Biochemical Characterization of Adeno-Associated Virus Rep68 DNA Helicase and ATPase Activities

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The adeno-associated virus (AAV) nonstructural proteins Rep68 and Rep78 are site-specific DNA binding proteins, ATP-dependent site-specific endonucleases, helicases, and ATPases. These biochemical activities are required for viral DNA replication and control of viral gene expression. In this study, we characterized the biochemical properties of the helicase and ATPase activities of homogeneously pure Rep68. The enzyme exists as a monomer in solution at the concentrations used in this study (<380 nM), as judged by its mobility in sucrose density gradients. Using a primed single-stranded (ss) circular M13 substrate, the helicase activity had an optimum pH of 7 to 7.5, an optimum temperature of 45°C, and an optimal divalent-cation concentration of 5 mM MgCl₂. Several nucleoside triphosphates could serve as cofactors for Rep68 helicase activity, and the order of preference was ATP > GTP > CTP = dATP > UTP > dGTP. The Kₘ values for ATP in both the DNA helicase reaction and the site-specific trs endonuclease reaction were essentially the same, approximately 180 μM. Both reactions were sigmoidal with respect to ATP concentration, suggesting that a dimer or higher-order multimer of Rep68 is necessary for both DNA helicase activity and terminal resolution site (trs) nicking activity. Furthermore, when the enzyme itself was titrated in the trs endonuclease and ATPase reactions, both activities were second order with respect to enzyme concentration. This suggests that a dimer of Rep68 is the active form for both the ATPase and nicking activities. In contrast, DNA helicase activity was linear with respect to enzyme concentration. When bound to ssDNA, the enzyme unwound the DNA in the 3′-to-5′ direction.

DNA unwinding occurred at a rate of approximately 345 bp per min per monomeric enzyme molecule. The ATP turnover rate was approximately 30 to 50 ATP molecules per min per enzyme molecule. Surprisingly, the presence of DNA was not required for ATPase activity. We estimated that Rep translocates processively for more than 1,300 bases before dissociating from its substrate in the absence of any accessory proteins. DNA helicase activity was not significantly stimulated by substrates that have the structure of a replication fork and contain either a 5′ or 3′ tail. Rep68 binds only to ssDNA, as judged by inhibition of the DNA helicase reaction with ss or double-stranded (ds) DNA. Consistent with this observation, no helicase activity was detected on blunt-ended ds oligonucleotide substrates unless they also contained an ss 3′ tail. However, if a blunt-ended ds oligonucleotide contained the 22-bp Rep binding element sequence, Rep68 was capable of unwinding the substrate. This means that Rep68 can function both as a conventional helicase for strand displacement synthesis and as a terminal-repeat-unwinding protein which catalyzes the conversion of a duplex end to a hairpin primer. Thus, the properties of the Rep DNA helicase activity suggest that Rep is involved in all three of the key steps in AAV DNA replication: terminal resolution, reinitiation, and strand displacement.

Adeno-associated virus (AAV) codes for two large nonstructural proteins, Rep68 and Rep78, that are synthesized from alternately spliced messages initiated by the viral p5 promoter (14, 24). Genetic studies have shown that these Rep proteins are indispensable for AAV DNA replication and transcriptional control of the viral promoters (4, 5, 25). Except for their C-terminal ends, the two proteins have the same amino acid sequence (24). Rep68 and Rep78 also exhibit the same set of biochemical activities. The first of these is a site-specific DNA binding activity which directs the enzyme to bind a 22-bp sequence, the Rep binding element (RBE), that is present in the AAV terminal repeats (TRs); the p5 promoter; and human chromosome 19 (1, 12, 19, 22, 29). Another Rep function is a site-specific endonuclease activity (5, 23). Once bound to a hairpinned AAV TR, Rep cuts a unique strand at the terminal resolution site (trs) to produce a nick at nucleotide 124 of the AAV sequence (5). During this process, a covalent tyrosine phosphate linkage is made between Rep and the 5′ phosphate at the nick (21). Genetic evidence suggests that tyr₁₁₅₂ is required for the tyr linkage, but direct evidence is still lacking (27). The nick is used to repair the TR to a blunt-ended duplex form in a process called terminal resolution (23) (Fig. 1). Since both the substrate and the product of terminal resolution have RBEs, the enzyme uses other cues to discriminate between the two kinds of TRs when cutting at the trs. The level of linear-TR nicking is 50- to 100-fold lower than that of hairpinned-TR nicking in vitro (1, 12, 22); this may be due in part to additional contacts that the enzyme makes with sequences within the TR but outside of the RBE (19). The site-specific endonuclease activity of Rep is unusual in that it requires ATP hydrolysis (5). Im and Muzyczka have shown that nonhydrolyzable ATP analogues do not support trs endonuclease activity (7). Using a maltose binding protein (MBP)-Rep fusion, Wonderling et al. (32) have shown directly that Rep68 is an ATPase that hydrolyzes ATP to AMP. A Rep68 mutant with histidine substituted for lysine at position 340, in the putative ATP binding site (K₃₄₀H), did not hydrolyze ATP, suggesting the presence of...

