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Escherichia coli DNA Helicase I Catalyzes a Unidirectional and Highly Processive Unwinding Reaction*

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Helicase I has been purified to greater than 95% homogeneity from an F^+ strain of *Escherichia coli*, and characterized as a single-stranded DNA-dependent ATPase and a helicase. The duplex DNA unwinding reaction requires a region of ssDNA for enzyme binding and concomitant nucleoside 5′-triphosphate hydrolysis. All eight predominant nucleoside 5′-triphosphates can satisfy this requirement. Unwinding is unidirectional in the 5′ to 3′ direction. The *length* of duplex DNA unwound is independent of protein concentration suggesting that the unwinding reaction is highly processive. Kinetic analysis of the unwinding reaction indicates that the enzyme turns over very slowly from one DNA substrate molecule to another.

The ATP hydrolysis reaction is continuous when circular partial duplex DNA substrates are used as DNA effectors. When linear partial duplex substrates are used ATP hydrolysis is barely detectable, although the kinetics of the unwinding reaction on linear partial duplex substrates are identical to those observed using a circular partial duplex DNA substrate. This suggests that ATP hydrolysis fuels continuous translocation of helicase I on circular single-stranded DNA while on linear single stranded DNA the enzyme translocates to the end of the DNA molecule where it must slowly dissociate from the substrate molecule and/or slowly associate with a new substrate molecule, thus resulting in a very low rate of ATP hydrolysis.

DNA helicases catalyze the unwinding of duplex DNA and play an essential role in the metabolism of nucleic acids in the cell. In *Escherichia coli* at least seven enzymes with helicase activity have been isolated and described (1–9). The reason for the variety of helicases is not understood, but presumably reflects multiple roles for these enzymes in the cell. The *E. coli* DNA helicases are known to play central roles in DNA replication (10), DNA mismatch repair (11), excision repair (12, 13), and recombination (14). In addition, *E. coli* helicases are essential for bacteriophage φX174 replication (15) and for bacterial conjugation (16).

Helicase I was the first DNA helicase isolated from *E. coli* (17, 18). This enzyme is a single polypeptide of M_r = 180,000 (17) encoded by the *traI* gene of the *E. coli* F factor (19). The F factor, a plasmid of approximately 100 kb,[1] is able to transfer its DNA from the host cell (F^+) to an F^- recipient cell which is in direct physical contact with the host (for a recent review, see Ref. 20). Helicase I is required at the DNA transfer stage of bacterial conjugation (21) and is though to be involved in unwinding the F plasmid from a site-specific nick at the origin of transfer (19). This unwinding of the F plasmid may provide the single-strand of DNA which is transferred to the recipient bacterium.

Helicase I has been purified and partially characterized biochemically (17, 18). It is a single-stranded (ss) DNA-dependent nucleoside 5′-triphosphatase (NTPase) and a helicase. As an NTPase, helicase I is markedly stimulated by a ssDNA cofactor and requires a divalent cation (either magnesium or calcium) for activity (17). The enzyme has been reported to function as a multimer as it (i) readily forms aggregates at low ionic strength, and (ii) shows very low ATPase activity at KCl concentrations above 150 mM, where the enzyme presumably exists in the monomeric state (17, 18, 22). ATP (dATP) appears to be the preferred substrate for the NTP hydrolysis reaction (17). The helicase I unwinding reaction requires concomitant NTP hydrolysis (18, 22, 23). When the unwinding reaction catalyzed by helicase I was measured by S1 nuclease digestion or by velocity sedimentation of the reaction products, helicase I was shown to be capable of unwinding DNA-DNA or RNA-DNA partial duplex structures (18). However, helicase I does not utilize RNA as an NTPase cofactor (17). The mode of action of the helicase appears to be processive (22, 23), and a region of ssDNA adjacent to the duplex DNA of approximately 200 nucleotides in length is necessary for helicase I to initiate an unwinding reaction (2, 23). Helicase I will not unwind completely duplex DNA molecules or nicked DNA molecules (22). Results with exonuclease eroded linear duplex DNA molecules have suggested that helicase I unwinds duplex DNA in the 5′ to 3′ direction with respect to the strand on which the enzyme is bound (23).

In this study we have extended the enzymatic characterization of helicase I both as an ATPase, and as a helicase using an assay which directly measures the unwinding reaction. Helicase I appears to translocate processively along a ssDNA effector using the energy released by NTP hydrolysis to fuel translocation. The enzyme can utilize all eight predominant NTPs as hydrolysis substrates in the helicase reaction. The unwinding of duplex DNA by helicase I is independent of protein concentration with respect to the *length* of duplex DNA unwound suggesting that the unwinding reaction is processive. Moreover, helicase I turns over extremely slowly from one DNA substrate molecule to another. In addition, we

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[1] The abbreviations used are: kb, kilobase pairs; ssDNA, single-stranded DNA; NTPase, nucleoside 5′-triphosphatase; NTP, nucleoside 5′-triphosphate; RF, replicative form; bp, base pairs; ATPγS, adenosine 5′-*O*-(thiotriphosphate); SDS, sodium dodecyl sulfate.

confirm the direction of unwinding as 5′ to 3′ with respect to the strand on which the enzyme is bound.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Purification of Helicase I—Helicase I was purified as described under "Experimental Procedures"; Table I summarizes the purification. The initial cell extract contained multiple DNA-dependent as well as DNA-independent ATPase activities making it impossible to estimate the total helicase I activity in crude extracts. For this reason, no estimate of overall yield is made. The phosphocellulose column resolves three peaks of DNA-dependent ATPase activity with helicase I eluting in the peak resolved at 250 mM NaCl. All subsequent chromatographic steps yield a single peak of DNA-dependent ATPase activity. The specific activity of helicase I calculated after the hydroxylapatite step of the purification varies from preparation to preparation. This variability is possibly due to an endonuclease which is sometimes present at this stage of the purification. If this endonuclease linearized the DNA substrate used in the ATPase assay the specific activity of helicase I would appear to drop dramatically. The activity of helicase I on linear DNA substrates is discussed in detail below. The final fraction of the helicase I purification contained a single polypeptide that migrated with a M_r = 180,000 on a polyacrylamide gel run in the presence of sodium dodecyl sulfate (Fig. 1). Fraction VI contained no detectable endo- or exonuclease activity as determined by lack of detectable degradation of the partial duplex DNA substrates used in the helicase assays.

Helicase and ssDNA-dependent ATPase Reactions—The unwinding reaction catalyzed by helicase I was originally characterized using either a coupled assay that measured the fraction of a radioactively labeled DNA substrate rendered susceptible to S1 nuclease, or by velocity sedimentation of the DNA substrate (18, 22, 23). We have extended this characterization using an assay that directly measures the ability of helicase I to unwind a partial duplex DNA molecule (24). This assay has been used to characterize several other DNA helicases (5, 24, 32, 33). The DNA substrate utilized in this assay consists of the complementary strand of a radioactively labeled DNA restriction fragment annealed to circular M13mp7 ssDNA as described under "Experimental Procedures" (see Fig. 4*A*). The helicase assay measures the fraction of the [^{32}P] DNA fragment displaced by the helicase.

