.7.7.7); DNA topoisomerase (EC 5.99.1.2); DNA ligase (ATP) (EC 6.5.1.1); polynucleotide 5′-hydroxyl-kinase (EC 2.7.1.78).

EXPERIMENTAL PROCEDURES

DNA oligodeoxyribonucleotides and nucleoside triphosphates. M13 ss DNA, M13 replicative form 1 DNA and total RNA from pea leaves were prepared as described [21]. NTP and adenosine 5′-[γ-thio]-triphosphate (ATP[S]) were obtained from Boehringer Mannheim. [γ-^{32}P]ATP (185 TBq/mmol), [α-^{32}P]dCTP (≈110 TBq/mmol) and [α-^{32}P]ATP (>15 TBq/mmol) were purchased from Amersham.

The oligodeoxyribonucleotides used to construct the DNA substrates were synthesized chemically by the International Centre for Genetic Engineering and Biotechnology nucleotide service and were purified electrophoretically. The sequences and details of the oligonucleotides were as follows: oligo 1, 47-nucleotides, 5′-T_{15}GTTTTCCCAGTCACGACT_{15}-3′, contains a sequence (nucleotides) 16–32 complementary to M13mp19 (+strand) DNA (Fig. 7A); oligo 2, 32 nucleotides, 5′-T_{15}GTTTTCCCAGTCACGAC-3′ (Fig. 7B); oligo 3, 32 nucleotides, 5′-GTTTTCCCAGTCACGACT_{15}-3′ (Fig. 7C); oligo 4, 17 nucleotides, 5′-GTTTTCCCAGTCACGAC-3′ (Fig. 7D); oligo 5, 32 nucleotides, 5′-TTCGAGCTCGGTACCCGGGGATCCTCTAGAGT-3′, complementary to M13mp19 (+strand) DNA and contains a *Sma*I site (Figs 7E and 8A, B); oligo 6, 17 nucleotides, 5′-GTCGTGACTGGGAAAAC-3′, complementary to oligo 4 (Fig. 7F).

Preparation of DNA helicase substrates. The substrate consists of partial DNA duplex of ^{32}P-labeled oligonucleotide annealed to M13mp19 ss DNA. Substrates were prepared as described previously [13, 15, 16]. 10 ng oligonucleotide was labeled at the 5′ end with T_4 polynucleotide kinase (5 U) and 0.925 MBq [γ-^{32}P]ATP (185 TBq/mmol) in 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine and 0.1 mM EDTA at 37 °C for 60 min and incubated for 2 min at 95 °C. The labeled oligonucleotide was annealed to M13mp19 ss DNA (2–4 μg) in 40 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM dithiothreitol. The mixture was heated

Table 1. Purification of chloroplast DNA helicase from pea leaves. The detailed purification procedure is described in Results. n.d., not determined due to the presence of nucleases.

Step	Volume	Protein	DNA helicase activity	
			total	specific
	ml	mg	U	U/mg
Chloroplast lysate (after dialysis)	150	1703	n.d.	n.d.
DE-52 cellulose	226	99	n.d.	n.d.
Heparin-Sepharose	56	3.94	n.d.	n.d.
ds-DNA–cellulose	11	0.22	4290	19 502
ss-DNA–cellulose	1.4	0.0028	933	333 214

