ATPase activity of *Escherichia coli* Rep helicase crosslinked to single-stranded DNA: Implications for ATP driven helicase translocation

(kinetics/mechanism/protein–DNA crosslinking/protein oligomerization/energy transduction)

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Communicated by Carl Frieden, Washington University School of Medicine, St. Louis, MO, May 22, 1996 (received for review March 24, 1996)

ABSTRACT To examine the coupling of ATP hydrolysis to helicase translocation along DNA, we have purified and characterized complexes of the *Escherichia coli* Rep protein, a dimeric DNA helicase, covalently crosslinked to a single-stranded hexadecameric oligodeoxynucleotide (S). Crosslinked Rep monomers (PS) as well as singly ligated (P₂S) and doubly ligated (P₂S₂) Rep dimers were characterized. The equilibrium and kinetic constants for Rep dimerization as well as the steady-state ATPase activities of both PS and P₂S crosslinked complexes were identical to the values determined for uncrosslinked Rep complexes formed with dT₁₆. Therefore, ATP hydrolysis by both PS and P₂S complexes are not coupled to DNA dissociation. This also rules out a strictly unidirectional sliding mechanism for ATP-driven translocation along single-stranded DNA by either PS or the P₂S dimer. However, ATP hydrolysis by the doubly ligated P₂S₂ Rep dimer is coupled to single-stranded DNA dissociation from one subunit of the dimer, although loosely (low efficiency). These results suggest that ATP hydrolysis can drive translocation of the dimeric Rep helicase along DNA by a “rolling” mechanism where the two DNA binding sites of the dimer alternately bind and release DNA. Such a mechanism is biologically important when one subunit binds duplex DNA, followed by subsequent unwinding.

DNA helicases are motor proteins that use the chemical energy of nucleoside 5′-triphosphate (NTP) hydrolysis to perform the mechanical work of disrupting the base pairs between complementary strands of duplex DNA to form single-stranded DNA (ssDNA) intermediates (1, 2). These molecular motors are essential components of most DNA metabolic and processing machineries in all organisms (2), and mutations in helicases involved in DNA repair processes have been linked to a number of human skin cancers (3–5). Despite the importance of these enzymes and the large number of DNA helicases that have been identified, mechanistic studies on these enzymes have only begun recently (for reviews, see refs. 1, 6, and 7).

DNA helicases must translocate along DNA to unwind DNA processively and thus have features in common with motor proteins such as cytoplasmic kinesin (8–10). DNA helicases appear generally to be oligomeric, primarily dimeric or hexameric (1, 6, 7); for example, the *Escherichia coli* Rep helicase functions as a dimer (11), with both subunits able to bind DNA and ATP (10, 12, 13). Of particular mechanistic interest is how helicases translocate along DNA and how this is coupled to NTP hydrolysis. In this regard, most helicases display a macroscopic “polarity” in DNA unwinding assays *in vitro*—i.e., unwinding by most helicases is stimulated if the DNA duplex possesses a 5′ ssDNA “tail” flanking the duplex (a 5′ → 3′ helicase) or a 3′ ssDNA tail (a 3′ → 5′ helicase). This observation has led to the suggestion that helicases couple the energy derived from ATP hydrolysis to

translocate unidirectionally along ssDNA, although direct evidence in support of this is lacking. On the other hand, we (14) have shown that unidirectional translocation along ssDNA is not essential for DNA unwinding by the *E. coli* Rep helicase, a 3′ → 5′ DNA helicase. Furthermore, we have proposed an active, rolling model for Rep-catalyzed DNA unwinding (13) based on our finding that one subunit of the Rep dimer binds directly to the duplex region to be unwound while the other subunit binds to ssDNA (13, 14), and we noted that such a rolling model could also be envisioned as a mechanism for translocation of the Rep dimer along ssDNA.

To distinguish between these mechanisms for helicase translocation along ssDNA, we reasoned that any directional bias in translocation requires coupling of ATP hydrolysis to movement. In particular, a strictly unidirectional sliding mechanism (no “slippage”), in which the same protein subunit maintains contact with the ssDNA during translocation, would require tight coupling to hydrolysis, while a “rolling” mechanism without directional bias would not. Toward this end, we characterized the ATPase activities of covalently crosslinked Rep–single-stranded–oligodeoxynucleotide complexes, reasoning that severe inhibition of the ATPase activities of such complexes would result if ATP-driven translocation occurs by a strictly unidirectional sliding mechanism. Such crosslinked Rep–ssDNA complexes will also provide a useful means for studying putative intermediates in the DNA unwinding reaction.