DNA Substrate Requirements for ATP Hydrolysis—The helicases characterized to date are all ssDNA-dependent ATPases (1). Table III summarizes the results of experiments performed using several different DNA molecules as effectors of the helicase I-catalyzed ssDNA-dependent ATPase reaction. Circular M13mp7 ssDNA proved to be the best effector of the DNA-dependent ATP hydrolysis reaction. Double-stranded linear (RF III) or supercoiled (RF I) DNA molecules could not substitute for ssDNA. Surprisingly, neither poly(dT) nor linear M13mp7 ssDNA could serve as effectors of the ATP hydrolysis reaction. Since both are ssDNA molecules we expected that they would substitute for circular ssDNA. These results suggest that DNA termini may inhibit the ssDNA-dependent ATPase reaction catalyzed by helicase I. The implications of this result will be discussed later in the text. The concentration of circular ssDNA required to achieve one-half-maximal ATPase reaction velocity (K_{eff}) was determined. K_{eff} for circular M13mp7 ssDNA was 0.51 μM.

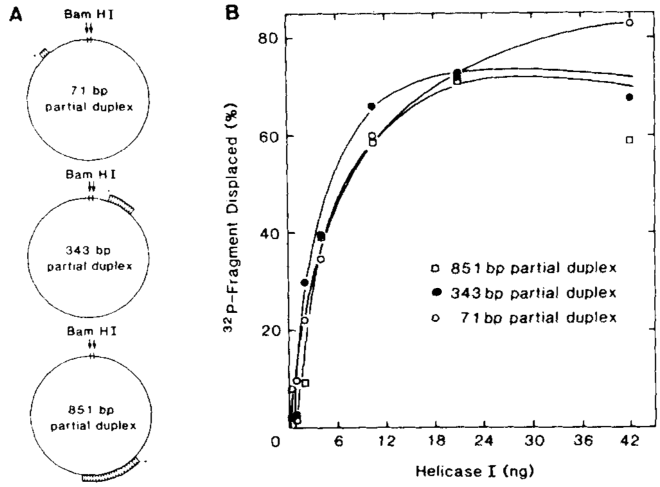

FIG. 4. **Protein concentration dependence of the helicase reaction.** *Panel A*, circular partial duplex helicase substrates. The 71-, 343-, and 851-bp helicase substrates were constructed as described under "Experimental Procedures." The DNA restriction fragment was labeled at its 3′ terminus on each substrate. *Panel B*, helicase reactions were as described under "Experimental Procedures" using 0.21, 1.05, 2.1, 4.2, 10.5, 21, and 42 ng of helicase I, respectively. The data presented represent an average of three or more experiments. ○, 71-bp partial duplex substrate; ●, 343-bp partial duplex substrate; □, 851-bp partial duplex substrate.

TABLE III

ATP hydrolysis in the presence of DNA

ATPase activity was measured in the standard ATP hydrolysis assay, using the indicated DNA effector, as described under "Experimental Procedures" using 10.5 ng of helicase I.

DNA effector	Nucleotide concentration	[^{3}H]ADP formed
	μM	*pmol*
M13mp7 circular ssDNA	3.0	654
M13mp7 linear ssDNA	3.4	≤40
M13mp7 RF I DNA[a]	3.5	54
M13mp7 RF III DNA[b]	3.3	49
Poly(dT)	3.6	28
No DNA	0.0	≤20

[a] Supercoiled.
[b] Duplex linear.

The Length of Duplex DNA Unwound Is Independent of Protein Concentration—To determine the effect of the length of duplex DNA on the unwinding reaction catalyzed by helicase I, three partial duplex DNA substrates were constructed as described under "Experimental Procedures" (Fig. 4*A*). Since the DNA substrate concentration was essentially the same for each substrate the results obtained with all three are directly comparable.

Helicase I displaced greater than 70% of the [^{32}P]DNA fragment from each of the three helicase substrates (Fig. 4*B*). The fraction of the [^{32}P]DNA fragment displaced from each substrate was directly proportional with enzyme concentration up to approximately 6 ng of helicase I. Interestingly, the same fraction of [^{32}P]DNA fragment was displaced from each helicase substrate at all enzyme concentrations tested. Since the 851-bp partial duplex substrate contains 12-fold more base pairs of duplex DNA than the 71-bp partial duplex substrate, and was unwound to the same extent, the unwinding reaction did not require input of additional protein to unwind longer regions of duplex DNA. Essentially the same results have been obtained using a helicase substrate contain-

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Tables I and II, and Figs. 1–3, and 9) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

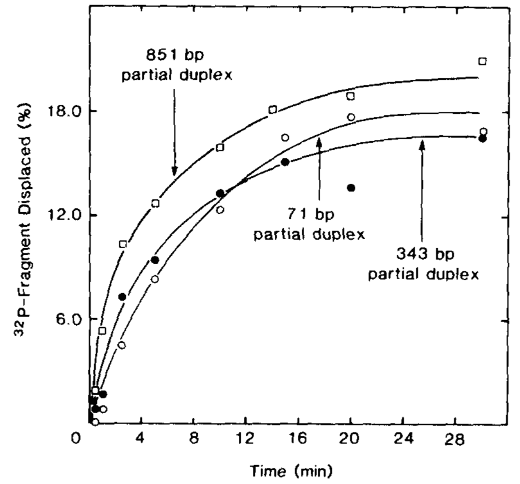

FIG. 5. **Rate of the unwinding reaction.** Helicase reactions were as described under "Experimental Procedures" with the following modifications. The reaction mixture volume was increased to 220 μl and 20-μl aliquots were removed for heat-denatured substrate and no helicase I controls. Helicase I (10.5 ng) was added to the remaining 180 μl and the reaction was placed at 37 °C. Aliquots (20 μl) were removed at the indicated times and the reaction stopped by the addition of EDTA and dyes as described under "Experimental Procedures." The data presented represents an average of three or more experiments. ○, 71-bp partial duplex circles; ●, 343-bp partial duplex circles; □, 851-bp partial duplex circles.

ing 2.5 kb of duplex DNA.[3] These results suggest that helicase I catalyzes a processive unwinding reaction. The enzyme is capable of moving through and unwinding at least 851 bp of duplex DNA.

In addition, a substantial fraction of the [^{32}P]DNA fragment was displaced at low concentrations of enzyme. Using 2 ng of helicase I/reaction mixture (2 helicase I molecules/DNA substrate molecule)[4] approximately 25% of the [^{32}P]DNA fragment was displaced in 10 min. Thus it is possible that helicase I is active as a monomer. Essentially complete unwinding of all three partial duplex substrates was obtained with a ratio of 10 helicase I molecules/DNA substrate molecule. This is in contrast to previous results (23) which suggested that 85 helicase I molecules were required to completely unwind up to 2.5 kb of duplex DNA.