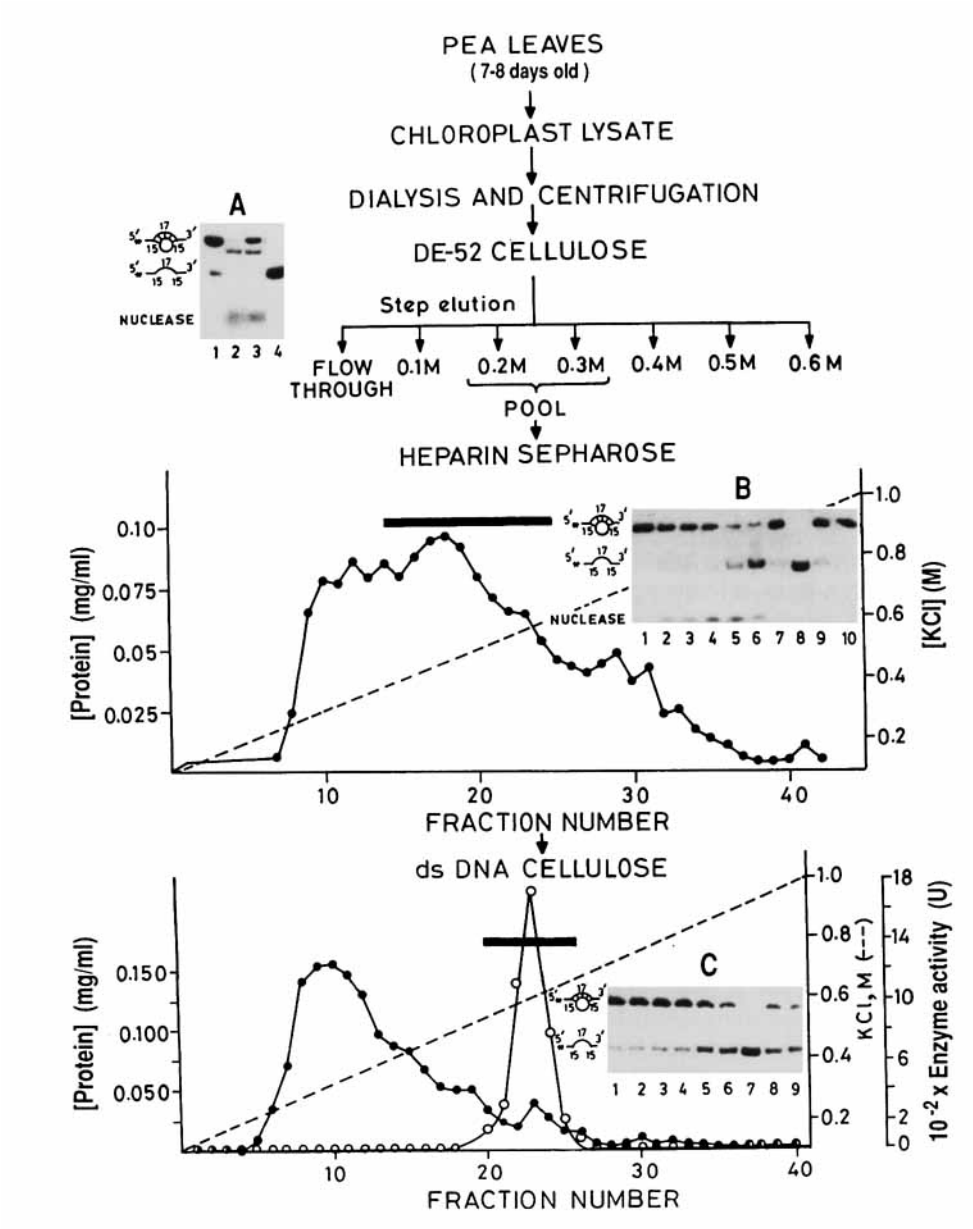

Fig. 1. Purification scheme and elution profile of pea chloroplast DNA helicase. The strategy used for fractionation of DNA helicase from pea chloroplast is shown. The dotted lines indicate the KCl gradients. The detailed description of the chromatographic procedures is given in the text. Pooled fractions are indicated by horizontal bars. (A) Autoradiogram of a gel that shows the DNA helicase activity of chloroplast lysate (lane 2) and of the pooled 0.2-M and 0.3-M KCl fractions from the DE-52 cellulose column (lane 3). Lanes 1 and 4 represent reactions without enzyme and with heat-denatured substrate, respectively. The structure of the substrate used is shown on the left of the gel. The smears at the bottom of the gel are due to nuclease action on the DNA substrate. The band of slower mobility in lanes 2 and 3 indicates that the preparation contained DNA-binding proteins which bound to the unwound DNA. (B) DNA helicase activity of the active fractions from the heparin-Sepharose column. Lanes 2–7, 9 and 10 show the activity of the fractions 14, 16, 18, 20, 22, 24, 26 and 28, respectively. Lanes 1 and 8 represent reactions without enzyme and with heat-denatured substrate, respectively. (C) DNA helicase activity of active fractions from the ds-DNA–cellulose column. Lanes 2–6, 8, 9, fractions 19–25, respectively. Lanes 1 and 7 show reactions without enzyme and with heat-denatured substrate.

at 95°C for 2 min, then allowed to anneal at 65°C for 20 min and cooled slowly to room temperature (3–4 h). The substrate was purified by gel filtration through a 2-ml Sepharose 4B column. The blunt-ended duplex DNA (Fig. 7F) was prepared by annealing 10 ng ^{32}P-labeled oligo 4 to 10 ng complementary oligo 7, as described above.

The direction-specific substrates were prepared as shown in Fig. 8A, B. For 3′-to-5′-direction study, oligo 5 was labeled at the 5′ end, then annealed to M13mp19 ss DNA. The annealed substrate was digested with *Sma*I and purified by gel filtration. For 5′-to-3′-direction-unwinding substrate, oligo 5 was annealed to M13mp19 ss DNA, then labeled at the 3′end as described [13].

Enzyme assays. The helicase assay measures the displacement of a labeled oligonucleotide from a partial duplex molecule. The assay mixture (10 µl) contained 20 mM Tris/HCl, pH 8.0, 1 mM $MgCl_2$, 4 mM ATP, 150 mM KCl, 8 mM dithiothreitol, 4% (mass/vol.) sucrose, 80 µg/ml BSA, 1 ng ^{32}P-labeled helicase substrate (≈1000 cpm) and the enzyme fraction. Incubation was performed at 37°C for 30 min, unless otherwise stated. The reaction was terminated by addition of 1.5 µl 75 mM EDTA, 2.25% SDS, 37.5% (by vol.) glycerol and 0.3% bromophenol blue, and the products were separated by 12% native polyacrylamide gel (8 cm×10 cm) electrophoresis in 89 mM Tris/borate, pH 8.2, 2 mM EDTA. The gel was dried and exposed to film for autoradiography. DNA unwinding was quantitated as described [13]. 1 U DNA helicase activity is defined as the amount of enzyme that unwinds 30% of the DNA helicase substrate at 37°C in 30 min.

The ss-DNA–dependent ATPase activity was assayed under the same conditions as the helicase as described by Tuteja et al. [15]. DNA topoisomerases were assayed according to Kaiserman et al. [22], except that the plasmid DNA used was pBluescript that contained a cDNA insert of 1kb of human cGMP phosphodiesterase [23]. DNA ligase activity was assayed as described previously [13]. DNA polymerase was assayed with activated calf thymus DNA [24].

Other methods. The glycerol-gradient [from 15% to 40% in 50 mM Tris/HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium bisulfite, 1 µM pepstatin, 20% (by vol.) glycerol (buffer A) containing 0.5 M KCl] centrifugation was performed with 200 U fraction V in the presence of standard protein markers for 21 h at 4°C at 48000 rpm in a SW60 rotor. Fractions of 0.2 ml were collected from the bottom of the tube and assayed for DNA helicase activity. For gel-filtration chromatography, 50 µl concentrated fraction IV (200 U) was applied to a calibrated column (24 mm×4 mm) of Sephadex G-150. The column was developed at 4°C with buffer A containing 0.5 M KCl. Fractions of 0.05 ml were collected and assayed for helicase activity. Protein concentration was determined by means of the Bradford-protein-assay kit of Bio-Rad. SDS/PAGE was performed by the method of Laemmli [25], and gels were silver stained with the Bio-Rad kit.

RESULTS

Purification of chloroplast DNA helicase. The DNA substrate used to measure the unwinding activity during purification and most of the characterization of the helicase consisted of a ^{32}P-labeled 47-base oligonucleotide annealed through its central 17 nucleotides (positions 16–32) to M13mp19 ss DNA. This duplex contained 5′ and 3′ overhanging tails. The results of the purification of DNA helicase are summarized in Table 1, and a flow diagram of the purification scheme and elution profiles of the chromatographic steps are shown in Fig. 1. All purification steps were carried out at 4°C.

