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The DNA unwinding reaction catalyzed by Rep protein is facilitated by an RHSP – DNA interaction

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ABSTRACT

The unwinding reaction catalyzed by the *Escherichia coli* Rep protein is stimulated by a small 15 kDa protein called Rep helicase stimulatory protein (RHSP)(1). The RHSP-stimulated unwinding reaction catalyzed by Rep protein proceeded at a rapid rate after a time lag of 1 – 2 min at 37°C. This time lag was eliminated by preincubating RHSP with the DNA substrate, indicating that stimulation resulted from an interaction between RHSP and DNA. RHSP was shown to increase the rate as well as the extent of the unwinding reaction catalyzed by Rep protein. RHSP bound both single- and double-stranded DNA with apparent equal affinity, forming an unusually stable complex. Electron microscopy illustrated that the RHSP-DNA complex consisted of large protein aggregates bound to DNA forming a highly condensed, aggregated DNA-protein complex. The protein aggregates were not observed in the absence of DNA and appeared to form cooperatively in the presence of DNA. NH_2-terminal amino acid sequence analysis suggested that RHSP was identical to *E. coli* ribosomal-protein L14. Binding assays showed that the interaction between RHSP and rRNA was similar to the RHSP-DNA interaction. Several models are put forth to explain the stimulation of the unwinding reaction catalyzed by Rep protein. In addition, the potential physiological significance of the RHSP-stimulated Rep protein unwinding reaction is discussed.

INTRODUCTION

DNA helicases are a class of enzyme which catalyze the unwinding of duplex DNA in a unidirectional, energy-dependent reaction (2,3,4). The tightly coupled nucleoside 5′-triphosphate (NTP) hydrolysis reaction presumably provides the energy for unidirectional translocation of the helicase as it catalyzes the disruption of the hydrogen bonds between strands of duplex DNA and/or for hydrogen bond disruption itself. At least nine helicases have been identified in *E. coli* (for reviews see 3,4,5) which participate in many facets of DNA metabolism including replication (6,7), repair (8,9,10) and recombination (10,11). Although different biochemical characteristics have been attributed to the purified enzymes, distinct physiological roles for each helicase have not been completely elucidated.

Rep protein was the first helicase to be genetically characterized (12). This enzyme is required for the replication of several small bacteriophage which infect *E. coli* including φX174 (13,14,15). However, a cellular role for Rep protein in DNA metabolism remains unknown. In φX174 replication, Rep protein interacts with the phage Cistron A (CisA) protein (14,16,17) acting as a helicase to unwind the φX174 chromosome. CisA protein nicks the double-stranded replicative form (RF) of φX174 at a specific site, providing the initiation point for the Rep protein unwinding reaction. Unwinding occurs in a 3′ to 5′ direction, with respect to the DNA strand bound by Rep protein, as the Rep-CisA protein complex travels around the φX174 chromosome ahead of the DNA polymerase III holoenzyme (15,16). Complete unwinding of the phage chromosome is achieved in a processive reaction, allowing replication of the genome to continue via a rolling circle mechanism (14). A role for Rep protein in *E. coli* DNA replication has been suggested by the study of *rep* mutants which show slowed replication fork movement (18). However, *E. coli* cells in which the *rep* gene has been deleted are viable (19). Moreover, the discovery that DnaB protein is a helicase (7) makes it unlikely that Rep protein is the primary replicative helicase.

We have characterized the unwinding reaction catalyzed by Rep protein *in vitro* (1,5). In the absence of additional proteins Rep helicase catalyzes a limited unwinding reaction using partial duplex DNA substrates. The unwinding reaction retains its 3′ to 5′ polarity and efficiently unwinds duplex DNA regions up to 71 base pairs (bp) in length. However, when the duplex region is increased to 343 bp, Rep helicase unwinds only a small fraction of the available partial duplex substrate. This reaction is not stimulated by the addition of *E. coli* single-stranded DNA binding protein (SSB). It has been suggested that Rep helicase does not function processively under these conditions (1,5). The role Rep helicase plays in the cell, therefore, may involve interactions with other proteins which enhance the unwinding reaction. Indeed, a small protein (M_r=15,000), called the Rep helicase stimulatory protein (RHSP), has been isolated from *E. coli* which stimulates the unwinding reaction catalyzed by Rep protein up to 40-fold (1). RHSP does not catalyze the hydrolysis of ATP,

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and does not stimulate the ATP hydrolysis reaction catalyzed by Rep protein. The amount of RHSP required to achieve maximal stimulation of the unwinding reaction suggests a stoichiometric interaction, perhaps with the DNA substrate.

In this report we present additional biochemical evidence for an interaction between RHSP and DNA which leads to the stimulation of the Rep helicase unwinding reaction. We show that formation of an RHSP-DNA complex alleviates a kinetic lag present in RHSP-stimulated unwinding reactions catalyzed by Rep protein. In addition, we show that RHSP binds to, and forms a stable complex with, both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). Sequencing of the 20 NH_2-terminal amino acids of RHSP suggests that it is identical to *E. coli* ribosomal-protein (r-protein) L14. Experiments with rRNA demonstrate that the RHSP-rRNA interaction is similar to the RHSP-DNA interaction.

MATERIALS AND METHODS

Enzymes

Restriction enzymes, T4 polynucleotide kinase and *E. coli* DNA polymerase I (large fragment) were purchased from New England Biolabs or US Biochemicals, Inc. RNasin was from Promega Corporation. Anti-rabbit IgG alkaline phosphatase conjugate was from Sigma.

Helicase II was purified from *E. coli* cells harboring a multicopy plasmid carrying the structural gene for helicase II (20). Rep protein was purified from a Rep overproducing strain, provided by Dr. D. T. Denhardt (University of Western Ontario), as previously described (21).

RHSP was purified as previously described (1) with the following modifications. Fractions throughout the purification procedure were analyzed by (i) Western blot utilizing antibodies directed against *E. coli* r-protein L14, (ii) stimulation of the Rep helicase unwinding reaction and (iii) visualization of the polypeptides on polyacrylamide gels run in the presence of sodium dodecyl sulfate (SDS). Fraction V was dialyzed against 10 mM KPO_4 (pH 6.5), 50 mM KCl, 10% glycerol, 0.5 mM EDTA, 5 mM β-mercaptoethanol, and was applied to a 1 ml MonoS column (Pharmacia) equilibrated in identical buffer. The column was washed with 10 column volumes of equilibration buffer and eluted with a 60 ml linear gradient from 50−750 mM KCl in the equilibration buffer. RHSP eluted at approximately 400 mM KCl. Active fractions were pooled (fraction VI) and dialyzed against 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol, 50% glycerol and stored at −70°C.