Kinetics of the Unwinding Reaction—Helicase I does not discriminate among DNA substrates utilized in a helicase reaction; circular helicase substrates with 71-, 343-, and 851-bp duplex regions were all utilized with approximately equal efficiency over a 40-fold range of protein concentration (Fig. 4*B*). Under the reaction conditions employed, the unwinding reaction was essentially complete in 10 min at 37 °C. The kinetics of the unwinding reaction were very similar for all three DNA substrates (Fig. 5). The rate of the unwinding reaction was linear for the first 6 min, then decreased until the rate of unwinding was essentially 0 after 15 min. Therefore, at early reaction times (less than 6 min), approximately 12-fold more base pairs were unwound on the 851-bp partial duplex substrate than on the 71-bp partial duplex substrate. Since the rate of the unwinding reaction was the same using all three DNA substrates it is likely that the rate-limiting step in the unwinding reaction is not the rate at which helicase I can unwind the duplex region, but the rate at which the enzyme (i) binds to the DNA, (ii) finds the duplex region, or (iii) associates to form multimers.

The extent of the unwinding reaction catalyzed by helicase I did not continue to increase until 100% of the [^{32}P]DNA fragment had been displaced, but reached a plateau after approximately 15 min (Fig. 5). This result was observed using all three partial duplex DNA substrates. However, when the concentration of helicase I in the reaction mixture was increased, the fraction of the [^{32}P]DNA fragment that was displaced increased proportionally (Fig. 6*A*). Thus the extent of the unwinding reaction was directly proportional with the helicase I concentration. This result does not appear to be due to helicase I inactivation. When helicase I was incubated at 37 °C for up to 50 min prior to initiating a reaction, the enzyme retained full activity (data not shown). Moreover, addition of helicase I to the reaction mixture once the rate of unwinding had leveled off (at the 15-min time point) resulted in renewed displacement of a [^{32}P]DNA fragment until a new plateau was reached (Fig. 6*B*). The extent of the combined displacement reactions was essentially proportional to the displacement expected from the total helicase I concentration added. These results suggest that helicase I does not rapidly turn over from one DNA substrate molecule to another.

Since helicase I does not appear to turn over from one circular DNA substrate molecule to another, we investigated the kinetics of the unwinding reaction using a linear helicase substrate (Fig. 7). On a linear DNA substrate the enzyme should encounter an end of the DNA molecule and may be forced to dissociate and seek a new DNA substrate. This may contrast with what occurs on a circular DNA molecule where the enzyme may be able to translocate indefinitely.

Linear helicase substrates were produced by taking advantage of the single *Cla*I restriction site in the duplex region of the circular 343-bp partial duplex substrate. Cleavage with this enzyme will generate a linear substrate with 141 bp of unlabeled duplex DNA at the 5′ end of the molecule and 202 bp of [^{32}P]DNA duplex at the 3′ end of the molecule separated by 6895 nulcleotides of ssDNA. As helicase I unwinds duplex DNA in a 5′ to 3′ direction, the [^{32}P]DNA fragment will be displaced from the 3′ end of the linear substrate. When kinetic experiments were carried out using this linear substrate (202 bp linear) there was no apparent increase in helicase I turnover (Fig. 7); the kinetics were the same as those observed using a 71-bp partial duplex circular DNA substrate.

To ensure that the presence of the duplex DNA ends was not inhibiting the enzyme, the same studies were carried out using linear DNA substrates with the duplex region located internally. These substrates were constructed by taking advantage of the short duplex hairpin loop created in the ssDNA by the polylinker region of M13mp7. The *Bam*HI restriction endonuclease site present in the hairpin was used to produce linear helicase substrates. Complete digestion at the *Bam*HI site produced a linear partial duplex DNA substrate from a circular substrate. The kinetics of unwinding on this 71-bp linear substrate were also identical to the unwinding kinetics observed using the 71-bp circular DNA substrate (Fig. 7). However, the extent of the unwinding reaction on linear substrates can be lower than on circular helicase substrates depending on where the duplex DNA is located relative to the ssDNA ends. Assuming that helicase I translocates 5′ to 3′ along ssDNA, a fraction of the helicase I molecules will bind to ssDNA and never encounter a region of duplex DNA on the 3′ side of the binding site. This results in apparent lower

[3] E. E. Lahue and S. W. Matson, unpublished results.

[4] The ratio of helicase I molecules/DNA substrate molecule assumes a molecular weight of 180,000 g/mol for helicase I. Since protein concentration was determined by the method of Lowry *et al.* (31) and DNA substrate concentration is estimated as described under "Experimental Procedures" the helicase I/DNA ratio must be considered an estimate. A potential error of as much as 2-fold in either direction is possible.

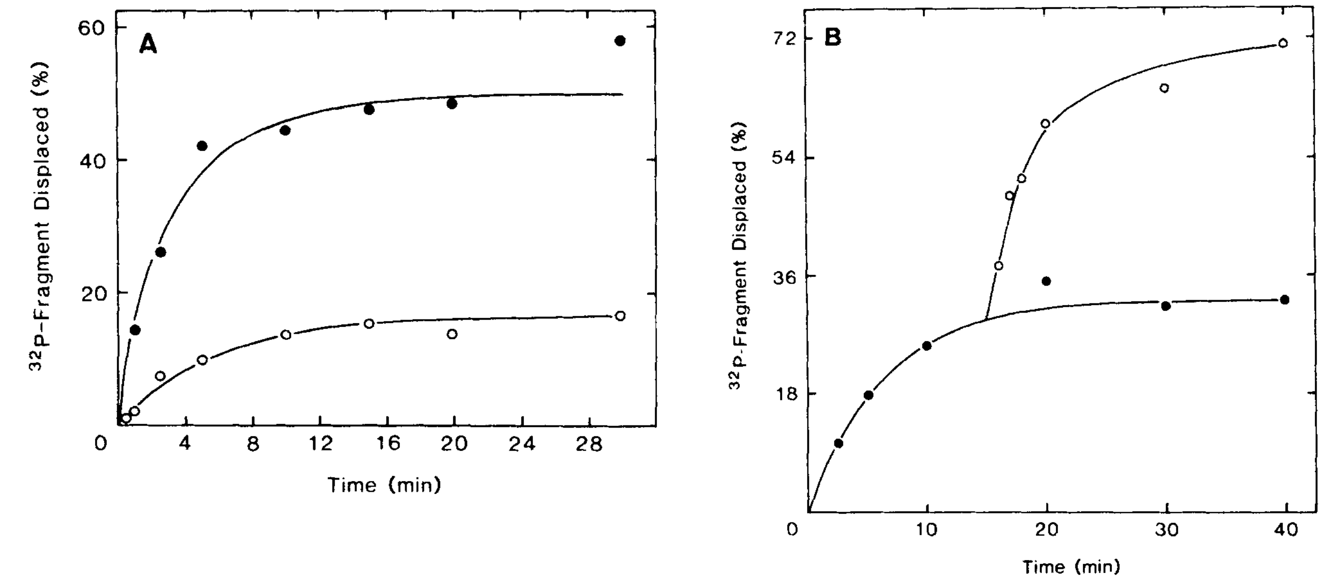

FIG. 6. **Extent of the unwinding reaction at different helicase I concentrations.** *Panel A*, kinetic analysis of the helicase I unwinding reaction using the 343-bp partial duplex substrate. Helicase reactions were as described under "Experimental Procedures" with the following modifications. The reaction volume was increased to 220 μl and 20-μl aliquots were removed for the heat denatured and no helicase I controls. O, 10.5 ng or ●, 27 ng helicase I was added to the remaining 180 μl and the reaction was placed at 37 °C. Aliquots (20 μl) were removed at the indicated times and the reaction was stopped by the addition of EDTA and dyes as described under "Experimental Procedures." *Panel B*, kinetic analysis of the helicase I unwinding reaction using the 851-bp partial duplex substrate. Helicase reactions were as described under "Experimental Procedures" with the following modifications. The reaction volume was increased to 300 μl and 20-μl aliquots were removed for the heat denatured and no helicase I controls. Helicase I (31 ng) was added to the remaining 260 μl and the reaction was placed at 37 °C. Aliquots (20 μl) were removed at the indicated times (●). At 15 min a parallel reaction was started by taking a portion (130 μl) of the reaction mixture and adding an additional 31 ng of helicase I to this aliquot. This second reaction mixture continued to incubate at 37 °C and 20-μl aliquots were removed at the indicated times (O). The data presented here represents an average of two experiments.

unwinding activity by helicase I on certain linear substrates (data not shown). The above data suggest that helicase I does not rapidly dissociate from the end of a linear DNA molecule and bind to a new DNA molecule. In fact, helicase I may remain bound at the end of the linear DNA molecule.