MATERIALS AND METHODS

Reagents and Buffers. [α -³²P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham. Spectrophotometric grade glycerol, HPLC grade methanol, and 99% triethylamine were obtained from Aldrich. Sulfo-*N*-succinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate (sulfo-SANPAH) was obtained from Pierce. Amino-modifier-C2-dT phosphoramidite was obtained from Glen Research (Sterling, VA). All solutions were made with reagent grade chemicals, except as noted above, using Milli-Q H₂O—i.e., distilled H₂O that was de-ionized using a Milli-Q Water Purification System (Millipore). All binding experiments and ATPase assays were carried out in BBM buffer [20 mM Tris-HCl, pH 7.5 at 4°C/6 mM NaCl/5 mM MgCl₂/10% (vol/vol) glycerol]. Triethyl-ammonium bicarbonate buffer (TEAB, 1 M) was made by bubbling CO₂ (g) derived from subliming dry ice into an aqueous solution containing >1 M triethylamine at 0°C for 4 hr or until a pH of 7.5 was achieved; Milli-Q H₂O was then added to achieve a final concentration of 1 M. Kinase buffer was 50 mM Tris-HCl (pH 7.5 at 25°C), 10 mM MgCl₂, and 10 mM 2-mercaptoethanol.

Proteins, Enzymes, and Oligodeoxynucleotides. *E. coli* Rep protein was purified to >99% homogeneity from *E. coli* MZ-1/

Abbreviations: ssDNA, single-stranded DNA; sulfo-SANPAH, sulfo-*N*-succinimidyl-6-(4′-azido-2′-nitro-phenylamino)hexanoate.

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pRepO (15) as described (16). Rep concentration was determined spectrophotometrically using $\epsilon_{280} = 7.68 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for Rep monomer (14). An ATPase-deficient mutant of Rep, K28I, in which Lys-28 was replaced with Ile, was constructed and purified to >99% purity (unpublished data). T4 polynucleotide kinase was purchased from United States Biochemical. Oligodeoxynucleotides dT₁₆, d(T₃C²T₁₂), d(T₇C²T₈), d(T₁₂C²T₃), where C² denotes amino-modifier-C2-dT, were synthesized using an Applied Biosystems model PCR-mate 391 DNA synthesizer and were purified to >99% homogeneity as described (17) and dialyzed (Spectra/Por 7 MWCO 1000) into Milli-Q H₂O for storage. Oligodeoxynucleotide concentrations were determined spectrophotometrically in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl at 25°C using $\epsilon_{260} = 1.29 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ per molecule.

Synthesis and Purification of d(T₇S²T₈). Solid sulfo-SANPAH was added to d(T₇C²T₈) (350 μM) in 0.5 M Na-carbonate (pH 9.0) buffer to a final concentration of 25 mM and allowed to react in the dark at 25°C for ≥ 4 h. Three volumes of ice-cold ethanol was added and the sample chilled at -20°C for 1 h to precipitate the DNA. The large bright orange pellet was collected by centrifugation at 4°C and rinsed with 1 volume of 1,4-dioxane, followed by 1 volume of 100% ethanol, then dried and resuspended in Milli-Q H₂O at 55°C for 5 min. The sample was chilled on ice for 30 min and centrifuged for 15 min at 4°C. The supernatant was transferred to a fresh microfuge tube, being careful to avoid an orange oily residue that clings to the old microfuge tube. The solution was then purified through a 25 ml Bio-Gel P2 column in 50 mM Na-carbonate buffer (pH 9.0). The faintly pink band eluting in the void volume was collected and contained d(T₇S²T₈). An equal volume of 1 M TEAB buffer (pH 7.5) was added to the pooled fractions and the sample loaded onto a 900 mg Maxi-Clean C₁₈ cartridge (Alltech Associates) activated with 1 ml methanol then equilibrated with 5 ml 10 mM TEAB (pH 7.5). Following washes of 5 ml 10 mM TEAB (pH 7.5) and 1 ml of Milli-Q H₂O, d(T₇S²T₈) was eluted with 50% (vol/vol) methanol and dried in the Speed-Vac. The purified DNA was >97% derivatized as determined spectroscopically using an extinction coefficient at 260 nm, $\epsilon_{260} = 1.4 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$. d(T₃S²T₁₂) and d(T₁₂S²T₃) were prepared in the same manner. Care was taken to avoid buffers that contained ammonia or primary amines—e.g., Tris or EDTA—and to minimize exposure of samples to light. However, the compound is stable in ambient light for at least 20 min.