DNA, RNA and Nucleotides

[α-^{32}P]dCTP (≈3000 Ci/mmol) was purchased from Amersham Corp. (methyl-^{3}H)thymidine (40−60 Ci/mmol) was from ICN Radiochemicals. 16S/23S rRNA (4μg/ml) was purchased from Boehringer Mannheim. Poly(dT) (≈1100 nucleotides) was from P−L Biochemicals, Inc. M13mp7 ssDNA and RFI DNA were isolated and purified as previously described (22).

DNA substrate preparation

The construction of partial duplex DNA substrates has been described (20). Briefly, a restriction fragment of defined length was isolated from a *Hae*III digest of M13mp7 RFI DNA. The complementary strand of the fragment was annealed onto M13mp7 ssDNA, radiolabelled at its 3′ terminus and purified by gel filtration. [^{32}P]poly(dT) was prepared from poly(dT) which was dephosphorylated and 5′ end-labelled using polynucleotide kinase as described (23).

M13mp7 ss[^{3}H]DNA and RFI [^{3}H]DNA were prepared as described (24,25). The ss[^{3}H]DNA had a specific activity of 33,000 cpm/μg and the RFI [^{3}H]DNA had a specific activity of 35,500 cpm/μg.

RNA substrate preparation

rRNA was 5′ end-labelled using polynucleotide kinase in an exchange reaction as described (23); 20 units of RNasin were included in the reaction mixture. Recovery was estimated to be 75% of the input RNA and the concentration was confirmed by the ethidium dot quantitation method (26). The integrity of the labelled rRNA was confirmed by running a sample on a 1% agarose gel and staining the RNA with ethidium bromide. The gel was subsequently fixed with 10% trichloroacetic acid, dried and analyzed by autoradiography.

Western Blot

Polypeptides were resolved on 12% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose as described (27). The filters were probed with rabbit antibodies generated against *E. coli* r-protein L14, generously donated by Dr. M. Nomura (Irvine, CA). The bound rabbit antibodies were visualized by incubating the filters with anti-rabbit IgG alkaline phosphatase conjugate, followed by a color reaction utilizing nitro blue tetrazolium (Sigma) plus 5-bromo-4-chloro-3-indolyl phosphate (Sigma), as described by Promega Corporation.

Helicase activity assays

The displacement of a [^{32}P]DNA fragment from a ssDNA circle was measured as previously described (20). Helicase reaction mixtures (20 μl) were as previously described (1).

DNA/RNA Binding Assay

The nitrocellulose filter binding assay used to detect binding of RHSP to DNA and RNA has been described (24,28). Binding reaction mixtures (20 μl) were identical to helicase reaction mixtures and contained 43 mM Tris-HCl (pH 7.5), 2 mM ATP, 2 mM $MgCl_2$, 7.5% glycerol (v/v), 5 mM dithiothreitol, 50 μg/ml bovine serum albumin, 0.015 mM EDTA, 0.75 mM 2-mercaptoethanol and 15 mM NaCl, unless otherwise indicated. The amount of RHSP and DNA or RNA in each reaction mixture is indicated in appropriate figure legends. Reaction mixtures were assembled on ice and incubated at 37°C for 5 min. The reaction mixtures were then diluted with either 3 ml or 1 ml of binding buffer (prewarmed to 37°C). Binding buffer was identical to the reaction mixture except that ATP was omitted. The diluted mixtures were filtered through a nitrocellulose filter at 2−4 ml/min. When the reaction mixtures had been diluted to 1 ml, each filter was washed twice with 1 ml of 37°C binding buffer. Nitrocellulose filters (Millipore, HAWP 0.45 μM pore size) were prepared on the day of the experiment by boiling in distilled water for 20 min and storing at room temperature in binding buffer until use. The filters were dried, and the radioactivity bound to the filter was determined in a liquid scintillation counter. 100% control values were determined for each experiment by spotting the appropriate amount of labelled substrate onto a pretreated filter. Background levels were subtracted from all reported experimental data points.

Electron microscopy

Electron microscopy was performed in the laboratory of Dr. Jack Griffith (University of North Carolina). The reaction mixtures (100 μl) were identical to those described for the binding assays except that Hepes (pH 7.5) replaced the Tris-HCl (pH 7.5). Reaction mixtures were incubated at 37°C for 5 min and then fixed by the addition of prewarmed glutaraldehyde to 0.6% final concentration. DNA complexes were isolated by gel filtration through a Sepharose 4B column (equilibrated with 10 mM Tris Acetate (pH 8.0), 0.5 mM EDTA). Aliquots of fractions containing DNA were direct mounted onto carbon-coated, glow-charged copper grids as described (30).