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only one catalytic ATPase site. Finally, Rep also contains an ATP-dependent DNA helicase activity (5).

Thus far, relatively little is known about the Rep helicase and ATPase activities. Snyder et al. (22) showed that ATP was not required for ts endonuclease activity provided that the sequence in the vicinity of the ts was single stranded (ss) rather than double stranded (ds). They hypothesized that the ATP requirement for nicking meant that it was necessary for the Rep helicase to unwind the ts prior to nicking. Wonderling et al. (32) demonstrated that the MBP-Rep fusion could unwind DNA-RNA duplexes as well as DNA-DNA duplexes. This reaction required the presence of ssDNA regions in the substrate, which Rep is known to bind (7).

In addition to its possible role in unwinding the ts prior to nicking, the Rep DNA helicase may be involved in at least three other steps during AAV replication. First, the helicase may unwind the hairpinned TR after the nick has been made (Fig. 1, terminal resolution). Second, following terminal resolution, the helicase may unwind the duplex linear TR to form the double-hairpin structure that is necessary to prime strand displacement synthesis. This reaction, which we have called reinitiation, would be possible if Rep could unwind DNA site specifically by binding to the RBE and creating a bubble within the TR (Fig. 1, reinitiation). In this respect, Rep would act like a TR isomerase that converts a duplex blunt end to a hairpinned primer. Finally, Rep may be a replicative DNA helicase which unwinds AAV DNA in front of the replication fork during subsequent elongation (Fig. 1, strand displacement).

Another question that remains is whether Rep activity requires multimeric forms of the protein. Several laboratories have shown by electrophoretic mobility shift assays that as many as six different species of enzyme-TR complexes can be identified in vitro (1, 6, 13, 17). Weitzman et al. (29) have shown that Rep can also act as a protein bridge between two TR substrates. Smith et al. (20) have shown that Rep can form multimeric complexes, including a hexamer and high-molecular-weight aggregates, in solution. As yet, however, none of these studies has demonstrated which, if any, of these structures are associated with specific enzymatic activities of Rep. Finally, Owens and colleagues (16, 32) have shown that a Rep ATP binding site mutant has a dominant-negative phenotype in vitro, inhibiting both ts nicking and helicase activities, when mixed with the wild type. Since ATP is not necessary for binding (6), this could simply reflect competition for substrate by an inactive enzyme. Alternatively, it could suggest that a multimeric form consisting of at least a dimer is required for enzymatic activity (16, 32).

In this study, we characterized some of the basic properties of the Rep helicase and ATPase activities. Our results provide evidence for the existence of a multimeric structure for active Rep endonuclease and ATPase activities, but not for helicase activity. They also show that Rep is capable of supporting the conversion of duplex TR to a hairpinned TR that is required for reinitiation. Finally, they show that the Rep ATPase activity is not DNA dependent.

MATERIALS AND METHODS

Materials. Ribonucleotides, deoxyribonucleotides, ATP analogues, HCl-washed charcoal, and polyethyleneimine (PEI) thin-layer plates were purchased from Sigma and Boehringer Mannheim. Radioactive nucleotides were purchased from ICN. Restriction and DNA-modifying enzymes were from New England Biolabs. M13mp18 DNA (both ss and ds) was prepared in accordance with standard protocols (10). Oligonucleotides were chemically synthesized on an Applied Biosystems oligonucleotide synthesizer, and the sequences are shown in Table 1. Chromatographic media were purchased from Sigma, Whatman, and Pharmacia.