Synthesis and Purification of Crosslinked Rep-d(T₇S²T₈). Rep protein was dialyzed extensively against 5 $\times$ binding buffer (100 mM Tris, pH 7.5/30 mM NaCl/5 mM EDTA/50% glycerol). Crosslinking was carried out at 1 μM Rep monomers and 5 μM d(T₇S²T₈) in 25 ml 2 $\times$ binding buffer (40 mM Tris, pH 7.5/12 mM NaCl/2 mM EDTA/20% glycerol) in a shallow 3-inch-diameter dish in the cold room. The sample was UV irradiated for 4–6 h. Buffer containing 50 mM Tris (pH 7.5) and 20% glycerol without salt was added to replace volume loss due to evaporation, and 5 M NaCl added to a final concentration of 100 mM. This was loaded onto a 2 ml Macro-Prep Hi-Q anion exchange column (Bio-Rad) equilibrated in 50 mM Tris (pH 7.5), 100 mM NaCl, and 20% glycerol, rinsed with 4 ml of the same buffer, and eluted with buffer containing 1 M NaCl. Fractions of six drops each were collected. Pink DNA containing fractions were pooled (≈ 3 ml) and carefully layered onto a 30 ml Bio-Gel P-6 column equilibrated with buffer containing 50 mM Tris (pH 7.5), 1 M NaCl, and 10% glycerol running at a 5 ml/h. Fractions of 1.5 ml were collected while monitoring the absorbance of the eluate at both 254 nm and 280 nm. Crosslinked complexes ($A_{280} > A_{260}$) eluted in the void volume which was collected and pooled. Buffer containing 50 mM Tris (pH 7.5) and 20% glycerol was added to the

sample to 25 ml and the sample was dialyzed for 6 h against 1 liter of the same buffer containing 100 mM NaCl. The dialysate was reloaded onto the Macro-Prep Hi-Q column that had been reequilibrated with 100 mM NaCl. The column was rinsed with 10 ml buffer containing 100 mM NaCl and eluted with 1 M NaCl as before. The fractions containing protein and DNA were pooled and dialyzed against 5 $\times$ binding buffer. Due to the thermodynamic stability of P₂S and the negative cooperativity of binding DNA to P₂S to form P₂S₂, the purified crosslinked mixture contained a significant fraction of uncrosslinked Rep subunits in the form of P₂S. However, all uncrosslinked DNA has been removed.

ATPase Assay. ATPase activity was determined at 4°C by measuring the initial rate of conversion of ATP to ADP using [α -³²P]ATP (Amersham) in BBM buffer. Reactions were typically initiated by addition of 10 μl of 10 mM ATP to 90 μl protein. At regular time intervals, 10 μl aliquots were removed and quenched into 10 μl 0.5 M EDTA (pH 8.5). Intervals between time points ranged from 5 to 20 s depending on the anticipated rate of hydrolysis. Eight to 10 time points were taken per assay, but only the linear portion of each time course, <60% product formation, was used to determine the initial velocity. The extent of ADP formation was monitored by spotting 1 μl onto polyethyleneimine-cellulose TLC plates (Merck). TLC plates were developed using 0.3 M potassium phosphate (pH 7.0) as the mobile phase, dried, and imaged on a Betascope 603 direct β -emission imager (Betagen). Spots corresponding to radiolabeled ATP and ADP were quantitated using software supplied by the manufacturer.

Concentration Dependence of the ATPase Activity of Crosslinked Rep-d(T₇S²T₈). Crosslinked Rep-d(T₇S²T₈) was serially diluted to span a range of concentrations from 2 μM to 27 nM. The solutions were allowed to equilibrate at 4°C in the cold room for 30 min and then assayed for ATPase activity as described above. The resulting isotherm was analyzed by nonlinear least-squares methods (18) and the following three equilibria:

For a mixture of native and crosslinked Rep monomers, the concentration of PS at any concentration of total Rep monomer, P_T , is given by the real nonnegative root of Eq. 1:

$$[\text{PS}]^3 + \frac{K_2(K_2K_3 + K_3 + 2K_2K_5)}{2K_2^2K_3K_5}[\text{PS}]^2 + \frac{K_2[1 - K_3P_T(2r - 1)] + 1}{2K_2^2K_3K_5}[\text{PS}] - \frac{rP_T}{2K_2^2K_3K_5} = 0 \quad [1]$$

where $r = 0.61$ is the fraction of crosslinked subunits. The concentrations of the other species are given by Eqs. 2a–d.

$$[\text{P}] = \frac{(1 - r)(2K_2^2K_5[\text{PS}] + K_2 + 1)[\text{PS}]}{(2r - 1)K_2K_3[\text{PS}] + r} \quad [2a]$$

$$[\text{PS}^*] = K_2[\text{PS}] \quad [2b]$$

$$[\text{P}_2\text{S}] = K_2K_3[\text{PS}][\text{P}] \quad [2c]$$

$$[\text{P}_2\text{S}_2] = K_2^2K_5[\text{PS}]^2 \quad [2d]$$

The fit is not sensitive to the isomerization between PS and PS*, however, the equilibrium was included, constraining $K_2 = 13$ as determined by Bjornson *et al.* (19). The specific ATPase

Kinetics of Transient Formation of P_2S_2 . For the experiments measuring the transient formation of P_2S_2 , 75 μ l of a Rep-d($T_7S^2T_8$) solution (2 μ M or 1.66 μ M) preincubated with 0.34 μ M Rep was mixed with 75 μ l of $2\times [dT_{16}]$ at $t = 0$. At 20-s intervals, 10 μ l aliquots were removed and mixed with 10 μ l $2\times [ATP]$ and allowed to react for 10 s. Ten microliters was then transferred into 10 μ l of 0.5 M EDTA to quench the reaction. In this experiment, the time courses were not linear beyond 10 s. However, within the first 10 s of the reaction, the rate of ATP hydrolysis was constant as determined by rapid

Data Analysis. FITSIM (21) was executed on an IBM PS/2 Model 76. NONLIN (18) was executed on a Hewlett-Packard 715. All other data analyses were performed using KALEIDAGRAPH (Synergy Software, Reading, PA) on an Apple Macintosh Quadra 700.