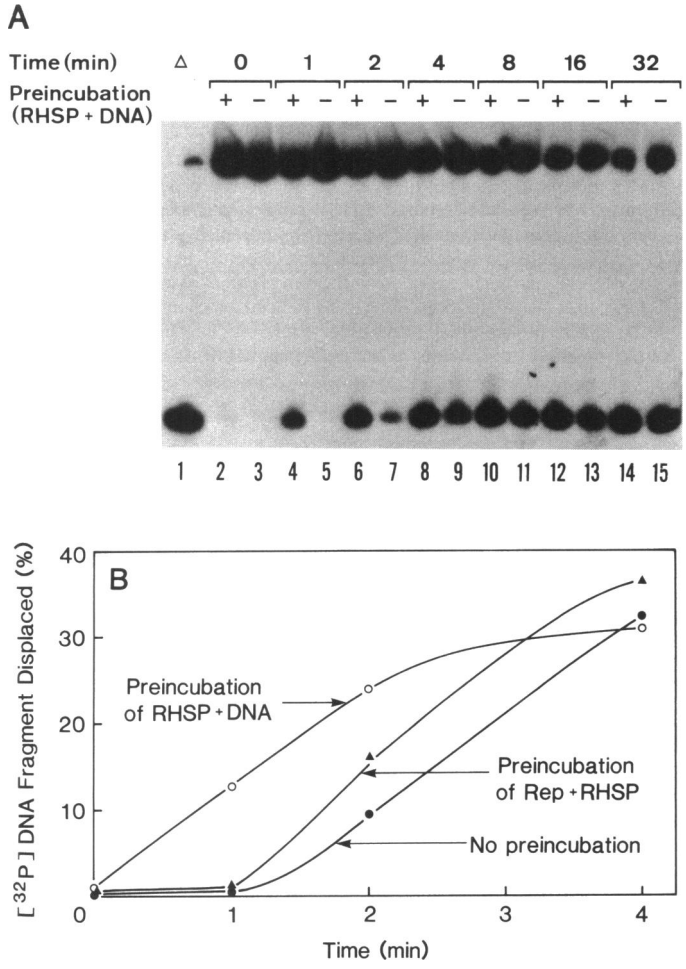

Fig. 1. A kinetic analysis of the RHSP-stimulated unwinding reaction catalyzed by Rep protein. Helicase reactions were as described under 'Materials and Methods' utilizing a 343 bp partial duplex DNA substrate. Reaction mixtures (160 μl) were assembled on ice with or without 270 ng RHSP and/or 220 ng Rep protein and then incubated at 37°C. At the indicated times, aliquots (20 μl) were removed and analyzed by gel electrophoresis. (**A**) In the odd numbered gel lanes, reaction mixtures were assembled without Rep protein or RHSP and incubated at 37°C for 5 minutes. Rep protein and RHSP were then added simultaneously to initiate the unwinding reaction. In the even numbered gel lanes, reaction mixtures containing both RHSP and the DNA substrate were preincubated at 37°C for 5 minutes. Rep protein was then added to initiate the unwinding reaction. (**B**) (●), quantitative data obtained from reaction mixtures as described for the odd numbered gel lanes in (**A**). (○), quantitative data obtained from reaction mixtures described for the even numbered gel lanes in (**A**). (▲), quantitative data obtained from reaction mixtures including Rep protein and RHSP preincubated at 37°C for 5 minutes. DNA was added to initiate the reaction. In each experiment, the 0 min time point was taken immediately following the addition of all the reaction components.

Protein Sequencing

The sequence of the amino terminal 20 amino acids of RHSP was obtained by K.Stone (Yale University) using RHSP isolated on a 12% SDS-polyacrylamide gel and subsequently transferred to an immobilon membrane filter (Millipore). The sequence from purified, acid precipitated RHSP was determined by R.Henry (University of North Carolina). In both laboratories, the amino terminal sequence was determined by Edman degradation using an automated gas-phase microsequencer (31).

Other methods

Protein concentrations were determined as described by Bradford (32) using bovine serum albumin as the standard. SDS-polyacrylamide gel electrophoresis was performed as described (33). DNA concentrations were determined by measuring the optical density at 260 nm and are expressed in nucleotide equivalents. The concentration of the helicase substrate was estimated from the concentration of DNA used in the annealing reaction assuming a 75% recovery from the gel filtration column.

RESULTS

RHSP interacts with the DNA substrate

Results obtained during the initial characterization of RHSP (1) suggested that RHSP stimulated the Rep protein unwinding reaction by interacting with the DNA substrate. An examination of the kinetics of the Rep-RHSP unwinding reaction revealed the presence of a time lag during the initial two min of incubation (Fig. 1A lanes 3,5,7; Fig. 1B). No unwinding was detected after one min of incubation followed by a rapid increase in the amount of DNA unwound during the next three min of incubation. The extent of unwinding increased slightly after 4 min, under these conditions, and reached a plateau between 6 and 8 min of incubation. This kinetic lag was relieved when an RHSP-DNA complex was formed by preincubating RHSP with the DNA

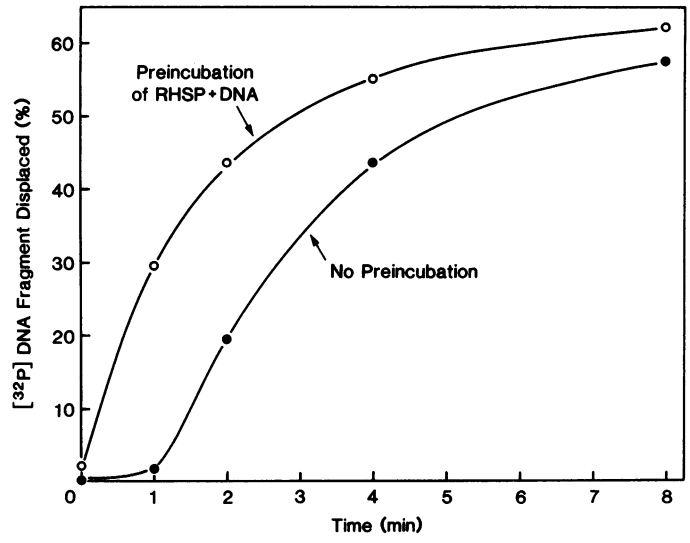

Fig. 2. A kinetic analysis of the RHSP-stimulated helicase II reaction. (●), quantitative data obtained from reaction mixtures identical to those described for the odd numbered gel lanes in Fig. 1A except that 120 ng helicase II was used and Rep protein was omitted. (○), quantitative data obtained from reaction mixtures identical to those described for the even numbered gel lanes in Fig. 1A except that 120 ng of helicase II replaced Rep protein.