Purification of baculovirus-expressed Rep68. Rep68 was purified to homogeneity from baculovirus-infected Sf9 cells as previously described (12, 15). Rep68 was purified by sequential chromatography on phenyl-Sepharose, ssDNA-cellulose, and DNA affinity matrices (fraction A) or by sequential chromatography on phenyl-Sepharose, ssDNA-cellulose, and DEAE-cellulose (fractions V and S). Both types of preparations were more than 99% pure as judged by sodium
TABLE 1. Sequences of oligonucleotides used to make M13 and duplex oligonucleotide substrates that contained 5′, 3′, or no ss tails

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence^a</th>
</tr>
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<tbody>
<tr>
<td>M13 primers</td>
<td></td>
</tr>
<tr>
<td>18-mer without tail</td>
<td>5′GTAAGAACGCGCCGACGTTG3′</td>
</tr>
<tr>
<td>30-mer with 12-base 5′ tail</td>
<td>5′tcagtcgcaatGTAAGAACGCGCCGACGTTG3′</td>
</tr>
<tr>
<td>30-mer with 12-base 3′ tail</td>
<td>5′GTAAGAACGCGCCGACGTTGgtgtgtaag3′</td>
</tr>
<tr>
<td>42-mer with 12-base 5′ and 3′ tails</td>
<td>5′tcagtcgcaatGTAAGAACGCGCCGACGTTGgtgtgtaag3′</td>
</tr>
</tbody>
</table>

| Oligonucleotide substrates        |            |
| 37-mer, bottom strand             | 5′CCGATTAAGGGATTAGGACGAGAAGAGCTACCGAG3′ |
| 37-mer, no top strand             | 5′CTCAGTGAGCGGAGAGCGGCAGAGGGAGTGCC3′ |
| 67-mer, top strand with 20-base 5′ tail | 5′ctctctgtagtgccggtcctgtaagcggctctgttcgctctgttc3′ |
| 67-mer, top strand with 20-base 3′ tail | 5′ctctctgtagtgccggtcctgtaagcggctctgttcgctctgttc3′ |

| RBE substrates                    |            |
| 37-mer RBE, top strand            | 5′GCCCACCTCCCTCTCCGGGCGGTGTCCTGATGAG3′ |
| 37-mer RBE, bottom strand         | 5′GCCCACCTCCCTCTCCGGGCGGTGTCCTGATGAG3′ |

^a Lowercase letters indicate bases that were ss in the final substrate.

dodecyl sulfate (SDS)-acrylamide gel electrophoresis and silver staining (12, 15). Using the standard trs endonuclease assay (see below), the specific activities of fractions A, V, and S had Rep68 protein concentrations of 46, 1, and 13 g/ml, respectively. In some experiments, Rep68 purified from AAV-infected HeLa cells was used for the protein concentrations obtained by the Bradford assay. Fractions A, V, and S had Rep68 protein concentrations of 46, 1, and 13 g/ml, respectively.

### RESULTS

DNA unwinding as a function of Rep68 protein concentration. The standard DNA helicase reaction was used to characterize some of the basic properties of the Rep68 DNA helicase activity. The substrate for the reaction was M13 circular ssDNA annealed to a 5′-labeled 69-base HaeIII fragment. Addition of increasing amounts of homogeneously pure Rep68 to this reaction revealed a linear relationship between the amount of enzyme added and the amount of unwound 63-base product (Fig. 2a). The reaction was linear until 60% of the substrate was unwound (Fig. 2a, inset). The lowest concentration of enzyme used (2.4 nM) was sufficient to unwind approximately 20% of the substrate. At this enzyme concentration, the molar ratio of enzyme to M13 substrate (calculated for M13 molecules) was approximately 16:1.

In contrast to the DNA helicase activity, trs endonuclease activity was second order with respect to enzyme concentration (Fig. 2b). Regression analysis of several enzyme titrations in-
that the active form of the enzyme for endonuclease activity was likely to be a dimer, provided that the enzyme was initially a monomer in solution. We had previously shown that at low concentrations of enzyme (approximately 1 μg/ml), Rep68 was a monomeric protein in solution (7). To confirm that the enzyme used here was also monomeric, we fractionated baculovirus-expressed Rep68 on a 10 to 40% sucrose gradient at a loading concentration of 380 nM (23 μg/ml). This concentration was more than twice the highest concentration used in these studies for any enzymatic reaction. When fractions from the gradient were examined by Western blotting, all of the Rep68 ran at a mobility consistent with a monomeric protein (Fig. 2c). Additionally, all of the trs endonuclease activity comigrated with the monomeric Rep peak. Thus, the results suggested that for the site-specific nuclease activity of Rep, the active form of the enzyme was a dimer. In contrast, our helicase titration results were consistent with a monomeric form of Rep being capable of catalyzing DNA helicase activity. 