Kinetics of the ssDNA-dependent ATPase Reaction—ATP hydrolysis was required for the unwinding reaction (see Table II) and it seems reasonable to assume that the two activities may be coupled. For this reason we investigated the kinetics of the ssDNA-dependent ATPase reaction catalyzed by helicase I using both circular and linear DNA effectors. After a brief lag phase the rate of the ATP hydrolysis reaction was linear with time for more than 30 min using either circular M13mp7 ssDNA (Fig. 8*A*) or the circular 851-bp partial duplex helicase substrate (Fig. 8*B*) as a DNA effector. This result offers a sharp contrast to what was observed when the unwinding reaction was monitored (see Fig. 5). The rate of the unwinding reaction leveled off to essentially zero after approximately 15 min. Clearly the enzyme continued to hydrolyze ATP after the unwinding reaction had ceased.

When linear M13mp7 ssDNA or the linear 851-bp helicase substrate were used as DNA effectors of the ssDNA-dependent ATPase activity, very little ATP hydrolysis was measured (Fig. 8, *A* and *B*). When the kinetics of the ATPase reaction using a linear DNA effector was compared to the kinetics of the unwinding reaction on the same linear molecule, an interesting contrast was seen. ATP hydrolysis in the presence of a linear DNA effector was negligible compared to ATP hydrolysis in the presence of a circular DNA effector. However, helicase activity on linear and circular DNA substrates was equivalent (see Fig. 7). One explanation for these results assumes that helicase I translocates unidirectionally in the 5′ to 3′ direction to the end of a linear molecule and stops, no longer requiring ATP hydrolysis for translocation or unwinding. On circular DNA substrates the enzyme may be able to translocate indefinitely resulting in a linear ATPase reaction.

The role of ATP hydrolysis in the helicase reaction is not clear. However, it is likely that the energy released by hydrolysis of ATP is utilized by helicase I for processive translocation along ssDNA and perhaps for unwinding the DNA duplex. A kinetic parameter, K_{eff}, has been utilized to define helicase reaction mechanisms on ssDNA. K_{eff} is defined as the amount of ssDNA required to achieve one-half the maximal rate of ATP hydrolysis. If the K_{eff} is substantially greater for linear DNA molecules than for circular DNA molecules, this can be interpreted as evidence for a processive translocation mechanism (24, 27).

To examine whether helicase I exhibits a processive translocation mechanism on ssDNA, K_{eff} values were determined for both circular and linear M13mp7 ssDNA. The K_{eff} value for M13mp7 circular ssDNA is 0.5 μM DNA (Fig. 9); the value for a linear DNA molecule cannot be determined as the rate of ATP hydrolysis on a linear DNA effector is at or below detectable limits under the conditions used. However, the results of unwinding assays using the linear helicase substrate indicated that the enzyme did indeed translocate over the linear DNA molecule. This suggests that helicase I is extremely processive, dissociating very infrequently from ssDNA. In fact, the enzyme apparently remains bound to the end of a linear DNA molecule even when the enzyme is not hydrolyzing ATP.

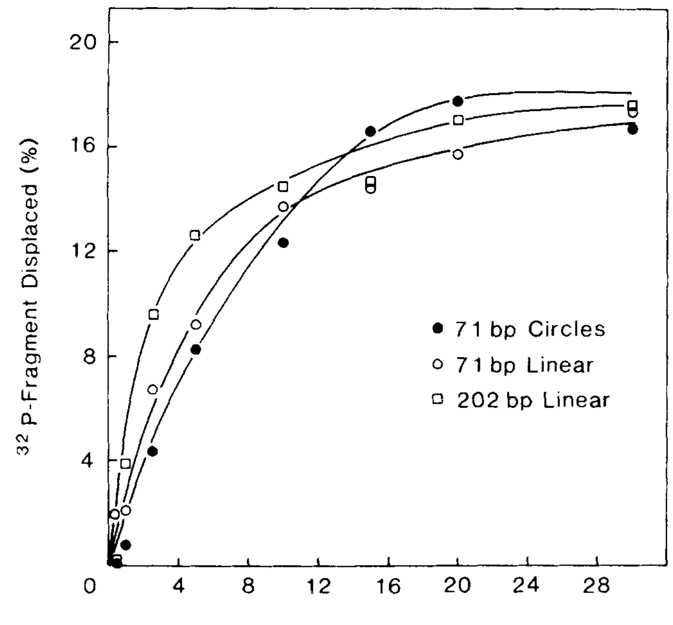

FIG. 7. **The kinetics of the unwinding reaction are the same on linear and circular helicase substrates.** Helicase reactions were as described under "Experimental Procedures" with the following modifications. The reaction volume was increased to 200 μl and 20-μl aliquots were removed for heat-denatured substrate and no helicase I controls. Helicase I (10.5 ng) was added to the remaining 180 μl and the reaction was placed at 37 °C. Aliquots (20 μl) were removed at the times indicated and the reaction stopped by the addition of EDTA and dyes as described under "Experimental Procedures." The data presented represent an average of three or more experiments. ●, 71-bp circular helicase substrate; ○, 71-bp helicase substrate linearized with *Bam*HI as described under "Results"; □, 343-bp helicase substrate linearized with *Cla*I to give a 202-bp linear helicase substrate as described under "Experimental Procedures."

DISCUSSION

Helicase I is believed to play a central role in the transfer of a single strand of F plasmid DNA from the donor cell to the recipient cell during bacterial conjugation (19, 21). The purified enzyme has two interrelated activities: (i) ssDNA-dependent ATPase activity and (ii) helicase activity, both of which are likely to be important for this role. In this study we have extended earlier biochemical studies of these two activities (17, 18, 22, 23) to show that helicase I catalyzes a unidirectional and highly processive unwinding reaction that is dependent on the hydrolysis of ATP. We have also described a modified purification procedure which may result in a preparation of helicase I that is more active than that described in previously published reports (17, 22). Essentially complete unwinding of partial duplex DNA substrates containing up to 851 bp of duplex DNA has been obtained with a molar ratio of approximately 10 helicase I molecules/DNA substrate molecule. Substantial unwinding of an 851-bp partial duplex molecule was also observed in unwinding reactions which contained a 1:1 ratio of helicase I protein molecules to DNA molecules. These results appear to differ from previous results which suggested that helicase I was active as a multimer of helicase I monomers (18, 22).