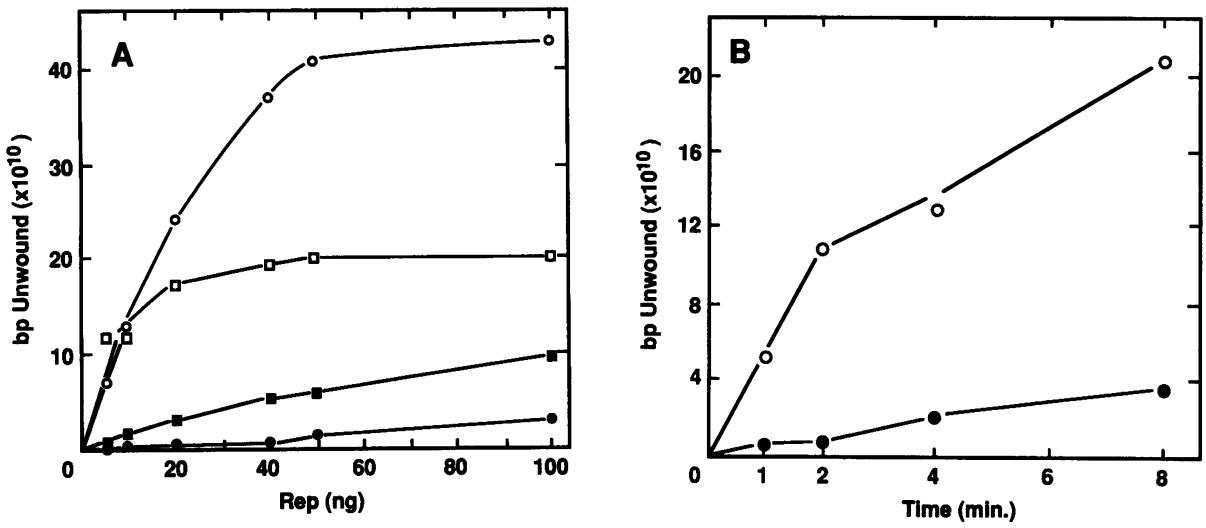

Fig. 3. RHSP increases the rate and the extent of the unwinding reaction catalyzed by Rep protein. The number of base pairs unwound was determined from the fraction of the [^{32}P]DNA fragment displaced. (**A**) Helicase reactions were as described under 'Materials and Methods' using the indicated amounts of Rep protein. (●), quantitative data obtained from reaction mixtures containing the 343 bp partial duplex substrate in the absence of RHSP. (○), quantitative data obtained using the 343 bp partial duplex substrate in reaction mixtures which contained the amount of RHSP required to achieve maximal stimulation (27 ng). (■), quantitative data obtained from reactions using the 71 bp partial duplex substrate in the absence of RHSP. (□), quantitative data obtained from reactions using the 71 bp partial duplex substrate in reaction mixtures which contained the amount of RHSP required to achieve maximal stimulation (27 ng). (**B**) Helicase reactions were as described under 'Materials and Methods' using the 343 bp partial duplex DNA substrate and 50 ng of Rep protein in the presence of 4 mM rATP. Aliquots (20 μl) were removed at the indicated times and analyzed on a polyacrylamide gel. (●), quantitative data obtained from reaction mixtures lacking RHSP; (○), quantitative data obtained from reaction mixtures containing 150 ng RHSP.

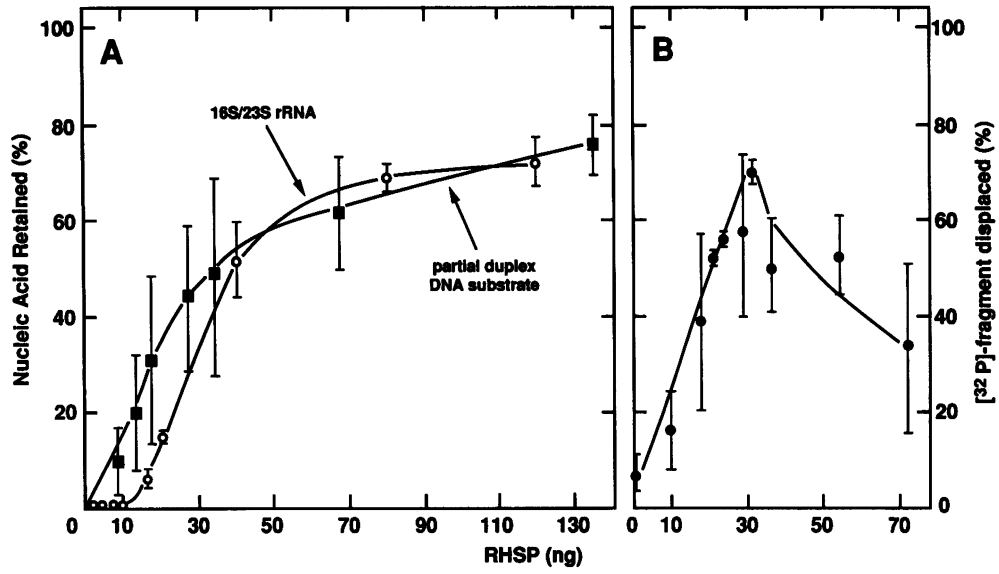

Fig. 4 RHSP binds the partial duplex DNA substrate and rRNA. (**A**) Nitrocellulose filter binding assays were as described under 'Materials and Methods' using the indicated amounts of RHSP and 2 μM 343 bp partial duplex DNA substrate (■) or 1.2 μM rRNA (○). (**B**) Helicase reactions were performed as described under 'Materials and Methods' using the 343 bp partial duplex substrate, 100 ng of Rep protein and the indicated amounts of RHSP.

substrate for 5 min in the reaction mixture prior to the addition of Rep protein (Fig.1A, lanes 2,4,6; Fig. 1B). Under these conditions, significant unwinding occurred during the first 2 min of the reaction. The fraction of the DNA substrate unwound continued to increase slightly before reaching a plateau between 6 and 8 min of incubation. RHSP-DNA complex formation was rapid and energy-independent since the kinetic lag was relieved to the same extent when the preincubation was shortened to 1 min, and when performed in the absence of ATP (data not shown). In addition, no effect on the initial unwinding reaction kinetics was observed if RHSP was incubated with Rep protein prior to the addition of DNA substrate (Fig. 1B) or if Rep protein and the DNA substrate were preincubated prior to the addition of RHSP (data not shown). In the former case, a slight increase in the extent of unwinding was evident at each time point after 1 min. Preincubation of RHSP with the DNA substrate at 0°C