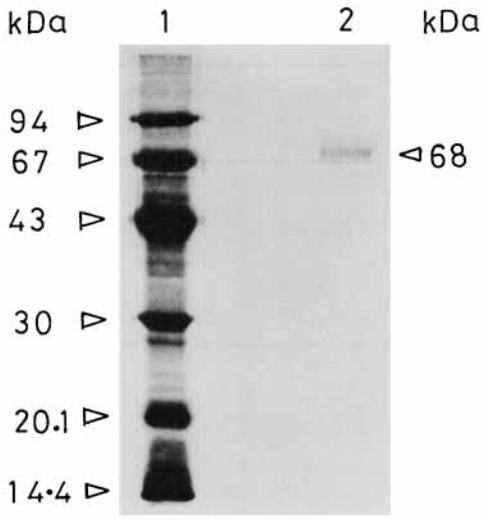

Fig. 2. SDS/PAGE of purified DNA helicase from pea chloroplast. Purified enzyme (100 ng, lane 2) and molecular-mass markers (lane 1) were separated on a 12% gel according to Laemmli and visualized by silver staining.

Preparation of chloroplast lysate. Approximately 750 g leaves from 7–8-d-old pea plants were homogenized in 2 l 0.5 M sucrose, 50 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.3 mM phenylmethylsulfonyl fluoride, 1 mM sodium bisulfite and 1 mg/ml benzamidine (buffer B). The homogenate was filtered through two layers of cheese cloth and two layers of Mira cloth. The filtrate was centrifuged for 10 min at 1000 *g* and the pellet suspended in 120 ml buffer B. To disrupt the chloroplast, 30 ml 12.5% Triton X-100 was added to the chloroplast suspension. The mixture was kept on ice for 45 min and stirred occasionally, then centrifuged at 3000 *g* for 15 min. The supernatant (chloroplast lysate) was collected and dialyzed for 8–10 h against buffer A. After dialysis, precipitated material was removed by centrifugation at 3000 *g* for 15 min, and the supernatant was used for further purification.

DE-52 cellulose chromatography. The dialyzed chloroplast lysate was loaded onto a 70-ml DE-52 cellulose column equilibrated with buffer A. The column was washed with at least 420 ml buffer A. The bound proteins were eluted in successive steps with 140 ml buffer A containing 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M or 0.6 M KCl (Fig. 1). Fractions eluted with 0.2 M or 0.3 M KCl contained helicase activity and DNA-binding activity which resulted in a reduction of the mobility of the unwound DNA (Fig. 1A). These fractions contained strong nuclease activity that interfered in the helicase assay. The fractions that contained the highest helicase activities were pooled.

Heparin-Sepharose chromatography. The pooled fractions from DE-52 cellulose were diluted to 556 ml with buffer A to 0.1 M KCl. This fraction was loaded onto a 17-ml heparin-Sepharose column equilibrated with buffer A containing 0.1 M KCl. The column was washed with 85 ml buffer A containing 0.1 M KCl and the bound proteins were eluted with a 180-ml linear gradient from 0.1 M to 1 M KCl in buffer A. The enzyme activity eluted from the column at approximately 0.4 M KCl (fractions 14–26; Fig. 1B). There was still some nuclease activity in these fractions and as such the helicase activity could not be quantitated precisely up to this step. The DNA-binding activ-