**Cofactor requirements for Rep68 helicase.** In addition to ATP, a number of other nucleoside triphosphates were capable of supporting DNA helicase activity (Fig. 3). GTP and ATP had equivalent abilities to act as cofactors in the reaction, and CTP retained 75% of the maximal activity. The order of preference was ATP > GTP > CTP > dATP > dUTP > dGTP. dCTP and dTTP failed to support Rep68 helicase activity. The order of nucleotide preference was essentially the same as that reported by us previously for the trs nicking reaction (7), except that GTP was preferred over CTP for the helicase reaction and the opposite was true for trs endonuclease activity. Wonderling et al. (32) saw a similar order of preference when Rep was assayed for RNA/DNA helicase activity. As expected, the non-hydrolyzable nucleotide analogues adenosine 5′-O-(3-thiotriphosphate) (ATPγS) and 5′-adenylylimido diphosphate (AMPNP) did not support helicase activity.

The Rep68 helicase activity was active over a broad range of Mg²⁺ concentrations tested, from 0.5 to 15 mM, and was partially active with Mn²⁺ in the narrow concentration range of 0.25 to 1.5 mM (Fig. 4d). Other divalent metal ions (Zn²⁺ and Ca²⁺) did not function as cofactors at any concentration. Similar divalent-cation requirements had been reported for the trs endonuclease reaction (7). The helicase assay was sensitive to NaCl at concentrations above 100 mM, and (NH₄)₂SO₄ inhibited the enzyme at all concentrations tested (Fig. 4a). The Rep68 helicase activity was active over a broad range of Mg²⁺ concentrations tested, from 0.5 to 15 mM, and was partially active with Mn²⁺ in the narrow concentration range of 0.25 to 1.5 mM (Fig. 4d). Other divalent metal ions (Zn²⁺ and Ca²⁺) did not function as cofactors at any concentration. Similar divalent-cation requirements had been reported for the trs endonuclease reaction (7). The helicase assay was sensitive to NaCl at concentrations above 100 mM, and (NH₄)₂SO₄ inhibited the enzyme at all concentrations tested (Fig. 4a). The

![FIG. 2. Helicase and trs endonuclease activity as a function of Rep68 protein concentration.](https://example.com/fig2)

(a) The standard helicase reaction mixtures (see Materials and Methods) contained 0.15 nM M13-69 substrate and the indicated concentrations of Rep68 fraction A. The insert plots only the data in the range from 0 to 10 nM enzyme, in which the reaction was linear with respect to enzyme concentration. (b) The standard nicking reaction mixtures contained 0.25 nM 5′-labeled hairpinned NE_BstXI substrate and the indicated concentrations of Rep68 fraction A. (c) Sucrose gradient centrifugation of Rep68 fraction A. The enzyme was loaded onto a 10 to 40% sucrose gradient at a concentration of 380 nM (23 μg/ml) and centrifuged at 200,000 × g for 40 h (see Materials and Methods for details). The triangles indicate the percentage of total trs endonuclease activity recovered in each fraction; the circles indicate the relative Rep68 concentrations as determined by Western blotting with anti-Rep52 monoclonal antibody. The arrows indicate the positions of alcohol dehydrogenase (150 kDa) and BSA (66 kDa). Rep68 has a calculated theoretical molecular mass of 61 kDa.

![FIG. 3. Nucleotide cofactor requirement for Rep68 helicase activity.](https://example.com/fig3)

The standard helicase reaction mixture described in Materials and Methods contained 0.15 nM (molecules) M13 circular ssDNA substrate annealed to the 69-base HaeIII fragment, 9.4 nM Rep68 (fraction A), and 0.5 nM nucleoside or deoxynucleoside triphosphate. The products of the reaction were electrophoresed on a 5% non-denaturing polyacrylamide gel. The percentage of substrate unwind that is listed beneath each lane was normalized to the activity seen with ATP, which was arbitrarily set to 100%.
pH optimum was 7.0, but at least 50% of the activity was retained over a broad pH range (7.0 to 9.0) (Fig. 4b). The optimum temperature was 43°C (Fig. 4c).