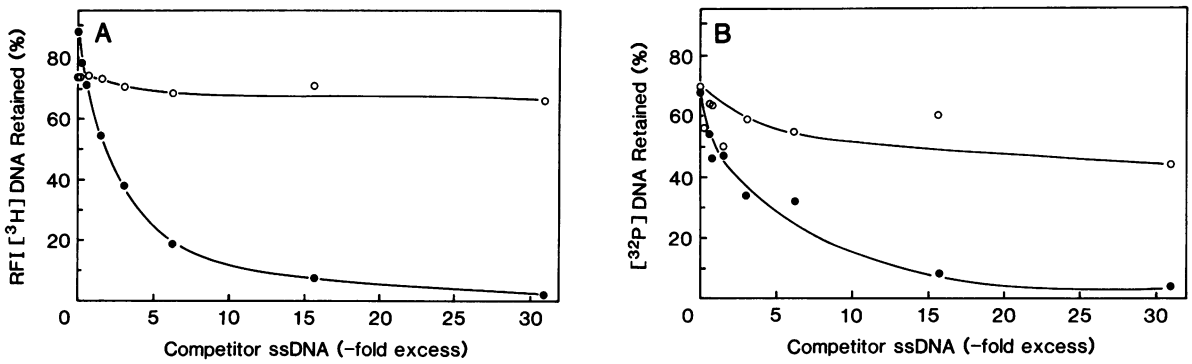

Fig. 5 ssDNA does not compete for RHSP bound to DNA. Nitrocellulose filter binding assays were performed as described under 'Materials and Methods'. (**A**) (○), 68 ng of RHSP was incubated with 4 μM M13mp7 RFI [^{3}H]DNA at 37°C for 5 minutes, prior to the addition of the indicated amounts of competitor M13mp7 ssDNA. (●), indicated amounts of competitor ssDNA and 4 μM RFI [^{3}H]DNA were present in the reaction mixture prior to the addition of 68 ng of RHSP. (**B**) (○), 68 ng of RHSP was incubated with 2 μM [^{32}P]DNA helicase substrate at 37°C for 5 minutes, prior to the addition of the indicated amounts of competitor ssDNA. (●), the indicated amounts of competitor ssDNA and 2 μM [^{32}P]DNA helicase substrate were present in the reaction mixture prior to the addition of 68 ng of RHSP.

did not relieve the kinetic lag (data not shown), although filter binding experiments demonstrated that RHSP bound DNA at 0°C (not shown). Therefore, the kinetic lag observed in unwinding reactions which included a 0°C preincubation was, most likely, due to the time required for the reaction mixture to reach 37°C in order for Rep protein to catalyze unwinding.

RHSP also stimulates the helicase reaction catalyzed by helicase II (1). As observed using Rep protein, the kinetics of the RHSP-helicase II unwinding reaction exhibit a kinetic lag (Fig. 2), with little detectable unwinding during the first min of incubation. The fraction of the substrate unwound increased rapidly from 2 min through 6 min of incubation, and the extent of unwinding did not increase appreciably after 8 min. The lag observed in the RHSP-helicase II unwinding reaction was also relieved by preincubating the DNA substrate with RHSP (Fig. 2), again suggesting the existence of an RHSP-DNA complex on which the unwinding reaction is enhanced. In this case, unwinding was substantial during the first min of incubation and reached a plateau after 8 min.

RHSP increases the extent and the rate of unwinding catalyzed by Rep protein

The addition of an optimal amount of RHSP markedly increased the extent of the helicase reaction catalyzed by Rep protein using either a 71 base pair (bp) or a 343 bp partial duplex substrate (Fig. 3A). This effect was evident at all concentrations of Rep protein tested. In the absence of RHSP, unwinding of a 343 bp partial duplex substrate was severely limited. In fact, fewer total bp of duplex DNA were unwound by Rep protein on the 343 bp substrate than on the 71 bp substrate at all Rep protein concentrations tested (Fig. 3A). In the presence of RHSP, however, essentially equal numbers of bp were unwound on each substrate at low concentrations of Rep protein. At higher Rep protein concentrations, the number of bp unwound on the 343 bp substrate continued to increase while the number of bp unwound on the 71 bp substrate reached a plateau due to depletion of the substrate.

To determine the effect of RHSP on the rate of the Rep protein unwinding reaction, a kinetic analysis was performed using optimal concentrations of both Rep protein and RHSP and the 343 bp partial duplex substrate (Fig. 3B). 4 mM rATP was included in the reaction mixtures to insure that the amount of ATP available was not limiting. The addition of RHSP markedly stimulated the rate of the unwinding reaction. RHSP increased the rate of the unwinding reaction approximately 20-fold during the first 2 min of incubation, and approximately 6-fold during the following 6 min of incubation. The reason for the apparent biphasic kinetics is unknown.

RHSP binds DNA

Previous results (1) and those presented above have led us to conclude that RHSP stimulates Rep protein and helicase II catalyzed unwinding reactions through an interaction with the DNA substrate. This prompted a more direct investigation of the interaction between RHSP and DNA. Nitrocellulose filter binding assays were employed to study the binding of RHSP to DNA. RHSP bound the 343 bp partial duplex DNA substrate in a concentration-dependent manner (Fig. 4A). RHSP exhibited apparently equal binding affinities for a variety of DNA topologies, as evidenced by the similar binding curves obtained utilizing M13mp7 circular ssDNA, RFI dsDNA or linear dsDNA (not shown). Interestingly, the amount of RHSP required for 50% binding also effected maximal stimulation of the Rep helicase unwinding reaction (Fig. 4B). The addition of more RHSP caused retention of greater amounts of DNA on the nitrocellulose filters, but inhibited the unwinding reaction catalyzed by Rep protein.

To insure that RHSP was not simply binding to secondary structure present in the M13mp7 ssDNA molecules, binding to poly(dT) was investigated. Gel mobility shift assays were employed because unbound poly(dT) was retained by nitrocellulose filters preventing the use of filter binding assays. The mobility of [^{32}P]poly(dT) through nondenaturing polyacrylamide gels (34) was altered upon the addition of increasing amounts of RHSP. A concentration of RHSP which caused 50% retention of DNA in the filter binding reactions resulted in a shifted migration of approximately 50% of the poly(dT) (not shown). Increasing amounts of RHSP caused all of the poly(dT) to exhibit the same altered mobility. RHSP, therefore, binds both ssDNA and dsDNA.