Although all of the DNA in the “purified” samples of Rep-d(T₇S²T₈) was crosslinked to Rep subunits, the sample also contained free Rep protein dimerized (noncovalently) to crosslinked Rep, because DNA binding induces Rep dimerization. We determined the fraction of crosslinked Rep subunits by directly titrating an aliquot of the purified sample, containing 200 nM total Rep protomers, with free Rep monomers (containing no DNA) while monitoring the ATPase activity (Fig. 2). The ATPase activity increased linearly on addition of free Rep monomers to a concentration of 42 nM and then plateaued at an activity of 9.5 s⁻¹ per total Rep monomer, corresponding to an activity of 19 s⁻¹ per crosslinked P₂S. This plateau value is identical within error to the rate of ATP hydrolysis by the uncrosslinked P₂S, indicating that the ATPase activity of P₂S was

Rep dimerization is induced upon DNA binding (11) such that the dimerization equilibrium constant increases by at least 4–5 orders of magnitude on binding DNA (12, 13) and dimerization increases Rep's ATPase activity dramatically (19, 20, 22). Consequently, Rep can form multiple complexes with a 16-base oligodeoxynucleotide, depending on the DNA and Rep concentrations. Rep monomers can be free (P) or bound to ssDNA (PS), and two types of Rep dimers can form, P₂S and P₂S₂, where one or both subunits are bound to ssDNA, respectively (12, 13). These

FIG. 2. Direct titration to determine fraction of crosslinked Rep subunits. The “purified” Rep-d(T7S2T8) sample contains a mixture of crosslinked and uncrosslinked Rep subunits. To determine the fraction of crosslinked Rep protomers in this mixture, Rep-d(T7S2T8) (200 μ M total protomers) was mixed with increasing amounts of free Rep monomers (BBM buffer at 4°C) and the ATPase activity was monitored.

are shown schematically in Table 1. Under the conditions of our experiments (BBM buffer at 4°C), Bjornson *et al.* (19) have shown that ssDNA-induced Rep dimerization proceeds according to the mechanism in Scheme 1 with kinetic and equilibrium constants shown in Table 2 (19, 22).

To determine if DNA crosslinking influenced the energetics of Rep dimerization, we first examined the kinetics of dissociation of the crosslinked P₂S dimer to form P and PS by monitoring the time-dependent loss of ATPase activity following the addition of a mutant Rep protein, K28I, to trap dissociated crosslinked Rep monomers (PS). K28I is defective in ATPase activity due to a Lys-28 to Ile substitution in the conserved GX₄GKT sequence of the putative ATP binding site (I.W., unpublished experiments). This mutant serves as an efficient trap for dissociated PS species because the heterodimer formed between a wild-type and a K28I protomer shows no steady-state ATPase activity (I.W., unpublished experiments). Since P₂S first undergoes rate-limiting dissociation to form PS and P before PS can then dimerize with K28I, the time-dependent loss of activity reflects the rate of dissociation of the dimer. The time course of the ATPase activity of Rep-d(T₇S²T₈) (0.4 μM total Rep protomer) mixed with excess K28I (2.0 μM monomer) is shown in Fig. 3A fitted to a single exponential with an observed rate constant of $2.5 \pm 0.04 \times 10^{-3} \text{ s}^{-1}$. This rate constant is identical to that measured for dissociation of native P₂S dimer ($2.7 \pm 0.8 \times 10^{-3} \text{ s}^{-1}$) by fluorescence stopped-flow experiments under identical solution conditions (19).

The change in the ATPase activity of the crosslinked Rep-d(T₇S²T₈) mixture as a function of concentration (27 nM to 2.0 μM total protomer) (Fig. 3B) showed further that the equilibrium constants for Rep dimerization were unaffected by ssDNA crosslinking. The increase in ATPase activity with increasing protein concentration was biphasic with an intermediate plateau region between 100 to 300 nM. Consistent with the negative cooperativity for DNA binding (12, 13, 22), the intermediate plateau region represents formation of P₂S while the second transition reflects formation of the P₂S₂ dimer. Due to the low solubility limits of Rep, it was not possible to saturate this second phase. From nonlinear analysis of the data, we determined a dimerization constant, $K_3 = 1.7 \pm 0.1 \times 10^8 \text{ M}^{-1}$, for the formation of P₂S from PS* and P and an apparent $k_{\text{cat}} = 18 \pm 1.2 \text{ s}^{-1}$ for ATP hydrolysis by P₂S. We were not able to resolve the dimerization constant, K_5 , for forming P₂S₂ from 2 PS*, due to the lack of an end-point for the second transition. We were, however, able to obtain a best fit value of $k_{\text{cat}} = 77 \pm 9 \text{ s}^{-1}$ for ATP hydrolysis by P₂S₂ by constraining K_5 to $1.6 \times 10^5 \text{ M}^{-1}$ as measured for native Rep (20). The population distribution of PS, PS*, P₂S, and P₂S₂ calculated from these values of K_2 , K_3 , and K_5 are shown overlaid on the fit to the data.