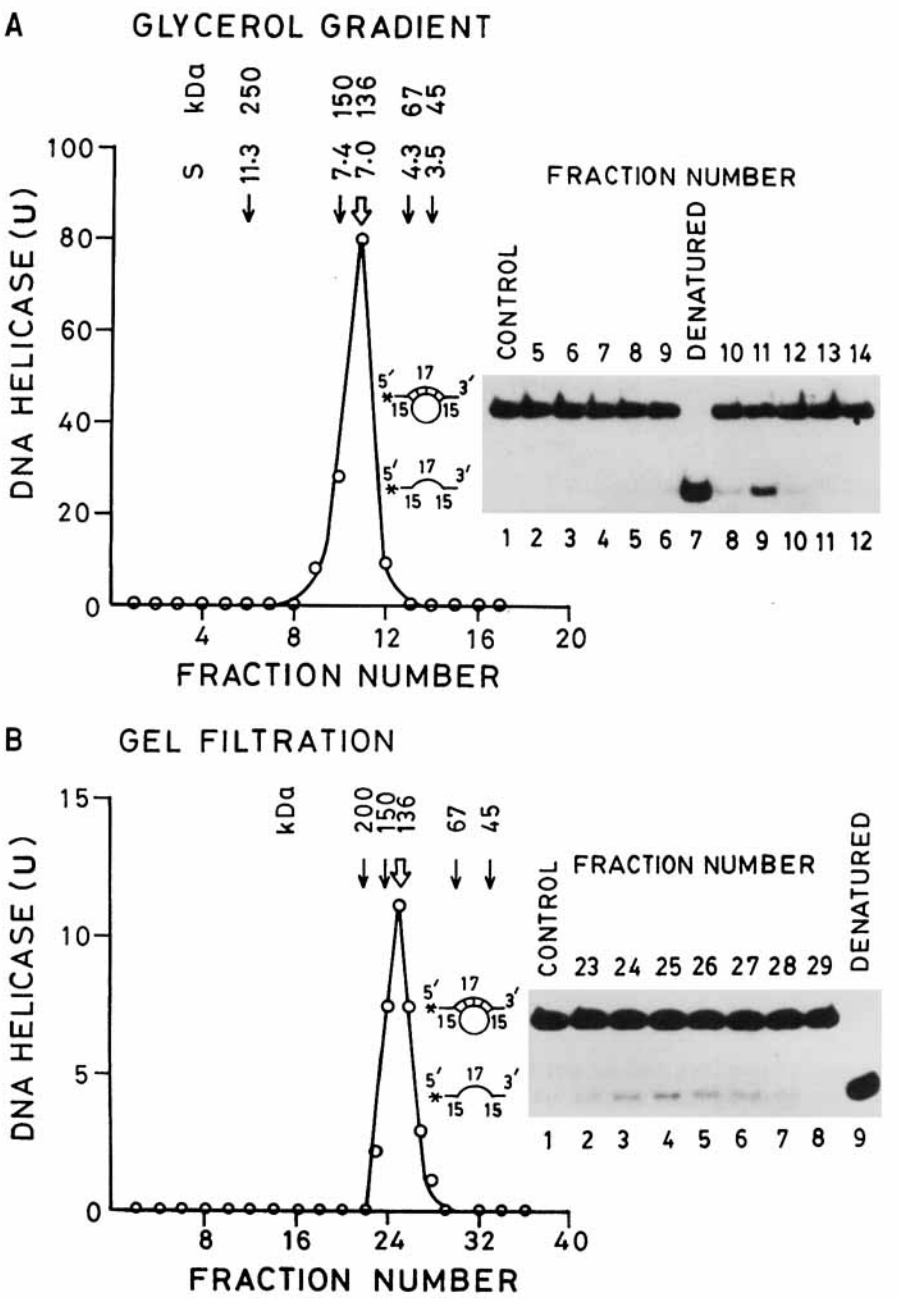

Fig. 3. Glycerol-gradient centrifugation and gel-filtration chromatography. (A) Glycerol-gradient (from 15% to 40%) centrifugation of 100 µl concentrated purified DNA helicase (200 U) at 48000 rpm for 21 h at 4°C in an SW 60 rotor. Fractions were collected from the bottom of the tube. The distribution of helicase activity and the position of sedimentation-coefficient and molecular-mass markers are shown. An autoradiogram of the gel of some fractions is shown on the right. Lanes 1 and 7 show controls without enzyme and with heat-denatured substrate, respectively. The markers were catalase (250 kDa, 11.3 S), alcohol dehydrogenase (150 kDa, 7.4 S), BSA (67 kDa, 4.4 S) and ovalbumin (45 kDa, 3.5 S). (B) Gel-filtration chromatography of 50 µl concentrated DNA helicase from the ds-DNA–cellulose column (200 U) on a Sephadex G-150 column (24 mm×4 mm). Molecular-mass standards were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (67 kDa) and ovalbumin (45 kDa). An autoradiogram of the gel is shown on the right. Lanes 1 and 9 show controls without enzyme and with heat-denatured substrate, respectively. The structure of the substrate used is shown on the left of the gel. The low level of activity was due to loss of activity during the column fractionation and dilution factor, since the pea chloroplast DNA helicase is very labile.

ity and some helicase activity was also detected in the fractions that did not bind to the column but these fractions were not analyzed in this study.

ds-DNA-cellulose chromatography. The pooled fractions from the heparin-Sepharose column were dialyzed against buffer A containing 0.1 M KCl, 1 mM ATP and 1 mM $MgCl_2$ (buffer C) and loaded onto a 4.5-ml ds-DNA–cellulose column equilibrated in buffer C. The column was washed with 22.5 ml buffer C and the bound proteins were eluted with a 40-ml linear gradient of KCl from 0.1 M to 1 M in buffer C. The DNA helicase activity eluted from the column with 0.6 M KCl. These fractions did not contain nuclease activity (Fig. 1 C).

ss-DNA–cellulose chromatography. The pooled fractions from the ds-DNA–cellulose column were dialyzed against buffer B and applied to a 1.5-ml ss-DNA–cellulose column equilibrated with buffer C containing 0.2 M KCl. After washing the column

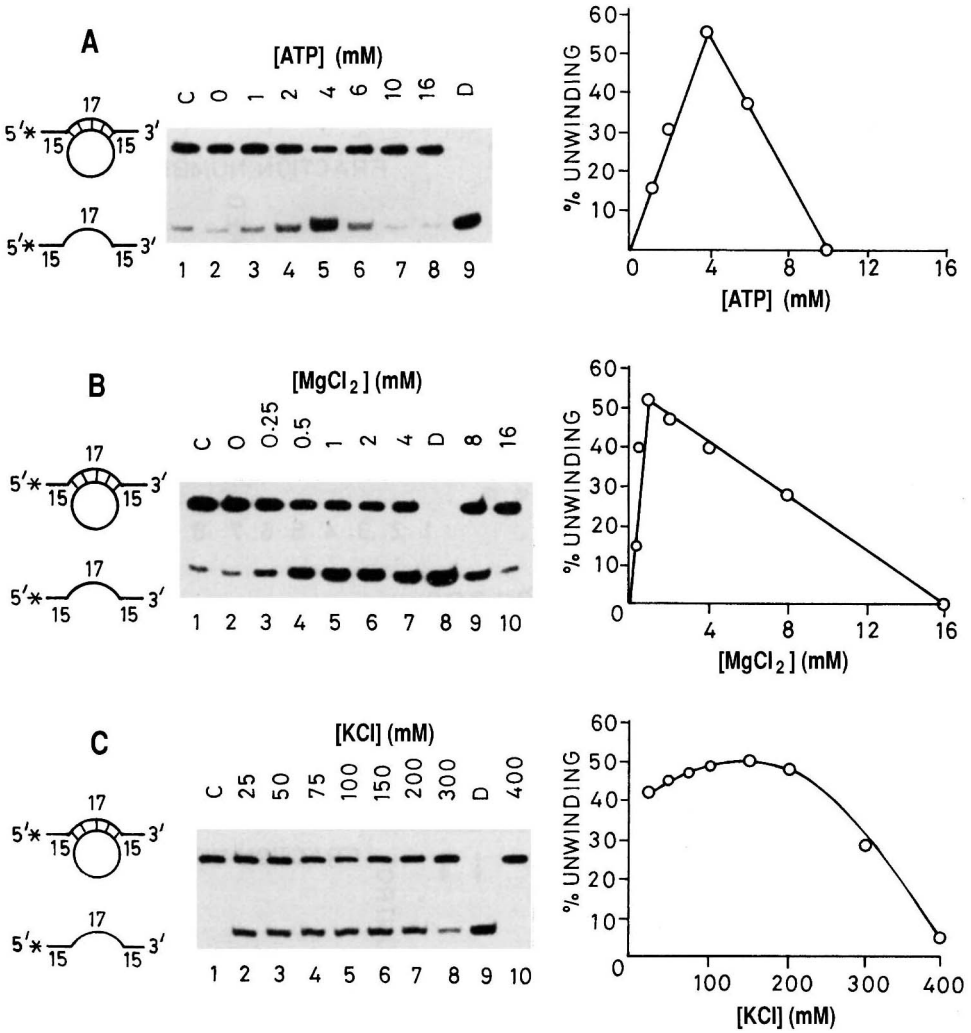

Fig. 4. Effect of ATP, $MgCl_2$ and KCl on DNA helicase activity. The structure of the substrate used is shown on the left of each autoradiogram and the quantitative data are displayed on the right. Asterisks denote the ^{32}P-labeled end. The helicase assay was carried out in varying concentrations of ATP (A), $MgCl_2$ (B) and KCl (C). The concentrations used are shown above each lane of the respective autoradiograms. Lane C, reaction without enzyme; lane D, reaction with heat-denatured substrate.

with 15 ml buffer C containing 0.2 M KCl the bound proteins were eluted in steps with 0.4 M, 0.6 M, 0.8 M and 1.0 M KCl in buffer C. The helicase activity was detected only in the 0.6 M KCl fraction.