The identification of RHSP as r-protein L14 (see below) prompted an investigation of the interaction between RHSP and rRNA under the conditions in which the Rep protein unwinding

reactions were stimulated. The binding of RHSP to a mixture of 23S and 16S rRNA was directly analyzed by nitrocellulose filter binding assays (Fig. 4A). The rRNA binding curve differed from the DNA binding curve at the lowest amounts of RHSP tested where very little rRNA was retained and 10–20% of the DNA was retained. The sigmoidal shape of the rRNA binding curve indicates that the interaction between RHSP and rRNA may be cooperative.

RHSP forms a stable complex with DNA

The stability of the RHSP-DNA complex was assessed in several challenge experiments (Fig. 5). RHSP was incubated with a labelled DNA substrate to form an RHSP-DNA complex. The complexes were then challenged with increasing amounts of competitor DNA. Up to a 30-fold molar excess of ssDNA molecules did not significantly disrupt RHSP-DNA complexes during 5 min of incubation. Similar results were obtained whether RHSP was bound to dsDNA (Fig. 5A) or the helicase substrate (Fig. 5B) regardless of whether the unlabelled competitor DNA was ssDNA or dsDNA (not shown).

In order to determine the rate of dissociation of RHSP from DNA, RHSP-DNA complexes were formed using the helicase substrate. A 50-fold molar excess of ssDNA molecules was added, the incubation continued and aliquots were removed at specific time points to 120 min. Excess competitor DNA molecules disrupted slightly less than 50% of the RHSP-DNA complexes during 120 min of incubation (data not shown). This suggests that the RHSP-DNA complex is extremely stable with a half-life greater than 120 min.

Although the RHSP-DNA complex formed under standard reaction conditions was quite stable, formation of the complex was very sensitive to NaCl concentration (data not shown). At 60 mM NaCl, the amount of DNA retained by RHSP on nitrocellulose filters was reduced to 20% of that bound under standard reaction conditions (15–25 mM). At concentrations of NaCl greater than 100 mM, essentially no binding of RHSP to DNA was detected. The effect of NaCl concentration on the RHSP-stimulated unwinding reaction catalyzed by Rep protein could not be determined because the Rep unwinding reaction is also inhibited by NaCl concentrations above 25 mM (data not shown).

Electron microscopic visualization

Electron microscopy (EM) was employed to visualize the RHSP-DNA complexes formed with M13mp7 circular ssDNA, M13mp7 RFI dsDNA and M13mp7 linear dsDNA. The reaction conditions were essentially identical to those used in helicase assays. The RHSP to DNA ratio was varied from 10-fold below that required for maximal stimulation to an RHSP to DNA ratio equal to that required for maximal stimulation. Figure 6A shows an example of the RHSP-DNA complexes observed with a protein to DNA ratio equal to half that required for maximal stimulation using M13mp7 RFI dsDNA. Similar complexes were found when the protein to DNA ratio was reduced to one tenth that required for maximal stimulation (Fig. 6B), indicative of cooperative protein-protein interactions. The complexes appeared identical using either RFI DNA or ssDNA (data not shown) at both RHSP to DNA ratios. ssDNA which is not complexed with protein was not visible under the conditions employed so micrographs using RFI DNA are shown illustrating the structure of the DNA regions which are protein-free. At protein to DNA ratios equivalent to that required for maximal stimulation of the unwinding reaction, some very large structures, possibly protein aggregates complexed with DNA, were seen (not shown). However, few DNA molecules (complexed with RHSP or naked) were found. It seems likely that large aggregates formed and failed to enter the sizing column used to purify the protein-DNA complexes prior to visualization by EM. In all samples utilizing lower RHSP to DNA ratios, many complexes were found and appeared very similar. The DNA became highly condensed and looped out around the protein. With the lowest protein to DNA ratios, more naked DNA was present than when the amount of RHSP approached that required for maximal stimulation in an unwinding

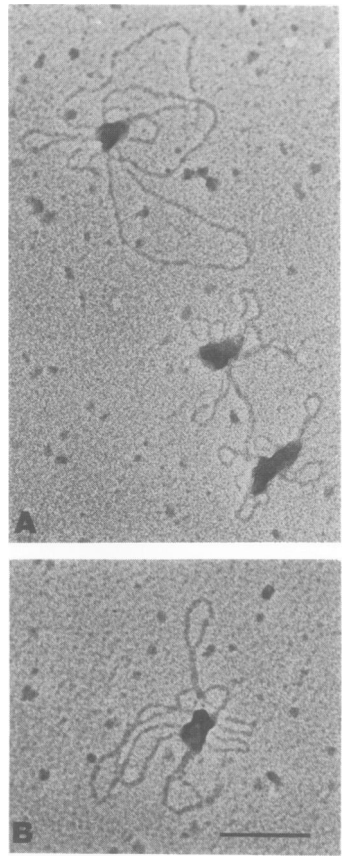

Fig. 6 Electron microscopic visualization of RHSP-DNA complexes. Reaction mixtures (100 μl) were as described under 'Materials and Methods'. Magnification = 250K. Bar represents 0.1 μm. (**A**) 20 μM M13mp7 RFI dsDNA and 425 ng RHSP; 0.5× that required for maximal stimulation of the Rep helicase unwinding reaction. (**B**) 20 μM M13mp7 linear dsDNA and 84 ng of RHSP, 0.1× that required for maximal stimulation of the Rep helicase unwinding reaction utilizing.

Table 1. The amino terminal 20 amino acids of RHSP are identical to those of r-protein L14.

L14	M	I	Q	E	Q	T	M	L	N	V	A	D	N	S	G	A	R	R	V	M
RHSP-1	x	I	Q	E	Q	T	M	L	N	V	A	D	n	s	G	A	i	x	V	m
RHSP-2	M	I	Q	E	Q	T	M	L	N	V	A	D	N	S	G	A	r	r	V	M

The amino terminal amino acid sequence of r-protein L14 is from published data (35). RHSP-1 is the amino terminal sequence determined from RHSP immobilized on immobilon membranes. RHSP-2 is the amino terminal sequence determined from purified, acid precipitated RHSP. Upper case letters indicate the amino acids determined from definitive degradation cycles. Lower case letters indicate the amino acids from somewhat ambiguous cycles. x's indicate cycles which did not yield an interpretable signal.

reaction. It should be noted that large aggregates were not found when RHSP was incubated in the reaction mixture without DNA, and that similar complexes were observed regardless of the fixation procedure used.