**Direction of DNA unwinding.** To determine the polarity of Rep68, we used a substrate described by Matson (11) (Fig. 5c). The substrate was constructed by annealing a 341-base primer to circular M13mp18 ssDNA. The partial duplex was digested with **ClaI**, which cuts the primer once, and the resulting product was labeled by using the Klenow fragment of DNA polymerase and [α-32P]dCTP to produce an ssDNA molecule containing a 202-base primer at the 5′ end and a 143-base primer at the 3′ end. A helicase translocating in the 3′-to-5′ direction would displace the 143-base primer, whereas translocation in the 5′-to-3′ direction would free the 202-base primer. Figure 5a shows that Rep68 displaced only the 143-base primer, indicating that Rep68 translocated in the 3′-to-5′ direction. To rule out the possibility that Rep68 has a limited processivity and thus could not displace the 202-base primer, we demonstrated that the helicase could unwind a larger primer of 343 bases if the **ClaI** digestion was omitted (Fig. 5a). Additionally, titration of Rep68 (Fig. 5b) displaced the 143-base fragment but not the 202-base fragment. In this experiment, a portion of the substrate had not been digested with **ClaI**, leaving a significant amount of intact circles bound to the 343-base fragment. As expected, these were unwound as well. We concluded that Rep68 has a 3′-to-5′ polarity.

**Rep68 helicase activity on DNA substrates with replication fork structures.** The activities of some DNA helicases require or are stimulated by replication fork structures. To determine if Rep is stimulated by fork structures, we assayed the Rep68 helicase activity on DNA substrates that did or did not contain preformed fork structures. These substrates were constructed by annealing synthetic oligonucleotides to M13 ssDNA circles. The complementary oligonucleotides formed duplex structures that contained an ss 5′ tail, a 3′ tail, both 5′ and 3′ tails, or no tail at all (Table 1). When these substrates were compared in the DNA helicase reaction, no significant difference was seen, suggesting that a replication fork structure did not stimulate helicase activity (Table 2).

**The rate of unwinding for Rep68 helicase.** To measure the rate at which Rep68 unwinds duplex DNA, we determined the kinetics of displacement by Rep68 of a 259-base primer annealed to M13mp18 ssDNA. Because the primers would not be detected as free molecules until they were completely displaced from the ssDNA circle, the rate of unwinding should be equal to the length of the primer divided by the minimum length of time required to detect displacement. We took the following factors into consideration: (i) the primer was short enough to be displaced by Rep and not reanneal rapidly to the ssDNA circles, (ii) the helicase was bound to the DNA substrate in the absence of ATP to eliminate any lag due to the formation of a possible multimeric enzyme form, and (iii) the amount of helicase in the assay was large enough (approximately one Rep molecule per 22 bases of M13 DNA) so that Rep molecules should have saturated the ss circular portion of the template. Under these conditions, it took 45 s to displace the 259-base primer (Fig. 6). Thus, Rep68 unwound DNA at a rate of approximately 345 bp/min.
Processivity of Rep68 helicase. To determine the processivity of the Rep68 helicase activity, two types of experiments were performed. In the first, we used a DNA substrate composed of primers of various lengths annealed to M13 ssDNA circles. DNA substrate was in excess so that the displacement of a primer would be the result of a single Rep68-substrate association event. Under optimal conditions, Rep68 displaced primers of at least 1,371 bases in length (Fig. 7a). Processivity measured by this method may be an underestimate because long primers may reanneal before they are completely unwound from the ssDNA template.

Processivity was also assessed in a pair of DNA substrate competition experiments (Fig. 7b). In one experiment, helicase was allowed to bind to its DNA substrate first and the reaction was then initiated in the presence of competitor DNA. In the converse experiment, the helicase was bound to competitor DNA and the reaction was then started in the presence of the labeled substrate DNA. Assuming that Rep binds to ssDNA by making contact with approximately 20 to 30 bases of DNA at a time (see below), the number of substrate binding sites in these experiments was in excess of the number of Rep molecules by a factor of 3 to 4. Thus, if the enzyme is highly processive, there should be a difference in the amount of substrate that is unwound depending on whether enzyme is first bound to labeled substrate or unlabeled competitor DNA. At competitor DNA-to-substrate DNA molar ratios ranging from 0.01:1 to 10:1, approximately 15% less substrate was unwound when Rep68 was prebound to competitor DNA rather than substrate DNA (Fig. 7b). This difference in activity, which is due to the order of addition of substrate and competitor, is an indication of the average amount of time that Rep is bound to its initial substrate before it dissociates and is available to bind new DNA molecules. When the assay was done in the absence of competitor DNA (but under otherwise identical conditions), it took 3 min for Rep68 to unwind 15% of the labeled substrate (data not shown). In 3 min, Rep68 would move 1,035 bases, assuming a rate of translocation of 345 bases/min as determined by the time course experiment (Fig. 6). This result is in good agreement with the estimate of 1,371 bases from the experiment in which Rep’s ability to unwind primers of various lengths was tested (Fig. 7a).