Molecular mass and purity of the DNA helicase. The purified DNA helicase was analyzed by SDS/PAGE with standard molecular-mass markers and visualized by silver staining (Fig. 2). A band of 68 kDa was observed and no other polypeptides were visible. The native molecular mass of the DNA helicase was determined from its hydrodynamic properties. A portion of the purified enzyme was mixed with markers (catalase, alcohol dehydrogenase, BSA and ovalbumin) and centrifuged on a glycerol gradient (from 15% to 40%) in buffer A in the presence of 0.5 M KCl. DNA helicase sedimented between alcohol dehydrogenase and BSA as a 136-kDa protein (Fig. 3A). The native molecular mass of pea chloroplast DNA helicase on gel filtration was also approximately 136 kDa (Fig. 3B). Thus the DNA helicase seems to be a 136-kDa dimer that consists of identical 68-kDa subunits. The purified DNA helicase contained no associated activities of DNA polymerase, DNA topoisomerase, DNA ligase or non-specific nucleases.

Reaction requirement and characterization. The reaction requirements of the enzyme are shown in Table 2. The enzyme was inactivated upon heating at 56°C for 1 min or after prolonged storage at 4°C. Trypsin destroyed the enzymatic activity. The enzyme was inhibited by EDTA (5 mM), ss DNA or ds DNA (10 μg/ml), RNA (10 μg/ml), potassium phosphate (100 mM) or ammonium sulfate (45 mM). The enzyme had a requirement for divalent cations; it did not show any activity in the absence of Mg^{2+}. The optimum concentration of $MgCl_2$ was 1 mM (Fig. 4B) and at 16 mM the unwinding activity was completely inhibited. In the presence of Mn^{2+} the enzyme had the same activity as in the presence of Mg^{2+}. Other divalent cations, such as Ca^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} or Cu^{2+}, were unable to support activity.

The enzyme was active over a broad range of concentrations of monovalent cations. The enzyme had similar activity in the range 25–250 mM KCl (Fig. 4C). At higher KCl concentrations (400 mM) the enzyme was inhibited.

The enzyme showed DNA-dependent ATPase activity. In the presence of 3 ng pure enzyme, 105 pmol ATP was hydrolyzed at 37°C in 30 min under standard assay conditions. DNA helicase activity was totally dependent upon ATP with an optimum con-

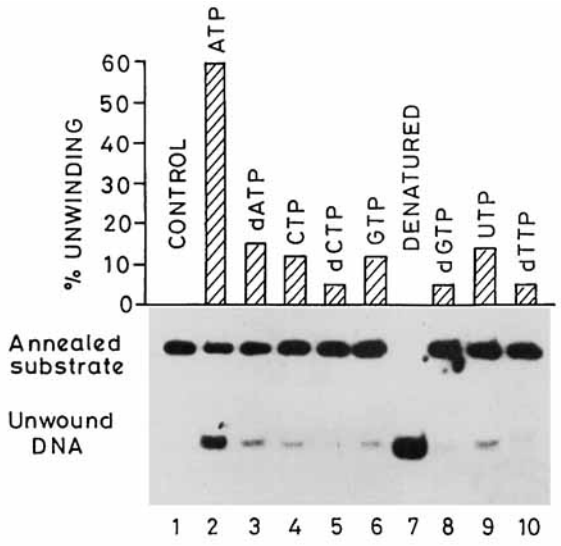

Fig. 5. Preference of nucleotide for DNA helicase activity. The strand-displacement assays were performed under standard conditions with 4 mM NTP or 4 mM dNTP. The amount of unwound DNA was quantitated and plotted as a histogram above the autoradiogram of the gel. Lanes 1 and 7, reactions without enzyme and with heat-denatured substrate, respectively. Lanes 2–6 and 8–10, reactions were performed in the presence of ATP, dATP, CTP, dCTP, GTP, dGTP, UTP and dTTP, respectively.

centration requirement of 4 mM (Fig. 4A). At higher ATP concentrations (10 mM) the enzyme was completely inhibited. Among the NTP or dNTP tested, ATP was the most active cofactor (Fig. 5). dATP supported approximately 25% of the activity while all other NTP or dNTP supported very little activity (Fig. 5). ADP, AMP and the poorly hydrolyzable ATP analog ATP[S] could not substitute for ATP to substitute enzyme activity (Table 2).

The kinetics of the helicase reaction under standard assay condition with 1.5 ng purified enzyme showed a linear rate up to 30 min (Fig. 6A) and deviated from linearity only with longer incubation. In the presence of 1 ng DNA substrate and increasing concentrations of enzyme, the activity was linear up to 60% unwinding with 3 ng protein (Fig. 6B).

DNA helicase activity with various substrates. The structures of the substrates used for assays are shown in Fig. 7. Approximately 1 ng substrate and 3 ng enzyme were used for each assay. The enzyme did not specifically require for its activity overhanging tails or replication-fork-like structures within the substrate. It could unwind a 17-bp duplex with overhanging tails at both the 5′ end and 3′ end (Fig. 7A) or at only one end, either 5′ (Fig. 7B) or 3′ (Fig. 7C). It could also unwind the tail-less substrate with the same efficiency (Fig. 7D). To determine whether the unwinding activity of the chloroplast DNA helicase is affected by the length of duplex region, a substrate with a longer duplex region (32 bp) was constructed. In the presence of this substrate there was a 12-fold reduction in helicase activity