RHSP is apparently identical to ribosomal protein L14

To determine the identity of RHSP, the amino terminal 20 amino acids were sequenced from two different preparations of RHSP. In both cases the sequence was identical to that of *E. coli* r-protein L14 (Table 1). To insure that the stimulatory activity was in fact due to the r-protein, and not due to an undetected protein present in the preparations, fractions throughout the purification procedure were monitored by Western blotting with antibodies directed against r-protein L14. In addition, each fraction was assayed for stimulatory activity and visualized on SDS-polyacrylamide gels. A representative elution profile from a monoS column is shown in Figure 7. The stimulatory activity and r-protein L14 coeluted in fractions which were collected from the phosphocellulose column (not shown), the DNA cellulose column (not shown) and the monoS column.

A sample of partially purified RHSP was applied to a Sephadex G75 gel filtration column. Stimulatory activity and r-protein L14 coeluted at a position correlating to a M_r between 13,000 and 18,000. In addition, partially purified RHSP was resolved on a 12% SDS-polyacrylamide gel, the protein was eluted and renatured using guanidine hydrochloride as described (35). Stimulatory activity was found in the sample eluted from the band previously identified as RHSP suggesting that the major protein present was responsible for the stimulatory activity (data not shown). No activity was detected in samples eluted from other sections in the gel.

Finally, RHSP was added to helicase reaction mixtures which contained Rep protein, the 343 bp partial duplex DNA substrate and increasing levels of rRNA. Stimulatory activity was inhibited by 50% when the molar concentration of rRNA was approximately equal to that of the DNA in the reaction mixture (data not shown). These data suggest that stimulation was due to a protein which interacted with rRNA as well as the DNA substrate.

RHSP does not bind rRNA preferentially

The affinity of RHSP for rRNA relative to that for DNA was assessed by adding RHSP to reaction mixtures which contained both the helicase substrate and varying concentrations of unlabelled rRNA (Fig. 8). The amount of RHSP added corresponded to the amount required to achieve 50% retention of the DNA in the filter binding assays. No preference for binding to rRNA was observed since binding to the labelled substrate was reduced by 50% when the number of rRNA molecules was nearly identical to the number of labelled DNA molecules present. Similar results were obtained when RHSP was added to reaction mixtures which contained the DNA substrate and various amounts of unlabelled DNA (not shown). Moreover, a 150-fold molar excess of rRNA molecules could not disrupt preformed RHSP-DNA complexes over a period of 120 min. (data not shown).

DISCUSSION

The dsDNA unwinding reaction catalyzed by either Rep protein or helicase II can be stimulated by a small basic protein we have called RHSP (1). When Rep protein or helicase II and RHSP were added simultaneously to initiate unwinding there was a short, but reproducible, kinetic lag in the unwinding reaction. This kinetic lag was eliminated by preincubating RHSP with the DNA substrate. Preincubation of RHSP with Rep protein had no effect. RHSP must, therefore, interact with the DNA substrate in order to stimulate the unwinding reaction catalyzed by Rep protein or helicase II. The kinetic lag observed when RHSP and Rep protein

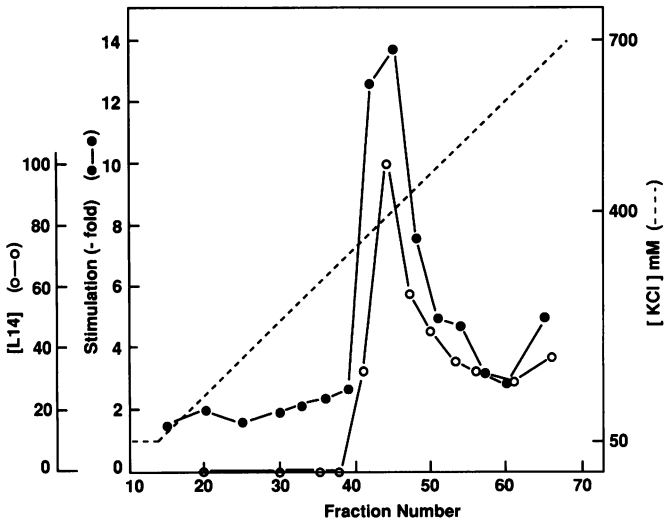

Fig. 7 R-protein L14 coelutes with RHSP. Fractions eluting from a MonoS column were assayed for (●) Rep helicase stimulatory activity using the helicase reaction as described under 'Materials and Methods' with 50 ng of Rep protein and 1 μl of a 1:10 dilution of the indicated MonoS fractions, and (○) the presence of r-protein L14 by Western blot as described under 'Materials and Methods' utilizing 10 μl of each fraction. The relative staining intensities were arbitrary units determined from a densitometric scan of a negative from the Western blotted filter.

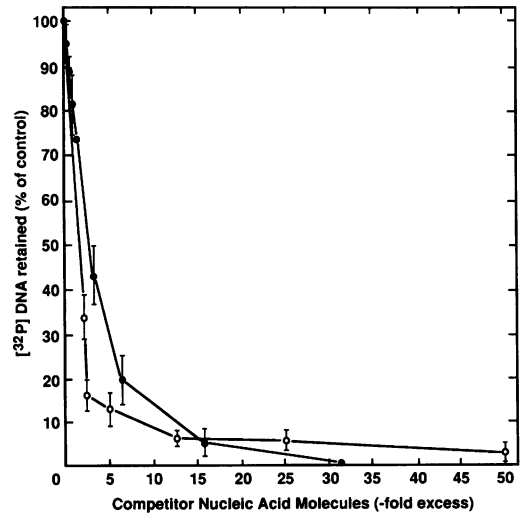

Fig. 8 RHSP does not exhibit a greater affinity for rRNA than for DNA. Nitrocellulose filter binding assays were as described under 'Materials and Methods' using 2 μM [^{32}P]DNA helicase substrate and 34 ng RHSP, plus the indicated amounts of either competitor rRNA (○) or competitor M13mp7 circular ssDNA (●). The level of RHSP used corresponds to the amount required for approximately 50% binding in the absence of competitor DNA. This value was adjusted to 100% to facilitate comparison.

are added simultaneously must be due to the time required for RHSP to form a complex with the DNA substrate. Once this complex has formed, the RHSP-stimulated unwinding reaction is able to proceed at an increased rate relative to reactions containing Rep protein alone. Filter binding studies and electron microscopy indicate that RHSP does indeed form a stable complex with both ssDNA and dsDNA. This is an unusually stable complex with a $t_{1/2}$ for dissociation greater than 120 min at 37°C.