We then measured the ATPase activities of each ssDNA ligation state of the crosslinked Rep to compare with those determined for uncrosslinked complexes (20). The monomer species, PS, was formed at 1 nM total subunit concentration; based on our previous studies of uncrosslinked Rep-dT₁₆ complexes as well as the value of K_3 determined in the above titration, only monomers, P and PS, are present at this concentration. Since

Table 2. Kinetic and equilibrium constants for protein dimerization of crosslinked versus native Rep

	Crosslinked	Native
PS* + P $\rightleftharpoons$ P ₂ S		
$k_3, \text{M}^{-1}\text{s}^{-1}$	$4.3 \pm 0.3 \times 10^5$	$4.5 \pm 0.3 \times 10^5^*$
k_{-3}, s^{-1}	$2.5 \pm 0.04 \times 10^{-3}$	$2.7 \pm 0.08 \times 10^{-3}^*$
K_3, M^{-1}	$1.7 \pm 0.1 \times 10^8$	$1.7 \pm 0.5 \times 10^8^*$
P ₂ S + S $\rightleftharpoons$ P ₂ S ₂		
$k_4, \text{M}^{-1}\text{s}^{-1}$	394 ± 6	$387 \pm 3^\dagger$
k_{-4}, s^{-1}	$<1.1 \times 10^{-3}$	$<1.1 \times 10^{-3}^\dagger$
2PS* $\rightleftharpoons$ P ₂ S ₂		
$k_5, \text{M}^{-1}\text{s}^{-1}$	$1.5 \pm 0.1 \times 10^3$	$1.3 \pm 0.2 \times 10^3^\dagger$
k_{-5}, s^{-1}	$6.9 \pm 0.1 \times 10^{-3}$	$7.9 \pm 0.1 \times 10^{-3}^\dagger$
K_5, M^{-1}	$2.2 \pm 0.2 \times 10^5$	$1.65 \pm 0.25 \times 10^5^\dagger$

Data are for BBM buffer at 4°C.

*From Bjornson *et al.* (19).

†From Wong *et al.* (20).

the ATPase activity of unligated Rep monomer, P, is 1000-fold lower than that of PS, its ATPase activity is not detectable (20). The ATP concentration dependence of the ATPase activity (BBM buffer at 4°C) of crosslinked PS normalized with respect to total PS concentration (Fig. 4A, ●) is well described by a rectangular hyperbola with best fit values of $k_{\text{cat}} = 2.29 \pm 0.07 \text{ s}^{-1}$ and $K_M = 2.3 \pm 0.19 \text{ μM}$, and is virtually indistinguishable from data obtained with uncrosslinked PS (○) ($k_{\text{cat}} = 2.17 \pm 0.04 \text{ s}^{-1}$ and $K_M = 2.05 \pm 0.1 \text{ μM}$) (20).

The steady-state ATPase activity of the crosslinked P₂S dimer, normalized to the total P₂S concentration is shown in Fig. 4B (●). The data was fitted to a rectangular hyperbola, with best fit values of $k_{\text{cat}} = 18.3 \pm 0.3 \text{ s}^{-1}$ and $K_M = 3.1 \pm 0.2 \text{ μM}$. Also shown are data previously obtained for uncrosslinked P₂S (○) fitted to $k_{\text{cat}} = 16.5 \pm 0.2 \text{ s}^{-1}$ and $K_M = 2.7 \pm 0.2 \text{ μM}$ (20). Therefore, crosslinked and uncrosslinked P₂S also have identical ATPase activities within experimental uncertainty.