The DNA unwinding reaction catalyzed by helicase I required the presence of: (i) a hydrolyzable NTP and (ii) a region of ssDNA to which the enzyme can bind (2, 22, 23). Substitution of the poorly hydrolyzed ATP analog, ATPγS, for ATP resulted in no detectable unwinding of the DNA substrate. This indicated a need for ATP hydrolysis concomitant with unwinding of duplex DNA. All eight of the commonly occurring predominant NTPs (dNTPs) were effectively utilized by helicase I as hydrolysis substrates in place of ATP in the unwinding reaction.

Helicase I requires a region of ssDNA for binding of the enzyme and does not unwind a fully duplex molecule (22). In fact, the ssDNA must be of a specific polarity in relation to the duplex DNA in order for an unwinding reaction to occur. This is consistent with the fact that all helicases known to date unwind duplex DNA with a specific directionality. The direction of the unwinding reaction catalyzed by helicase I is 5′ to 3′ with respect to the strand on which the enzyme is bound. This was demonstrated using a linear ssDNA molecule with duplex ends (see Fig. 3*A*). Thus helicase I unwinds duplex DNA in the same direction as the *E. coli* DnaB protein (5) and helicase III (1) and in the opposite direction of Rep protein (3), helicase II (28), and the *E. coli* 75-kDa helicase (7).

The unwinding reaction catalyzed by helicase I was independent of protein concentration with respect to the *length* of the duplex region unwound. This was demonstrated using three partial duplex substrates with duplex regions ranging from 71 to 851 bp in length. A specified concentration of helicase I displaced the same fraction of [^{32}P]DNA fragment from each partial duplex substrate. Thus the fraction of DNA substrate molecules unwound by helicase I is independent of the *length* of the duplex region on the substrate. In fact, a duplex region of 2.5 kb in length could be unwound to a comparable extent using the same concentration of helicase I.[3] Since no additional protein was required to unwind longer duplex DNA regions the mechanism of the unwinding reaction appears to be processive.

Consistent with a processive unwinding mechanism is the apparent slow turnover of helicase I molecules from one DNA substrate to another. The extent of the reaction, as defined by the fraction of substrate unwound when the plateau was reached, was directly proportional with helicase I concentration on both circular and linear partial duplex DNA substrates. Since the enzyme remained active for more than 40 min and the DNA substrate was competent to be further unwound, we interpret this result as indicating that helicase I does not turn over to a new DNA substrate at any significant rate. The results were the same even when a DNA terminus was provided, as on the linear partial duplex molecules.

The kinetics of the ssDNA-dependent ATP hydrolysis reaction were quite different on linear and circular DNA effectors (see Fig. 8, *A* and *B*). The ATP hydrolysis reaction was linear with time for more than 40 min on a circular DNA molecule; ATP hydrolysis was barely detectable on a linear DNA molecule. This was true of both ssDNA effectors and partial duplex DNA helicase substrates used as effectors. Since ATP hydrolysis is required for unwinding of duplex DNA and helicase I has been shown to unwind a duplex region on a linear molecule, it seems reasonable to conclude that ATP hydrolysis fuels the 5′ to 3′ translocation of helicase I along ssDNA as well as movement through duplex DNA.

A comparison of the results of ATP hydrolysis assays and unwinding assays on both linear and circular DNA molecules presents an interesting contrast. A linear partial duplex DNA substrate was fully functional as a helicase substrate but did not appear to be a functional effector for ATP hydrolysis. Circular DNA molecules, on the other hand, provide good helicase substrates and were effectors of the ATP hydrolysis reaction. This conflict can be explained if helicase I remains bound on a circular DNA molecule for an indefinite period of time and continues to translocate and hydrolyze ATP. When

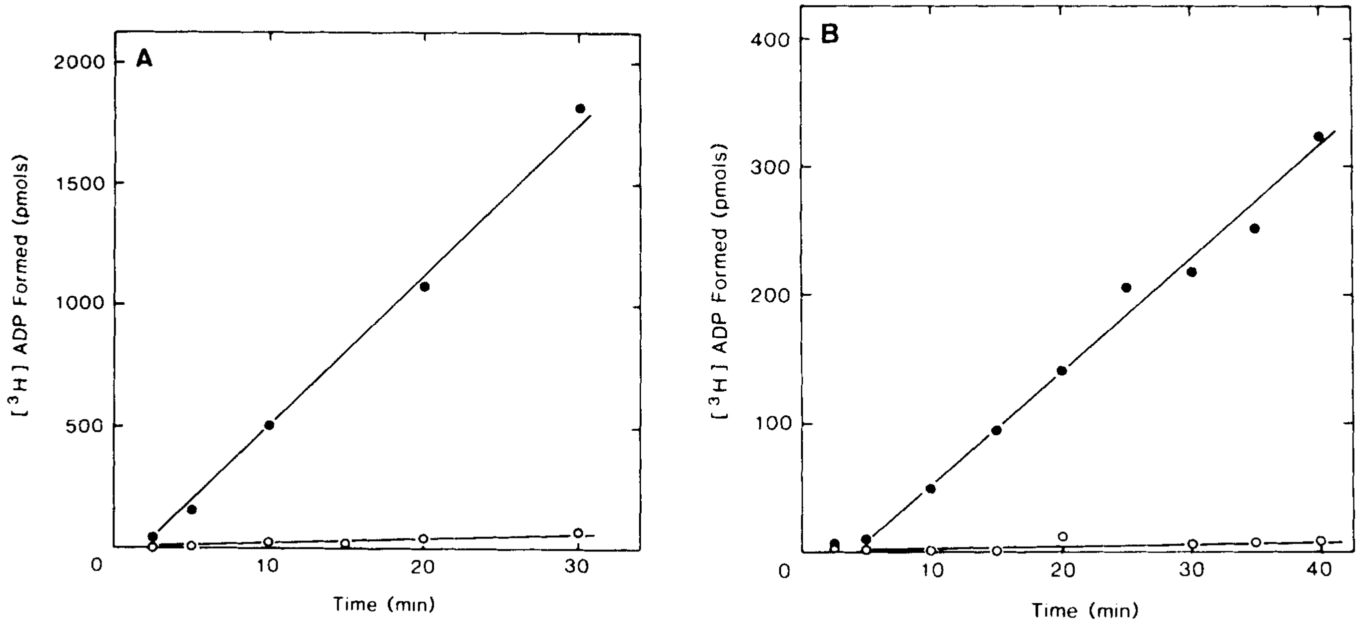

FIG. 8. **ATP hydrolysis in the presence of ssDNA effectors.** ATP hydrolysis was measured as described under "Experimental Procedures." *Panel A*, ATP hydrolysis *versus* time using M13mp7 ssDNA as an effector. The volume of the reaction mixture was increased to 40 μl and a 5-μl aliquot was removed for a no helicase I control. Helicase I (21 ng) was added to the remainder of the reaction mixture and the reaction was placed at 37 °C. Aliquots (5 μl) were removed at the indicated times and the reaction stopped as described under "Experimental Procedures." ○, M13mp7 ssDNA linearized with *Bam*HI, ●, M13mp7 ssDNA circles. *Panel B*, ATP hydrolysis *versus* time using the 851-bp partial duplex substrate as an effector. The volume of the reaction mixture was increased to 60 μl and a 5-μl aliquot was removed for a no helicase I control. Helicase I (3.6 ng) was added to the remainder of the reaction mixture and the reaction was placed at 37 °C. Aliquots (5 μl) were removed at the indicated times and the reaction stopped as described under "Experimental Procedures." ○, 851-bp partial duplex substrate linearized with *Bam*HI as described under "Results"; ●, circular 851-bp partial duplex substrate. The 851-bp partial duplex substrate concentration in the reaction mixtures was approximately 1.8 μM for both linear and circular substrates. Data presented is an average of two or more experiments. Background values have been subtracted for all data presented.