Repet preferentially unwinds a duplex DNA substrate that contains an RBE. To see if Rep68 could unwind completely duplex substrates, we tested its activity on oligonucleotide substrates with defined sequences (Table 1). Each oligonucleotide substrate contained a duplex region of 37 bp containing a sequence from M13. One contained blunt ends, while the other two contained either a 5′ or a 3′ ss tail of 30 bases in length (Table 1 and Fig. 8a). As expected, the 3′-tailed oligonucleotide was partially unwound while the 5′-tailed substrate was inactive. No activity was seen with the substrate that was completely duplex and had no tails.
labeled substrate (0.15 nM) was added along with ATP to start the reaction. Enzyme was first preincubated for 15 min with unlabeled competitor, and then first. In the second set of experiments (open circles) (competitor first), the percentage of substrate unwound was then plotted (closed circles) (substrate first). In the second set of experiments (open circles) (competitor first), the percentage of substrate unwound was then plotted (closed circles) (substrate first). The experiments described above suggested that Rep68 binds ssDNA but not duplex DNA unless the substrate contains an RBE. To confirm this, we tested the affinity of Rep68 for ss and ds M13 DNA. Using the standard helicase reaction, we added increasing amounts of ss or ds M13 DNA as a competitor. At a competitor-to-substrate ratio of 1, ssDNA reduced helicase activity by 50%. In contrast, dsDNA, even when present at a 100-fold molar excess, did not significantly affect the helicase activity (Fig. 8c). This confirmed that Rep68 binds dsDNA poorly.

Both helicase and trs endonuclease activities are second order with respect to ATP concentration. The $K_m$ for ATP was determined by assayising helicase activity at various ATP concentrations (Fig. 9b) in the standard DNA helicase reaction. Surprisingly, even though helicase activity was linear with respect to enzyme concentration, it was second order with respect to ATP concentration. The $K_m$ for ATP was 180 μM. Similar results were obtained when we assayed Rep68 that had been purified from AAV-infected HeLa cells in the DNA helicase assay (data not shown). Finally, we also determined the $K_m$ for ATP in the $trs$ endonuclease assay, using the HeLa cell-derived ssDNA Rep68 fraction. The $K_m$ for ATP was 155 μM, and, as in the case of the DNA helicase activity, the nicking activity of Rep68 was second order with respect to the ATP concentration (Fig. 9b).

The ATP turnover number for Rep68. The rate of ATP hydrolysis catalyzed by Rep68 was measured in the standard M13 helicase reaction in the presence of $[γ-^3P]ATP$ at a concentration equal to the $K_m$. ATP hydrolysis was linear with respect to both time and enzyme concentration (Fig. 9a). Assuming that the molecular mass of Rep68 is 61 kDa, the Rep68 ATPase turnover number was 14 ATP molecules hydrolyzed per min per enzyme molecule at the $K_m$ for ATP. At saturating ATP concentrations (0.4 mM or higher), the turnover number was approximately twice that amount, 30 to 50 ATP molecules/min/enzyme molecule (Fig. 9a).

The Rep68 ATPase activity is DNA independent. Virtually all DNA helicases have DNA-dependent ATPase activity. To determine whether the Rep68 ATPase was DNA dependent, we assayed the ATPase activity in the presence of increasing amounts of M13 ssDNA (Fig. 10a) or the RBE-containing 37-bp duplex oligonucleotide described earlier (Fig. 10b and Table 1). Surprisingly, ATPase activity appeared to be independent of DNA concentration and did not require the presence of DNA. In the case of M13 ssDNA, there was essentially no change in ATPase activity at M13 ssDNA concentrations of from 0 to 80 nM (approximately 500 times the normal substrate concentration in the helicase reaction). In the case of the RBE oligonucleotide, there was a modest (30 to 40%) increase.

We also tested a completely duplex, blunt-ended oligonucleotide substrate that contained the 22-bp RBE within the AAV TR, as well as flanking AAV sequences. Like the blunt-ended M13 oligonucleotide described above, the RBE-containing oligonucleotide was also 37 bp long. However, in contrast to the M13 DNA-containing oligonucleotide, the RBE-containing substrate was readily unwound. Thus, Rep68 was capable of unwinding a duplex substrate, provided that it contained an RBE. We note that Rep68 was much less active on the 3′-tailed oligonucleotide containing an M13 sequence than on the blunt-ended RBE-containing oligonucleotide. This is apparently because the 30-base ss 3′ tail in the M13 oligonucleotide was close to the minimum size required for binding of Rep to ssDNA.