Lastly, we measured k_{cat} for ATP hydrolysis by the fully ligated Rep dimer, P₂S₂, with one or both of the Rep subunits crosslinked to ssDNA. Due to the negative cooperativity for DNA binding to the Rep dimer, it is difficult to populate a sufficiently large fraction of P₂S₂ at equilibrium. However, we have previously shown that the ATPase activity of P₂S₂ can be accurately determined by measuring the ATPase activity as a function of time following the addition of excess dT₁₆ to a solution of P₂S (20) since this results in a transient accumulation of P₂S₂ prior to re-equilibration to PS (Fig. 5). The results from a series of such experiments performed at 0.5 μM P₂S and 6.25, 12.5, 25, 50, and 100 μM dT₁₆ are shown in Fig. 5A. The observed transient increase in ATPase activity was dependent on [dT₁₆] and correlates well with transient formation of P₂S₂. The subsequent loss of activity corresponds to dissociation of the dimer to yield an equilibrium mixture of PS*, PS, and P₂S₂ as shown previously (20). The [dT₁₆] dependence of the rate of the transient increase gives the apparent association rate constant, k_4 , for binding ssDNA to P₂S. The amplitude of this transient is directly proportional to the difference between the k_{cat} values of P₂S and P₂S₂. The rate

Table 1. ATPase activities of ssDNA ligation states of crosslinked versus native Rep

		$k_{\text{cat}}, \text{s}^{-1}$		$K_M, \text{μM}$	
		Crosslinked	Native	Crosslinked	Native
	P		$2 \times 10^{-3}^*$		$7 \times 10^{-3}^*$
	PS	2.30 ± 0.07	$2.17 \pm 0.04^\dagger$	2.30 ± 0.19	$2.05 \pm 0.1^\dagger$
	P ₂ S	18.3 ± 0.3	$16.5 \pm 0.2^\dagger$	3.1 ± 0.2	$2.7 \pm 0.2^\dagger$
	P ₂ S ₂	68 ± 2	$71 \pm 2.5^\dagger$		

Data are for BBM buffer at 4°C.

*From Moore and Lohman (23).

†From Wong *et al.* (20).

FIG. 3. Kinetics and thermodynamics of crosslinked dimers. (A) An excess ($2.0 \mu\text{M}$) of Rep mutant, K28I, was added to Rep-d($\text{T}_7\text{S}^2\text{T}_8$) ($0.4 \mu\text{M}$). PS formed from the dissociation of P_2S to PS and P is trapped by rapid rebinding to this mutant to form heterodimers which are defective in ATPase activity. The time dependence of the loss of activity therefore corresponds to the rate of P_2S dimer dissociation and fits to a single exponential with a rate constant of $2.5 \pm 0.04 \times 10^{-3} \text{ s}^{-1}$. (B) The ATPase activity of Rep-d($\text{T}_7\text{S}^2\text{T}_8$) is very sensitive to protein concentration due to the linkage to Rep dimerization. The solid line represents the nonlinear least-square best fit of the ATPase activities at different protein concentrations using k_{cat} values of 2 s^{-1} , 18 s^{-1} , and $77 \pm 9 \text{ s}^{-1}$ for the ATPase activities of PS + PS^* , P_2S , and P_2S_2 , respectively. Equilibrium dimerization constants K_3 and K_5 for formation of P_2S from $\text{PS}^* + \text{P}$ and P_2S_2 from 2PS^* were $1.7 \pm 0.1 \times 10^8 \text{ M}^{-1}$ and $1.5 \pm 0.1 \times 10^5 \text{ M}^{-1}$, respectively. The isomerization equilibrium for PS to PS^* was fixed at $K_2 = 13$. The species distributions are also shown.

of decay of the transient gives the rate of dissociation of P_2S_2 to form 2PS^* . These data were globally fitted together with data obtained at a different protein concentration (not shown) by FITSIM (21) according to Scheme I. Solid lines represent best fits using $k_4 = 3.94 \pm 0.06 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$, $k_5 = 1.5 \pm 0.1 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, $k_{-5} = 6.9 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$, and $k_{\text{cat}} = 68 \pm 2 \text{ s}^{-1}$. The dissociation rate of DNA from P_2S_2 , k_{-4} , was not resolved beyond an upper limit of $1.1 \times 10^{-3} \text{ s}^{-1}$. The rate constant for the dissociation of P_2S to form PS^* and P, k_{-3} , was fixed at 0.0025 s^{-1} as determined in the mutant trapping experiment, and the association rate constant, k_3 , was fixed at $4.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, calculated as $k_{-3}K_3$ using a value of $K_3 = 1.7 \times 10^8 \text{ M}^{-1}$ as determined in the titration described above. Fig. 5B shows the predicted species population distribution during the time course of the reaction shown in the top curve of Fig. 5A based on these rate constants, which confirms that the transient rise in ATPase activity results from the transient accumulation of P_2S_2 which then dissociates to form 2PS .