a duplex region of DNA is encountered, the duplex is unwound but the enzyme remains bound to the ssDNA and does not turn over to a new DNA substrate molecule. On a linear DNA molecule the enzyme migrates in the 5′ to 3′ direction utilizing ATP hydrolysis to fuel translocation, but stops when an end is reached. At this point ATP hydrolysis also ceases. If duplex DNA is encountered during the 5′ to 3′ migration an unwinding reaction takes place. The enzyme subsequently dissociates from the end of the linear DNA molecule or associates with a new DNA molecule very slowly. Alternatively the active enzyme species could be a multimer which must dissociate to form monomers prior to binding a new substrate molecule. The result of this slow step is that ATP hydrolysis is barely detectable when a linear DNA molecule is used as an effector of ATP hydrolysis. Interestingly, this provides an explanation for why linear homopolymer DNA molecules were not effective in stimulating the ATP hydrolysis reaction catalyzed by helicase I (17). In addition, this also suggests that only a low level of ATP hydrolysis is required to fuel translocation along ssDNA and for unwinding regions of duplex DNA. Whether the energy released in hydrolyzing ATP is utilized solely in reaching duplex DNA or is also required for the separation of duplex DNA strands is not clear at present. However, it should be noted that no substantial increase in ATP hydrolysis was observed when linear partial duplex substrates were used as effectors of the ATP hydrolysis reaction as compared to linear ssDNA (see Fig. 8).

The biochemical properties of helicase I are suitable for the role it is thought to play in bacterial conjugation. The enzyme is a highly processive helicase capable of unwinding long regions of duplex DNA. However, the purified enzyme will not initiate an unwinding reaction on a nicked DNA molecule (22). Many DNA helicases have shown a dependence on, or interactions with other proteins in order to provide optimal helicase activity (3, 5, 9, 14). As knowledge of the enzymology of bacterial conjugation increases it will be interesting to see whether a protein involved in the replication or transfer of the F factor will be required to aid in the functioning of helicase I. Perhaps an enzyme will be found which enables helicase I to unwind the F factor from the strand-specific nick known to occur at the origin of transfer.

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SUPPLEMENTARY MATERIAL TO

ESCHERICHIA COLI DNA HELICASE I CATALYZES A UNIDIRECTIONAL AND HIGHLY PROCESSIVE UNWINDING REACTION

Elaine E. Lahue and Steven W. Matson

EXPERIMENTAL PROCEDURES

Materials

Enzymes - Helicase I was purified as described below. Restriction endonucleases were purchased from either Boehringer Mannheim or New England Biolabs. Reaction conditions were those suggested by the supplier. DNA polymerase I (large fragment) was purchased from US Biochemicals. Lysozyme was purchased from Sigma Chemicals. Helicase II was purified as described (24) and was the kind gift of J.W. George (Univ. of North Carolina).

DNA and Nucleotides - Phage M13mp7 ssDNA and replicative form I DNA were prepared as described (25). All unlabeled nucleotides were purchased from P-L Biochemicals. [α-^{32}P]dCTP was purchased from ICN Radiochemicals, [^{3}H]ATP was obtained from New England Nuclear.

Buffers - Buffer A contained 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 20% glycerol. Buffer B contained 10 mM KPO_4 (pH 7.2), 1 mM sodium citrate, 5 mM 2-mercaptoethanol, 20% glycerol.

Methods

Purification of helicase I - Helicase I was purified using a modification of the procedure of Abdel-Monem and Hoffmann-Berling (17). Ten liters of *E. coli* 71.18 (F+) (26) was grown at 37°C in the following media: tryptone (11 g/l), yeast extract (23 g/l), thymine (40 μg/ml), 50 mM NaCl, 0.5% glycerol, 0.1 M KPO_4 (pH 7.4). Cells were grown to late log phase. Cells were harvested (308 g) and resuspended in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 10% sucrose (2 mls per gram wet weight). Resuspended cells were frozen in a dry ice/ethanol bath and stored at -20°C until use. The following steps were carried out at 0-4°C unless noted. Frozen cells (400 mls, 150 g) were thawed on ice, transferred to centrifuge tubes and lysed by the addition of NaCl to a final concentration of 0.5 M and lysozyme to a final concentration of 0.2 mg/ml. The suspensions were held on ice for 45 minutes then quick frozen in a dry ice/ethanol bath. The frozen cells were then thawed in a 37°C bath with gentle mixing. The freeze-thaw procedure was repeated four times. The cell lysate was centrifuged at 39,000 x g for one hour. To the resulting supernatant (fraction I) solid ammonium sulfate (0.3 g/ml) was added slowly over 45 min. with constant stirring in the cold. Stirring was continued for an additional 15 min after the ammonium sulfate had dissolved. The precipitate was collected by centrifugation at 27,000 x g for 15 min. The resulting pellet was resuspended in 80 mls of buffer A containing 0.28 g/ml ammonium sulfate using a Dounce homogenizer. The precipitate from this resuspension was collected as above and the pellet resuspended in 80 mls of buffer A containing 0.2 g/ml ammonium sulfate. The precipitate was collected and the pellet resuspended in 12 mls of buffer A. The final resuspension was dialyzed overnight against buffer A (600 mls). Any precipitate in the dialysate was removed by centrifugation at 27,000 x g. If necessary the conductivity of this fraction (Fraction II) was adjusted to that of buffer A by dilution with buffer A.

Fraction II was loaded onto a phosphocellulose column (2.5 cm x 5.5 cm) equilibrated with buffer A. The column was subsequently washed with two column volumes of buffer A and eluted with a 500 ml linear gradient from 0 to 0.5 M NaCl in buffer A. Fractions were assayed for ssDNA-dependent ATPase activity as described below. Three peaks of ssDNA-dependent ATPase activity eluted from this column; the first at 100 mM NaCl, the second at 250 mM NaCl, and the third at 425 mM NaCl. The peak of activity which eluted at 250 mM NaCl represents helicase I and was pooled (Fraction III).

Fraction III was dialyzed against buffer A containing 50 mM NaCl and loaded onto a ssDNA cellulose column (1 cm x 5 cm) which had been equilibrated with buffer A containing 50 mM NaCl. The DNA cellulose column was washed with two column volumes of buffer A plus 50 mM NaCl and eluted with a 40 ml linear gradient from 50 mM to 1 M NaCl in buffer A. The ssDNA-dependent ATPase activity eluted at 250 mM NaCl. Active fractions were pooled and dialyzed overnight against buffer B (Fraction IV).