The RBE oligonucleotide is similar to the substrate used in the $trs$ endonuclease reaction in that both substrates contain the 22-bp RBE and some of the flanking sequences. To see if helicase activity was second order with respect to enzyme concentration, we titrated Rep68 in a helicase reaction using the RBE substrate (Fig. 8b). The reaction was linear with respect to enzyme concentration, and the enzyme-to-substrate ratio needed to unwind approximately 20% of the substrate was 9.4:1.

In the second set of experiments (open circles) (competitor first), the percentage of substrate unwound was then plotted (closed circles) (substrate first). In the second set of experiments (open circles) (competitor first), the percentage of substrate unwound was then plotted (closed circles) (substrate first).
in ATPase activity when DNA was added (Fig. 10b). However, significant levels of ATPase activity were present when no DNA was added. Wonderling et al. (32) observed a similar phenomenon when they assayed the ATPase activity of a Rep68-MBP fusion protein in an RNA/DNA helicase assay.

The baculovirus-expressed Rep68 used in this study had been purified by chromatography on ssDNA-agarose and (for some preparations) a DNA affinity column containing an RBE oligonucleotide. We therefore considered the possibility that Rep68 binds DNA so tightly that it copurifies with short DNA fragments or that some DNA eluted from the DNA-containing chromatographic columns along with the enzyme. In either case, adding additional DNA might have no effect on ATPase activity. To test this possibility, we did the following experiment. We treated a portion of the enzyme with calf intestinal phosphatase and terminal transferase in an attempt to label a DNA contaminant if it were present. We reasoned that if DNA were present in the enzyme, this procedure should remove any 3'-phosphate residues that might exist and allow the DNA contaminant to be radioactively labeled with terminal transferase and [α-32P]deoxynucleoside triphosphates. Attempts to label a potential DNA contaminant in Rep68 fraction V by these methods were unsuccessful. In contrast, when we added 1 pmol of the 69-base oligonucleotide primer (used as the M13 DNA helicase substrate) to the enzyme, the DNA was readily labeled by this method (data not shown). We concluded that the ATPase activity of Rep68 did not require DNA for activity.

Finally, since Rep68 activity was sigmoidal with respect to ATP concentration, we titrated the enzyme in the ATPase reaction to determine how the ATPase activity behaved with respect to enzyme concentration (Fig. 10c). The ATPase activity, when assayed at approximately two times the $K_m$ for ATP, was sigmoidal with respect to enzyme concentration.

**DISCUSSION**

**Biochemical characteristics of Rep and their role in AAV DNA replication.** We have defined some of the biochemical characteristics of the Rep DNA helicase and ATPase activities. Rep appears to have a conventional DNA helicase activity, being capable of binding to ssDNA and of unwinding duplex DNA in a 3'-to-5' direction (Fig. 5). In this respect, it is similar to simian virus 40 (SV40) T antigen, a well-characterized 3'-to-5' replicative helicase (3). Like T antigen, Rep binds duplex DNA poorly, and duplex DNA does not significantly inhibit helicase activity (Fig. 8c). Wonderling et al. (32) also presented evidence that Rep requires ssDNA for unwinding an RNA/DNA duplex. Additionally, neither helicase is stimulated by a 5' tail, a structure that mimics a replication fork (30) (Fig. 8 and Table 2). Both helicasewnd DNA at comparable velocities, approximately 100 bases per min for T antigen and 345 bases per min for Rep (30) (Fig. 6). Both helicasewnd DNA at comparable velocities, approximately 100 bases per min for T antigen and 345 bases per min for Rep (30) (Fig. 6). Both helicasewnd DNA at comparable velocities, approximately 100 bases per min for T antigen and 345 bases per min for Rep (30) (Fig. 6). Both helicasewnd DNA at comparable velocities, approximately 100 bases per min for T antigen and 345 bases per min for Rep (30) (Fig. 6). Both helicasewnd DNA at comparable velocities, approximately 100 bases per min for T antigen and 345 bases per min for Rep (30) (Fig. 6). Both helicasewnd DNA at comparable velocities, approximately 100 bases per min for T antigen and 345 bases per min for Rep (30) (Fig. 6). Both helicasewnd DNA at comparable velocities, approximately 100 bases per min for T antigen and 345 bases per min for Rep (30) (Fig. 6). Both helicasewnd DNA at comparable velocities, approximately 100 bases per min for T antigen and 345 bases per min for Rep (30) (Fig. 6).