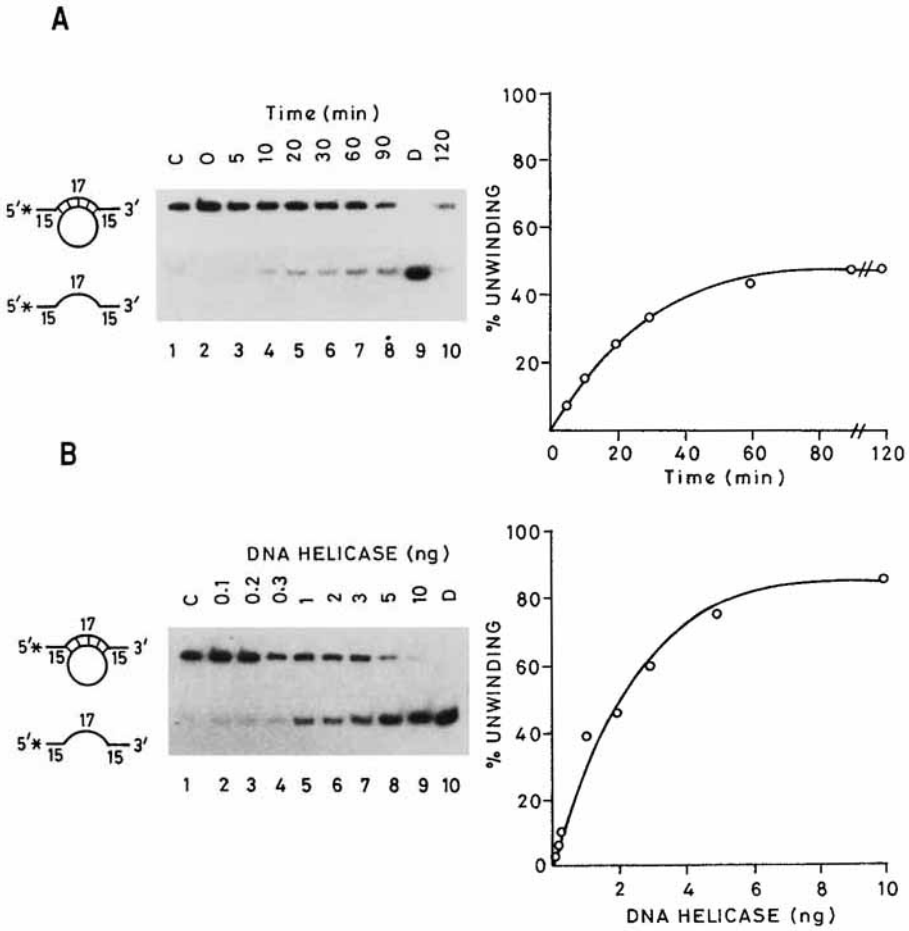

Fig. 6. Time dependence and concentration dependence of DNA helicase. The enzyme activity data from the autoradiograms (left) were quantitated and shown on the right. The structure of the substrate used is shown on extreme left. Asterisks denote the ^{32}P-labeled end. (A) The standard reaction was carried out with 1.5 ng purified DNA helicase at the times indicated. (B) The increasing amount of purified DNA helicase indicated on top of each lane was used in the standard helicase assay. Lanes C and D, reactions without enzyme and with heat-denatured substrate, respectively.

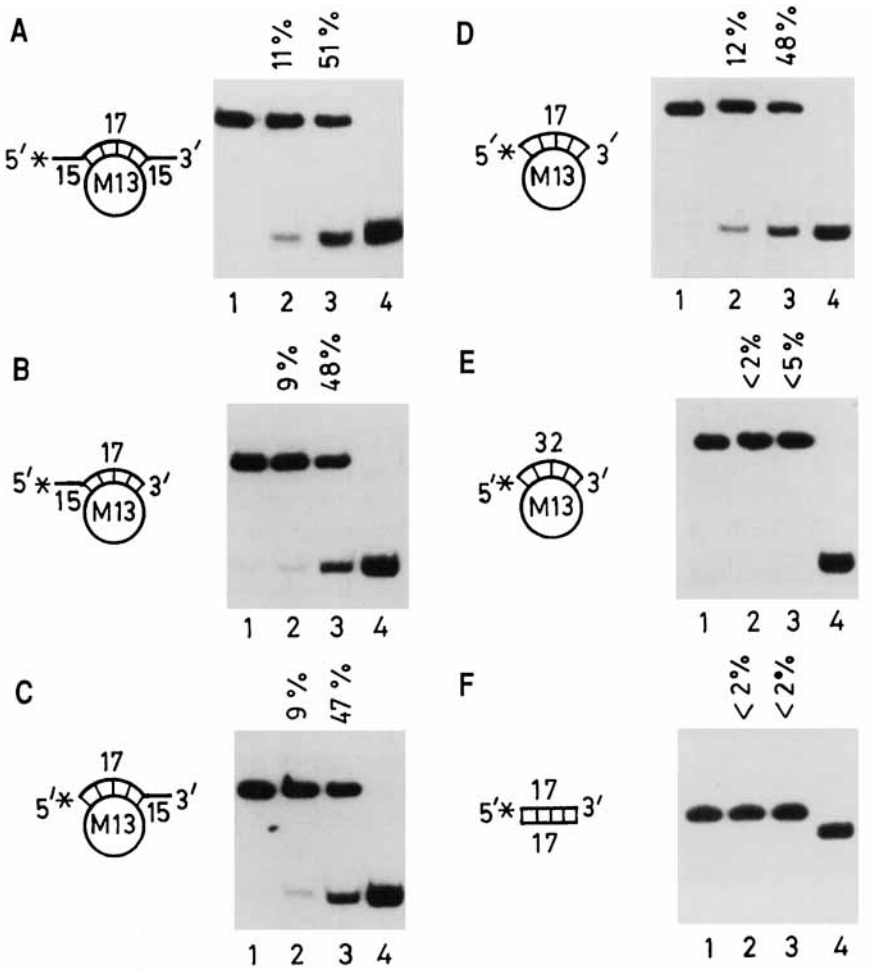

Fig. 7. Unwinding activity of DNA helicase with various substrates. The helicase reaction was performed under standard conditions. Each panel shows the structure of the substrate used and the autoradiogram of the gel. Asterisks denote the ^{32}P-labeled ends. Lanes 1 and 4, reactions without enzyme and with heat-denatured substrate, respectively. Lanes 2 and 3, reactions performed with 0.6 ng and 3.0 ng of purified DNA helicase, respectively. The extent of unwinding (%) is shown above lanes 2 and 3.

Table 2. Reaction requirements of purified chloroplast DNA helicase. The DNA helicase reaction was performed with 3 ng pure protein and 1 ng DNA substrate with overhanging tails (Fig. 7A) as described in Experimental Procedures.