An analysis of unwinding reactions catalyzed by Rep protein revealed that fewer bp of duplex DNA were unwound using the 343 bp partial duplex substrate than the 71 bp partial duplex substrate. Apparently, as the length of the duplex region increases the ability of Rep protein to move through that duplex region decreases. This suggests that Rep protein, in the absence of additional proteins, does not catalyze a processive unwinding reaction. If the unwinding reaction was processive in mechanism, similar fractions of both the 71 bp and 343 bp substrates would be expected to be unwound. This would result in a 5-fold increase in the total number of bp unwound on the 343 bp substrate, which was not observed. However, the addition of RHSP caused an equal number of base pairs to be unwound utilizing the 343 bp substrate and the 71 bp substrate. This is similar to the protein concentration-dependent unwinding reaction catalyzed by helicase II (20). Interestingly, the unwinding reaction in the presence of RHSP is not processive, further suggesting that the interaction between Rep protein and CisA protein in ϕX174 DNA replication is crucial for the processive character of the Rep unwinding reaction in that system. Binding of RHSP to the partial duplex DNA substrate caused an increase in the rate, as well as the extent, of the unwinding reaction catalyzed by Rep protein. This effect was most evident when the 343 bp partial duplex substrate was used. The RHSP-DNA complex may, therefore, provide an environment which allows Rep protein to proceed through longer duplex regions more rapidly, thereby catalyzing the unwinding of more bp with each interaction between Rep protein and the DNA substrate.

There are several ways in which an RHSP-DNA complex might stimulate unwinding by Rep protein or helicase II. i) RHSP could bind preferentially to the single-stranded region of the partial duplex substrate, reducing the sites to which Rep protein could bind nonproductively. ii) Excess RHSP could bind to the unwound strands behind an advancing Rep protein molecule to prevent reannealing of the DNA subsequent to the unwinding event. In either case, Rep protein would, in effect, become a more efficient helicase. However, both of these models are based on the notion that RHSP binds with higher affinity to ssDNA versus dsDNA. However, no difference was detected in the binding of RHSP to ssDNA or dsDNA by filter binding assays. Moreover, EM has illustrated the binding of RHSP to both dsDNA and ssDNA under helicase reaction conditions. We note that even a slightly higher affinity for ssDNA may be enough to cause RHSP to bind to the ssDNA region initially. Cooperative protein-protein interactions would then cause all the ssDNA regions to be coated so that Rep protein could be preferentially directed to the dsDNA region of the DNA molecule. Once enough protein is present to coat the entire molecule, the Rep protein unwinding reaction would be inhibited, which has been observed (1). This model might also be valid in the absence of any preference for ssDNA. There is so little dsDNA on the substrate molecule employed that random, cooperative binding by RHSP may preferentially mask the ssDNA region thereby targeting Rep protein to the dsDNA in the same manner. Thus, a targeting effect of RHSP cannot be excluded.

Alternatively, or in addition to a targeting effect, the RHSP-DNA complex may stabilize the Rep protein-DNA complex. This model is intriguing because Rep protein exhibits low processivity, in the absence of additional proteins (1), yet is able to processively unwind the ϕX174 chromosome ahead of the advancing DNA polymerase III holoenzyme in the presence of the phage CisA protein (14,16). However, unlike the phage encoded CisA protein, 400−600 molecules of RHSP per DNA circle are required to effect maximal stimulation (1). Furthermore, preincubation of RHSP with Rep protein, in the absence of DNA, did not affect the initial kinetics of unwinding suggesting that a specific Rep-RHSP interaction did not play a significant role in increasing the rate of the unwinding reaction. The slight increase in the extent of unwinding which is observed after 2 min when RHSP is preincubated with Rep protein may suggest that protein-protein interactions do have a slightly positive effect on the extent of the unwinding reaction. Specific protein-protein or protein-DNA-protein interactions are suggested by the fact that RHSP stimulates the unwinding reactions catalyzed by Rep protein and helicase II, but has no effect on helicase IV or helicase I (1).

Another alternative considers the differential energetics of particular DNA structures. Binding of RHSP to DNA may strain the double-stranded region of the substrate and lower the energy required for the disruption of the hydrogen bonds, making it more favorable for Rep protein to unwind the duplex region. The energetics of a torsionally strained dsDNA helix have been shown by computer modelling to favor disruption of base paired regions (36). EM indicated that RHSP formed a large structure with DNA and caused the DNA to become highly condensed and looped. If held rigidly, it is plausible that this structure imparts strain on the DNA which might be relieved by unwinding duplex regions.

It is difficult to determine what functional significance the RHSP-stimulated Rep helicase reaction might have in the cell. A role for Rep protein in *E. coli* cellular metabolism has not been determined, so it is not known whether stimulation of the unwinding reaction is physiological. The apparent identity of RHSP as r-protein L14, as well as genetic data showing that *rep/rho* double mutants are not viable (37), present the formal possibility that Rep protein may play a role in transcription and translation. Preliminary data indicate that Rep protein is capable of unwinding short RNA·DNA partial duplexes, and that RHSP stimulates this reaction (unpublished observations, this laboratory). In cells deficient for the Rho protein, Rep protein may somehow compensate for the Rho protein RNA·DNA unwinding activity. Alternatively, a similar activity may be required to remove transcription complexes ahead of an advancing replication fork. RHSP may interact with Rep protein to promote unwinding of the mRNA·DNA hybrid in order to release the transcription complex and allow replication to proceed normally.

A complete understanding of the RHSP-stimulated unwinding reactions catalyzed by Rep protein or helicase II will require further investigation. Until a physiological role for Rep protein is uncovered, the significance of stimulation by RHSP will remain obscure. Current work is underway to investigate the possibility that Rep protein participates in transcription regulation. Once the function of Rep protein is better understood, a more complete analysis of the RHSP-stimulated unwinding reaction will be achieved.

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