DISCUSSION

Tables 1 and 2 summarize the kinetic and equilibrium constants for Rep dimerization and the k_{cat} values for ATP hydrolysis by the crosslinked Rep-d($\text{T}_7\text{S}^2\text{T}_8$) complexes, PS, P_2S , and P_2S_2 , and compares them with those measured for the uncrosslinked complexes formed with dT₁₆ (19, 22). All parameters measured for the crosslinked and the uncrosslinked species are identical within experimental error. Neither the Rep dimerization energetics nor the initial velocity steady-state ATPase activities of any of the ssDNA ligation states are perturbed by DNA crosslinking. The ATPase activity of Rep was also not changed upon crosslinking to either d($\text{T}_3\text{S}^2\text{T}_{12}$) or d($\text{T}_{12}\text{S}^2\text{T}_3$) (data not shown). These results demonstrate that ATP hydrolysis by Rep is not tightly coupled to DNA disso-

FIG. 4. Steady-state ATPase activity of crosslinked Rep monomer (PS) and singly ligated Rep dimer (P_2S). (A) Crosslinked PS was formed by performing experiments at 1 nM total protomers and ATPase activity was assayed as a function of $[\text{ATP}]$. The solid line represents best fit of the data ($\bullet$) to a rectangular hyperbola with $k_{\text{cat}} = 2.3 \pm 0.07 \text{ s}^{-1}$ and $K_M = 2.3 \pm 0.19 \mu\text{M}$. $\circ$, Data for uncrosslinked PS obtained at 1 nM Rep and 50 nM dT₁₆. The dotted line represents best fit of the uncrosslinked data to $k_{\text{cat}} = 2.17 \pm 0.04 \text{ s}^{-1}$ and $K_M = 2.05 \pm 0.1 \mu\text{M}$. (B) Free Rep monomers ($0.07 \mu\text{M}$) were preincubated with $0.33 \mu\text{M}$ crosslinked mixture to form $0.2 \mu\text{M}$ crosslinked P_2S and assayed for ATPase activity as a function of $[\text{ATP}]$. The solid line represents best fit of the data ($\bullet$) to a rectangular hyperbola with $k_{\text{cat}} = 18.3 \pm 0.08 \text{ s}^{-1}$ and $K_M = 3.1 \pm 0.2 \mu\text{M}$. $\circ$, Data for uncrosslinked P_2S obtained at $0.5 \mu\text{M}$ Rep monomers and of 3 nM dT₁₆ ($>99\%$ Rep is P_2S under these concentrations). The dotted line represents the best fit of the uncrosslinked data to $k_{\text{cat}} = 16.5 \pm 0.2 \text{ s}^{-1}$ and $K_M = 2.7 \pm 0.2 \mu\text{M}$.

ciation or translocation, thus ruling out any mechanisms for helicase translocation that require tight coupling.

We also have shown that the time courses of ATP hydrolysis by both crosslinked and uncrosslinked P_2S dimers remain linear until $>60\%$ of the starting ATP has been converted to ADP. This result is consistent with the hypothesis that the ssDNA does not dissociate from the P_2S dimer during steady-state ATP hydrolysis. Net dissociation of ssDNA from P_2S would result in dimer dissociation and a resulting loss of linearity in the time course of ATP hydrolysis because the rate of dimerization to reform P_2S would be rate-limiting under

FIG. 5. Transient kinetic formation of P_2S_2 and determination of its ATPase activity. (A) Crosslinked P_2S ($0.5 \mu\text{M}$) was mixed with 6.25 ($\blacktriangle$), 12.5 (∇), 25 ($\blacktriangledown$), 50 ($\circ$), and $100 \mu\text{M}$ ($\bullet$) dT₁₆ to form transiently crosslinked P_2S_2 at $t = 0$. At 20-s intervals, the ATPase activity was measured and was observed to increase transiently followed by a slower decay. Solid lines represent best fits to all data sets using the rate constants shown in Tables 1 and 2 according to Scheme I. (B) The simulated population distribution of species is displayed for the top curve in A showing transient formation of P_2S_2 from P_2S and its dissociation to PS.

these reaction conditions. However, because of the steady-state nature of the ATPase assay, this observation of linearity alone does not constitute proof that dissociation does not occur as there could be other compensating effects such as from changes in the protein oligomeric state (20). The crosslinking results, however, provide strong corroborating evidence that the ATPase activity of P_2S is not coupled to DNA dissociation. The results are therefore most consistent with the ssDNA remaining bound to Rep in both its PS and P_2S complexes during multiple rounds of ATP hydrolysis.

Because we have ruled out ATP coupled dissociation of ssDNA from both P_2S and PS, the only remaining possible mode of translocation in these ligation states, if it occurs at all, would be via sliding (i.e., translocation while the same protein subunit maintains contact with the DNA). A related question is whether ATP hydrolysis fuels unidirectional sliding as a mechanism for helicase translocation along ssDNA (24–26). The extent of any directional bias during translocation must be directly related to the degree of coupling between ATP hydrolysis and movement. In one extreme, strictly unidirectional translocation without “slippage” would require a high coupling efficiency between ATP hydrolysis and translocation such that tethering of the DNA to the helicase by crosslinking would be expected to severely inhibit its ATPase activity. In the other extreme, if ATP hydrolysis is independent of DNA movement, then it could not possibly fuel a directionally biased translocation. Therefore, our results indicate that translocation does not occur by a strictly unidirectional sliding mechanism since the tethered single-stranded oligodeoxynucleotide would restrict translocation and thus inhibit ATP hydrolysis. Although we cannot completely rule out a biased directional sliding mechanism, this would have to occur with low efficiency. Furthermore, the fact that the same ssDNA stimulated ATPase activity can be obtained with either dT_{16} or poly(dT) (20) also indicates that biased directional translocation is not required for maximal ATPase activity.