Reaction conditions	Helicase activity
	% unwinding
Complete reaction	60
− enzyme	<2
+ heated enzyme (56 °C, 1 min)	<2
− ATP	<2
− ATP + ATP[S] (4 mM)	<2
− ATP + ADP (4 mM) or AMP (4 mM)	<2
− $MgCl_2$	<2
− $MgCl_2$ + $MnCl_2$ (1 mM)	62
− $MgCl_2$ + $CaCl_2$ (1 mM) or $ZnSO_4$ (1 mM) or $NiCl_2$ (mM) or $CoCl_2$ (1 mM) or $CuCl_2$ (1 mM)	<2
+ KCl (200 mM) or NaCl (200 mM)	58
+ EDTA (5 mM)	<2
+ M13 ss DNA (10 μg/ml)	<2
+ M13 RF1DNA (10 μg/ml)	<2
+ pea leaves total RNA (10 μg/ml)	<2
+ potassium phosphate (pH 8.0, 100 mM)	<2
+ ammonium sulfate (45 mM)	7
+ trypsin (1 U)	<2

(Fig. 7E). The enzyme failed to unwind the blunt-ended 17-bp duplex (Fig. 7F).

DNA helicase unwinds DNA unidirectionally from 3′ to 5′. For directional studies, two substrates with long linear ss DNA that had short stretches of duplex DNA at both ends were constructed. The construction and structure of the 3′-to-5′-direction and 5′-to-3′-direction specific substrates are shown in Fig. 8. The results showed that the DNA helicase moves unidirectionally from 3′ to 5′ along the DNA strand to which it binds (Fig. 8A). The enzyme cannot unwind in 5′-to-3′ direction (Fig. 8B).

Inhibition of DNA helicase by DNA-interacting ligands. When 10 μM nogalamycin or daunorubicin was included in a standard helicase reaction with 1 ng substrate and 3 ng purified protein, the intercalators completely inhibited the reaction (Fig. 9). Histone H1 (1 μg/ml) was also an inhibitor of the DNA helicase activity (Fig. 9). Aphidicolin (50 μM) had no effect on the unwinding activity of the enzyme (Fig. 9).

DISCUSSION

The DNA helicases, also called unwinding enzymes, have been isolated and characterized from a number of prokaryotes

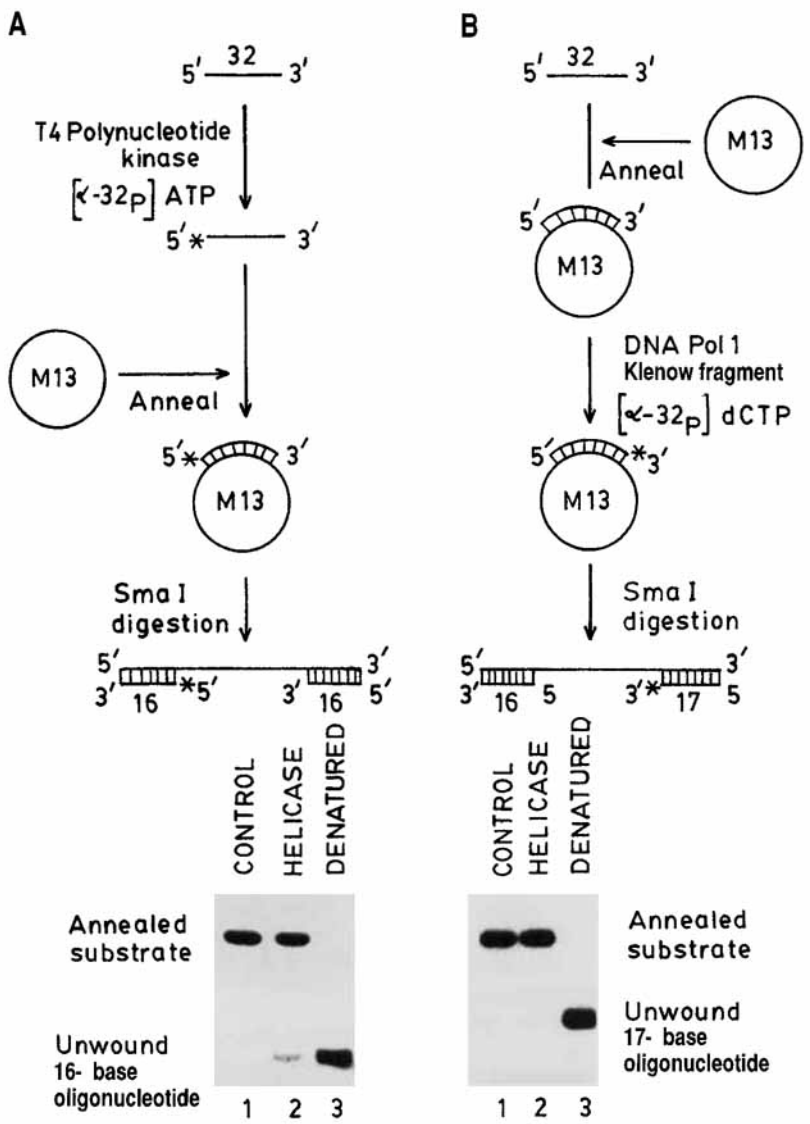

Fig. 8. Direction of unwinding by DNA helicase. Schematic representation of the construction of the linear substrates used to detect 3′-to-5′ (A) and 5′-to-3′ (B) unwinding. Asterisks denote ^{32}P-labeled ends. In each gel, lane 1, control; lane 2, in presence of purified DNA helicase; and lane 3, heat-denatured substrate.

and eukaryotes [11–12]. Very little, however, is known about DNA helicases in plants. The first reported enzyme from plants was the unwinding protein from meiotic cells of *Lilium* [19]. This unwinding protein had ATP-dependent DNA unwinding activity, bound to duplex DNA and could initiate unwinding from the ends of linear molecules or from nicks in linear and circular forms [19]. The specific direction of unwinding by DNA helicases is still unknown. The second report from plant was of a DNA helicase from chloroplast of *Glycine max* [20]. Neither of these helicases were well characterized or purified to near homogeneity. By means of a strand displacement-assay for measurement of the DNA helicase activity, we have purified a pea chloroplast DNA helicase to apparent homogeneity.