Fraction IV was loaded onto a hydroxylapatite column (0.5 cm x 0.65 cm), washed with two column volumes of buffer B, and eluted with an 8 ml linear gradient from 10 mM to 500 mM KPO_4 (pH 7.2). The ssDNA-dependent ATPase activity eluted at 150 mM KPO_4. Active fractions from the hydroxylapatite column were pooled (Fraction V) and if the volume was less than 2 mls the pool was loaded directly onto a Sephacryl S-200 column (1 cm x 56 cm). If the volume was greater than 2 mls fraction V was concentrated prior to loading by packing solid polyethylene glycol around dialysis tubing containing the pooled fraction. The Sephacryl S-200 column was equilibrated with buffer A plus 250 mM NaCl and was run at approximately 2 mls per hour. Helicase I ssDNA-dependent ATPase activity eluted in the void volume (Fraction VI). At this step in the purification the protein was greater than 95% pure as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The M_r was approximately 180,000 and the preparation was free of nuclease activity. The purified protein was dialyzed into buffer A containing 50% glycerol and 100 mM NaCl and stored at -20°C. Helicase I is stable for more than one year under these conditions.

ATPase assay - The ssDNA-dependent ATPase assay measures the conversion of [^{3}H]ATP to [^{3}H]ADP catalyzed by helicase I. The standard reaction mixture (20 μl) contained 40 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 3 μM M13mp7 ssDNA, 0.55 mM [^{3}H]ATP, and the indicated amount of helicase I. Incubations were performed at 37°C for 10 min. Aliquots (5 μl) were removed and added to 5 μl of 6 mM ATP, 6 mM ADP and 33 mM EDTA to terminate the reaction. Reaction products were analyzed as described previously (27). One unit of ATPase activity is defined as the amount of enzyme necessary to catalyze the production of 1 nmole of rADP in a 5 μl aliquot of the 20 μl reaction mixture under the standard assay conditions.

Helicase assay - The helicase assay measures the displacement of a [^{32}P]DNA fragment annealed to a circular M13mp7 ssDNA molecule. The construction of these DNA substrates has been described (24,28). Briefly, the substrates used in the unwinding assays were constructed using purified M13mp7 replicative form I *Hae*III restriction fragments of varying lengths (69 base pairs (bp), 341 bp, 849 bp) which were denatured and annealed on M13mp7 ssDNA circles. These partial duplex molecules were then 3'-end labeled using [α-^{32}P]dCTP and DNA polymerase I large fragment. Three different partial duplex DNA molecules were used in this study; one with 71 bp of duplex DNA, one with 343 bp of duplex DNA, and the third with 851 bp of duplex DNA. All of the helicase substrates were circular DNA molecules unless stated that a linear substrate was used. All helicase substrates contained substantial ssDNA to facilitate enzyme binding. In addition, it should be noted that the location of the duplex structure was different for each DNA substrate (see Fig. 4A). The standard helicase reaction mixture (20 μl) contained 40 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, approximately 2 μM DNA substrate, 1.8 mM ATP and the indicated amount of helicase I. Incubations were at 37°C for 10 min unless otherwise indicated. The reaction was stopped by the addition of 10 μl of 50 mM EDTA, 40% glycerol, 0.6% SDS, 0.1% bromophenol blue, 0.1% xylene cyanole and the samples loaded directly onto a nondenaturing polyacrylamide gel (8% for 71 bp substrate, 6% for 343 bp and 851 bp substrates). Electrophoresis was at 50 or 150 V overnight depending on fragment length. Gels were analyzed by film autoradiography or by slicing the gel into 1 cm sections and determining cerenkov radiation in a liquid scintillation counter. Circular helicase substrates were linearized as described under "Results". The DNA substrate used to determine the direction of the unwinding reaction was constructed as previously described (28).

Other methods - DNA concentrations were determined by measuring the absorbance at 260 nm and are expressed as nucleotide equivalents. The helicase substrate concentration is estimated based on the known ssDNA concentration in the annealing reaction, assuming a 75% recovery from the gel filtration column. Nondenaturing polyacrylamide gel electrophoresis was performed as described (29). Polyacrylamide gels were run in the presence of sodium dodecyl sulfate utilizing the method of Laemmeli (30). Protein concentration was determined by the method of Lowry (31) using bovine serum albumin as a standard.

RESULTS

Helicase reaction requirements - The reaction requirements for helicase I unwinding activity were determined using the 343 bp partial duplex substrate and are shown in Table II. The complete helicase reaction components are listed under "Experimental Procedures." In the absence of added $MgCl_2$ the helicase reaction was reduced by about 30%, while the addition of 4 mM EDTA resulted in no detectable displacement of the [^{32}P]DNA fragment. The enzyme has optimal unwinding activity at

$MgCl_2$ concentrations up to 8 mM (data not shown). The helicase reaction also required the presence of a hydrolyzable NTP. None of the nonhydrolyzable ATP analogs tested were capable of substituting for ATP in the unwinding reaction (Table II), suggesting that the helicase reaction requires concomitant ATP hydrolysis. The helicase reaction was also inhibited by NaCl concentrations at or above 75 mM.

NTP requirement - As shown in Table II, the unwinding reaction catalyzed by helicase I required concomitant ATP hydrolysis. To determine which of the eight predominant NTPs could satisfy this requirement, each NTP was tested in a helicase reaction at two different enzyme concentrations (Fig. 2). Suprisingly, all eight NTPs can satisfy the requirement for a hydrolyzable NTP. We infer from this result that helicase I hydrolyzed all eight NTPs although this has not been directly tested. This wide range of NTP utilization has not been observed for any of the other E. coli DNA helicases.

ssDNA-dependent ATPase reaction requirements - The ssDNA-dependent ATPase reaction catalyzed by helicase I displayed reaction requirements similar to those of the unwinding reaction (Table II). However, the ssDNA-dependent ATPase activity was more sensitive to $MgCl_2$ concentration than the helicase activity (data not shown). As conditions were initially chosen to optimize the unwinding reaction, we have used helicase assay conditions when measuring ssDNA-dependent ATPase activity for the purpose of comparison. All ssDNA-dependent ATPase reactions reported here use 4 mM $MgCl_2$ unless otherwise noted. Using 4 mM $MgCl_2$, ssDNA-dependent ATPase activity was approximately 25% of that seen when 1 mM $MgCl_2$ was used. Due to the unusual interactions between helicase I and magnesium we were unable to determine a K_m for ATP in the ssDNA-dependent ATPase reaction. High concentrations of $MgCl_2$ severely inhibit the ssDNA-dependent ATPase activity even in the presence of high concentrations of ATP. Optimal ssDNA-dependent ATPase activity was observed in the presence of 1 mM $MgCl_2$ (data not shown). The effect of added NaCl on the ssDNA-dependent ATPase activity was the same as observed for the helicase activity.