If ATP hydrolysis by a P_2S Rep dimer does not stimulate DNA dissociation or translocation by sliding, then how might a Rep dimer translocate along DNA? Unlike the P_2S dimer, ATP hydrolysis by the doubly ligated P_2S_2 Rep dimer is linear for only the first ≈ 10 s, after which it decreases significantly, indicating that continued ATP hydrolysis by the P_2S_2 dimer leads to changes in its DNA ligation and/or dimerization states (20, 27). In fact, recent stopped-flow fluorescence measurements show that dissociation of a single-stranded oligodeoxynucleotide from one subunit of the P_2S_2 Rep dimer is enhanced ≈ 60 -fold during the course of ATP hydrolysis (27). Therefore, the rate of DNA dissociation from one subunit of P_2S_2 , but not P_2S or PS, is enhanced during ATP hydrolysis, indicating that ATP hydrolysis is coupled to DNA dissociation from P_2S_2 . However, even for this reaction, an average of ≈ 150 ATPs are turned over for each net dissociation of DNA indicating a low coupling efficiency ($\leq 1\%$) under our conditions (BBM buffer at 4°C). Therefore, DNA dissociation from P_2S_2 is not obligatory during each ATPase cycle. This is consistent with our result that the initial steady-state ATPase activity of the P_2S_2 dimer is unaffected by DNA crosslinking.

Based on these results we propose a “rolling” model of translocation for the dimeric Rep helicase that takes advantage of the two distinct DNA binding sites of the dimer (12, 13). In this model, net movement results from transient binding of ssDNA to the unligated subunit of P_2S followed by release of ssDNA from the first subunit. This is the same type of translocation mechanism that we previously proposed for the dimeric Rep helicase during its DNA unwinding reaction (13), the only difference being that translocation during unwinding occurs upon binding duplex DNA, D, into the free Rep subunit to form a P_2SD intermediate; this duplex DNA is then unwound to transiently form a P_2S_2 complex. Translocation along ssDNA by this mechanism need not occur with any net directionality. As we have previously proposed

(13), the macroscopic $3' \rightarrow 5'$ “polarity” of DNA unwinding by the Rep helicase may originate from the asymmetry inherent at the unwinding junction. This is supported by the results reported here because a random diffusion process would not require a tight coupling of energy derived from ATP hydrolysis. Such a rolling mechanism also provides a means for processive translocation of the dimeric helicase. Whereas the increase in the rate of dissociation of ssDNA from one subunit of the P_2S_2 Rep dimer upon ATP hydrolysis would result directly in an increased rate of translocation, the lack of an ATP-stimulated DNA dissociation from the P_2S dimer would ensure that one subunit of the dimer (although not always the same subunit) remains tightly bound to the ssDNA lattice throughout the translocation cycle.

There is currently no direct evidence to support the common assumption that unidirectional sliding of helicases along ssDNA is crucial to their mechanism of DNA unwinding. Interestingly, observations that an increase in the length of the ssDNA lattice results in a net increase in the ATPase activity of DNA helicases have been cited as support for ATP-driven unidirectional translocation (25). In this context, the crosslinked Rep–ssDNA complexes described here would approximate the limit of Rep bound to an infinitely long ssDNA lattice, yet we observe no net increase in ATPase activity in these crosslinked complexes relative to uncrosslinked complexes of Rep bound to dT_{16} , a single-stranded oligodeoxynucleotide approximately equal in length to the site size for the Rep monomer (12). Furthermore, we have also shown that the ≈ 2 -fold increase in ATPase activity of Rep bound to poly(dT) versus dT_{16} is due to an increase in the transient population of the P_2S_2 species (20).

We have previously proposed an active “rolling” model for Rep helicase-catalyzed DNA unwinding in which the dimer simultaneously binds to both duplex and ssDNA (13). Our current findings also rule out a strict unidirectional sliding along ssDNA as a mechanism for a targeted search by the Rep helicase for duplex DNA. We now propose, based on these findings, that the Rep helicase can translocate along ssDNA via a similar rolling model without need for an explicit directional bias, although this may occur by an as yet unknown mechanism. The same rolling or subunit switching mechanism can be readily extended to account for translocation of the ring-like hexameric helicases with each subunit providing a potential DNA binding site (1).

We thank Bill Van Zante for oligodeoxynucleotide synthesis and purification, and Keith Bjornson and Janid Ali for critical discussions and Carl Frieden and Peter Burgers for comments on the manuscript. This work was supported by grants from the National Institutes of Health (GM 45948) and the American Cancer Society (NP-756B). I.W. received partial support from an American Cancer Society Postdoctoral Fellowship (PF-3671).

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