Chloroplast DNA helicase is an ATP-dependent unwinding enzyme and is a single polypeptide of 68 kDa on SDS/PAGE. Glycerol-gradient sedimentation and gel filtration suggest that the active enzyme has an apparent molecular mass of 136 kDa, which indicates that pea chloroplast DNA helicase consists of two, presumably identical, 68-kDa polypeptides. *E. coli* DNA helicase III has also been shown to be a homodimer [11]. The chloroplast DNA helicase also contains DNA-dependent ATPase activity but is free of DNA polymerase, DNA ligase, topoisomerase and nuclease activities. This enzyme is present in low abundance in plant; from about 750 g of pea leaves we recovered only 2.8 μg pure protein.

Tuteja and coworkers have identified and purified six DNA helicases from human cells [13–18]. Human DNA helicase II has been identified as the Ku autoantigen [14] and human DNA helicase IV has been identified as nucleolin [26]. Human DNA helicase I has a similar molecular mass to chloroplast DNA helicase (on SDS/PAGE) but antibodies against human helicases do not cross-react with chloroplast DNA helicase on Western blots (data not shown). The antibody against the 69-kDa topoisomerase from pea chloroplast [9] also did not cross-react with the chloroplast DNA helicase.

The chloroplast helicase from pea leaves does not require a replication-fork-like structure in the substrate for activity. The enzyme has similar activity whether the substrate contains tails at 3′ end or 5′ end or both, or no tails. These properties are similar to those of the previously reported chloroplast helicase from *Glycine max* [20], calf thymus DNA helicase F [27] human DNA helicases I, IV and V [13, 16, 18] and DNA-dependent ATPase B from mouse [28]. However, a hydroxyl group at the 3′ end of the DNA is required to effect binding with lily-unwinding

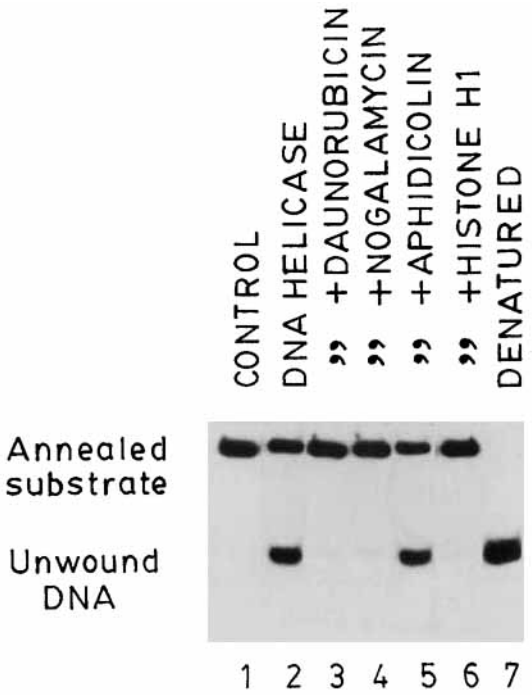

Fig. 9. Inhibition of DNA helicase activity by DNA-interacting ligands. The standard helicase assay was performed with 3 ng purified enzyme in the presence of 10 μM daunorubicine (lane 3), 10 μM nogalamycin (lane 4), 50 μM aphidicolin (lane 5), or 1 μg/ml histone H1 (lane 6). Lane 1, without enzyme; lane 2, with purified enzyme (3 ng); and lane 7, with heat-denatured substrate.

protein [19] and a replication-fork-like structure in the substrate is required for maximum activity of human DNA helicases II, III and VI [14–15, 18] and δ helicase from calf thymus [29].

The pea chloroplast DNA helicase moves in the 3′-to-5′ direction along the bound strand similar to the previously described human DNA helicases I, II, III, V and VI [13–15, 17, 18] and simian virus (SV)-40 T antigen [30]. Most prokaryotic DNA helicases that are involved in origin activation and growing-fork movement move in the 5′-to-3′ direction with respect to the strand to which they are bound.

The helicase reaction is an energy-requiring process, the energy being supplied by the hydrolysis of NTP or dNTP by the intrinsic ATPase activity of the helicases [1, 31]. The chloroplast helicase required ATP as a cofactor for optimal activity. However, all other NTP or dNTP could also be utilized but to a lesser extent, as reported for the human DNA helicase II [14] and mouse helicase [28]. Mg^{2+} is essential for the activity of chloroplast DNA helicase. Mn^{2+} can replace Mg^{2+} with no effect on the optimal activity, as reported for human DNA helicases I and IV [13, 16], mouse helicase [28] and SV-40 T-antigen [32]. In this respect it differs from chloroplast DNA helicase of *Glycine max* [20]. For the latter enzyme Mn^{2+} can replace Mg^{2+} only partially [20]. The optimum concentration of $MgCl_2$ was found to be 1 mM for pea chloroplast helicase, while for chloroplast helicase of *Glycine max* the optimal $MgCl_2$ concentration was reported to be 10 mM. The pea chloroplast helicase was completely inhibited by 100 mM potassium phosphate similar to human DNA helicase IV [16] and SV-40 T-antigen [32].

Intercalating ligands which bind to DNA are also known to inhibit helicase reactions by blockade of the translocation of the helicase. Nogalamycin and mitoxantrone inhibit *E. coli* DNA helicase II [33]. Nogalamycin was also observed to inhibit the unwinding activity of pea chloroplast helicase. We found that daunorubicine, an inhibitor of topoisomerase II, also inhibits pea chloroplast helicase.

The *in vivo* roles of most DNA helicases isolated from *E. coli* have been determined [11]. In eukaryotes, little is known about the role of DNA helicases except for a few reports. Rad3 DNA helicase of yeast *Saccharomyces cerevisiae* has a role in DNA repair [34]. Among virus-encoded DNA helicases, the T-antigen–associated helicase activity has been shown to play a role in DNA-replication initiation and fork advancement [30–32]. A DNA-repair helicase has been shown to be a component of basic transcription factor 2 (TFIIH) [35]. The E1 protein from bovine papilloma virus, which is required for viral DNA replication, was identified as an origin-binding DNA helicase [36]. Recently, the Bloom's syndrome gene product has been shown to have sequence similarity with RecQ helicases, a subfamily of DEXH-box-containing DNA and RNA helicases [37]. We plan to investigate the possible role of pea chloroplast DNA helicase in replication by means of plasmids that contain the chloroplast origin of replication, and the *in vitro* replication system [4, 6].

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