Helicase I unwinds DNA in a 5′ to 3′ Direction

Previous results using linear duplex DNA molecules eroded by exonucleases to provide a ssDNA binding region suggested that helicase I unwinds duplex DNA in a 5′ to 3′ direction (2). We have confirmed this result using an assay which directly determines whether the polarity of the unwinding reaction is 5′ to 3′, 3′ to 5′, or bidirectional (28). The DNA substrate used in this study was a linear ssDNA molecule with duplex ends which differ in length (Fig. 3A). Helicase I requires ssDNA for initial binding to the DNA substrate (22,23) and the substrate depicted in Fig. 3A contains a long region of ssDNA suitable for this purpose. Binding to the single-stranded region and unwinding in the 5′ to 3′ direction should result in displacement of a 202 nucleotide (nt) DNA fragment. Unwinding in the 3′ to 5′ direction should result in the displacement of a 143 nt fragment.

When this linear DNA substrate was incubated with helicase I the enzyme catalyzed the displacement of the 202 nt DNA fragment (Fig. 3B, lane 5). No displacement of the 143 nt DNA fragment was observed. This suggests that helicase I unwinds duplex DNA in the 5′ to 3′ direction. Helicase II was used as a control as it unwinds DNA in a 3′ to 5′ direction (28). In Fig. 3B, lanes 6-8, helicase II was incubated with either the circular 343 bp partial duplex substrate (lane 7) or the linear partial duplex substrate shown in Fig. 3A (lane 8). Helicase II catalyzed the unwinding of the 143 nt DNA fragment confirming that both DNA fragments can be displaced under the reaction conditions used. We conclude from these results that helicase I unwinds duplex DNA unidirectionally in the 5′ to 3′ direction.

TABLE I

Purification of Helicase I

FRACTION	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (units)	SPECIFIC ACTIVITY (units/mg)
I. Cell Lysate	nd	nd	nd
II. $(NH_4)_2SO_4$ ppt.	133.0	1×10^6	7,500
III. Phosphocellulose	0.880	79,900	90,800
IV. DNA-cellulose	0.226	25,700	114,000
V. Hydroxylapatite	0.095	6,600	69,500
VI. Sephacryl S-200	0.042	13,200	314,000

Protein and ATPase assays were performed as described under "Experimental Procedures."

The cell lysate was obtained from 150 g of cells resuspended to a final volume of 400 mls using lysis buffer as described under "Experimental Procedures."

[nd] not determined

TABLE II

Requirements for the Helicase and ATPase Reactions

Reaction Components	$[^{32}P]$ DNA Fragment Displaced (%)	$[^3H]$ADP formed (nmoles)
Complete	75	0.65
$-MgCl_2$	50	0.23
$-MgCl_2$, + 4mM EDTA	<3	nd
-ATP	<3	nd
-ATP, +ATP(γ)S[a]	<3	nd
-ATP, + β, γ-mATP[b]	<3	nd
-ATP, +AMP-PNP[c]	<3	nd
+75mM NaCl	<3	<0.02

Helicase activity was measured in the standard helicase assay, with the indicated modifications, as described under "Experimental Procedures" using the 343 bp partial duplex substrate.

ATPase activity was measured in the standard ATPase assay, with the indicated modifications, as described under "Experimental Procedures."

10.5 ng of helicase I was used in all reactions.

[nd] not determined

[a] adenosine 5′-O-3′-(thiotriphosphate)

[b] β, γ-methylene rATP

[c] adenosine 5′-(β, γ-imido)triphosphate

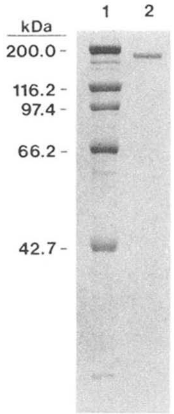

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified helicase I. Coomassie Blue stained 8% polyacrylamide gel. Lane 1, molecular weight standards: ovalbumin, 42.7 kDa; bovine serum albumin, 66.2 kDa; phosphorylase B, 97.4 kDa; β-galactosidase, 116.2 kDa; myosin, 200 kDa. Lane 2, 5 g of purified helicase I (Fraction VI).

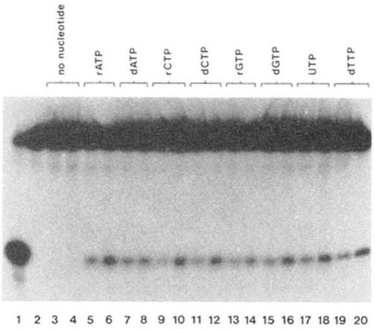

Fig. 2. Helicase I utilizes all NTP's in the unwinding reaction. Helicase reactions were as described under "Experimental Procedures" using 2.1 ng (lanes 3,5,7,9,11,13,15,17,19) or 4.2 ng (lanes 4,6,8,10,12,14,16,18,20) of helicase I and the 343 bp partial duplex DNA substrate. Lane 1 was the heat denatured 343 bp substrate; lane 2 was the no helicase I control. Lanes 3 and 4 contained no added nucleotide. Nucleotide concentrations were 1.8 mM rATP, 1.6 mM dATP, 1.0 mM rCTP, 1.5 mM dCTP, 1.0 mM rGTP, 1.4 mM dGTP, 1.3 mM UTP, and 1.3 mM dTTP.

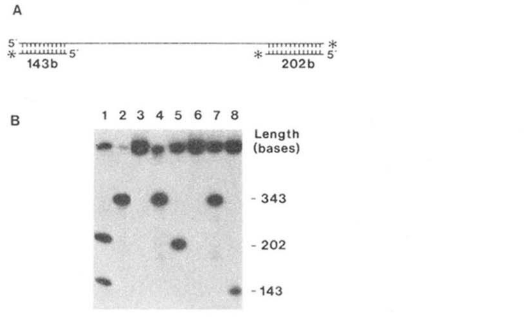

Fig. 3. Helicase I unwinds DNA in a 5′ to 3′ direction. Panel A: Linear partial duplex DNA substrate used to determine the direction of the unwinding reaction. Asterisks denote the position of the radioactive label. Panel B: Helicase reactions were as described under "Experimental Procedures." Lane 1, heat denatured direction substrate shown in panel A; lane 2, heat denatured 343 bp circular partial duplex DNA substrate; lane 3, no helicase I and the 343 bp substrate; lane 4, 343 bp substrate and 23 ng helicase I; lane 5, direction substrate and 23 ng helicase I; lane 6, no helicase II and the direction substrate; lane 7, 343 bp substrate and 17 ng helicase II; lane 8, direction substrate and 17 ng helicase II.

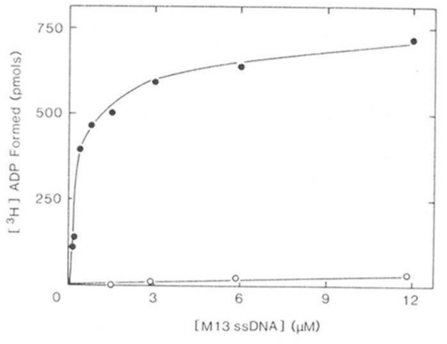

Fig. 9. Helicase I is highly processive. ATP hydrolysis was measured as described under "Experimental Procedures" using 10.5 ng of helicase I. The DNA effector was either a circular (•) or linear (o) M13mp7 ssDNA molecule, at the concentration indicated. The K_{eff} value for circles was determined from a Lineweaver Burke plot of initial reaction rate versus DNA effector concentration. Lines were determined by linear regression analysis.