Rep also shares another property with SV40 T antigen. Both are capable of binding duplex DNA that contains a specific sequence and then unwinding that sequence. In the case of the SV40 ori, T antigen unwinds duplex DNA to form an ss bubble that initiates DNA replication (3, 26). In the case of the RBE, our results suggest that Rep helicase activity is necessary for catalyzing two key steps in AAV DNA replication, terminal resolution and reinitiation (Fig. 1).
We have shown previously that ATP is not essential for \textit{trs} endonuclease activity, provided that the sequence in the immediate vicinity of the \textit{trs} is ss. Here we have shown that when Rep binds the RBE within the TR, it can function as an ATP-dependent helicase. Together, these facts suggest that the unusual requirement for ATP during the \textit{trs} nicking reaction reflects either a need for the Rep helicase to unwind the \textit{trs} site prior to nicking or a need to use the translocation machinery of the Rep helicase to position the \textit{trs} in the endonuclease active site. In either case, it appears that the RBE-dependent Rep DNA helicase activity is essential for \textit{trs} endonuclease activity.

Once the hairpinned TR is repaired to a linear duplex conformation, the next step in AAV DNA replication requires that the TR be unwound and allowed to form a double-hairpin intermediate that will prime strand displacement synthesis (Fig. 1, reinitiation). This step, which involves the isomerization of 125 bp of terminal sequence, cannot occur spontaneously. We and others (15, 28) have shown that \textit{DpnI}-resistant full-length products are not synthesized in vitro on AAV templates unless Rep is present in the reaction mixture. The implication of this finding is that Rep is necessary to reinitiate strand displacement synthesis, converting a duplex TR to a hairpin. Recently, Willwand et al. (31) presented evidence that the related NS1 protein of minute virus of mice (MVM) performs a similar function during DNA replication. This group showed that initiation of MVM DNA replication was highly stimulated by the MVM nonstructural protein NS1. Addition of ATP\textsubscript{S} blocked the initiation of DNA replication but not the extension of preexisting hairpin primers formed in the presence of NS1 alone. Our results showing efficient and selective unwinding of RBE-containing duplex DNA (Fig. 8) demonstrate directly that the RBE-dependent Rep helicase activity is capable of carrying out this step of converting a duplex TR to a hairpin.

Thus, the helicase activity of Rep appears to be capable of carrying out three different steps during AAV DNA replication. First, the RBE-dependent helicase activity is necessary for modifying the hairpin TR prior to nicking at the \textit{trs}. Second, the RBE-dependent helicase is capable of converting the linear TR to a hairpin form. Finally, Rep can act as a replica.
tive helicase during strand displacement synthesis. Note that although our results show that Rep is capable of carrying out these steps in DNA replication, further studies in vitro with purified components are necessary to determine whether Rep is the only DNA helicase required during AAV replication. Additionally, during the terminal resolution step, Rep becomes covalently attached to the RBE (21). It is not clear from our studies whether covalently attached Rep remains functional for helicase activity.

What is the active form(s) of the Rep68 helicase-ATPase-endonuclease? As noted earlier, many groups have shown that Rep68 can form multimeric binding complexes on substrates containing the RBE (1, 6, 13, 17). As yet it is not clear which of these complexes have functional or enzymatic activity. The results presented here suggest that the Rep68-associated endonuclease and ATPase activities are sigmoidal with respect to enzyme concentration, indicating that two or more Rep molecules must form a complex to be active. It is worth noting that Rep68, as well as Rep78, can form multimers and high-molecular-weight aggregates when the protein concentration is above 100 μg/ml (14a, 20). In the studies presented here, Rep was maintained at a concentration which ensured that it was primarily a monomer in solution (Fig. 2c). Thus, the second-order relationship between enzyme concentration and both the endonuclease and ATPase activities (Fig. 2b and 10c) suggests that these two functions require the formation of a dimer Rep68 complex.

Consistent with this notion is the fact that DNA helicase and nicking activities were both found to be second order with respect to the ATP concentration (Fig. 9b). Wonderling et al. (32) have shown that a point mutation in the known ATP binding motif of Rep eliminates ATPase activity. This suggests that a single molecule of Rep contains only one ATPase active site and that efficient ATPase activity requires the formation of a dimer of Rep, even in the absence of a DNA substrate (Fig. 10c).

Most of the previously studied DNA helicases are active as multimers, either as dimers or hexamers (9). These helicases have a common feature, namely, their helicase and ATPase activities have a sigmoidal dependence on protein concentration (3, 18). It is surprising, therefore, that the Rep DNA helicase activity is linear with respect to enzyme concentration regardless of the substrate used (Fig. 2a and 8b). Furthermore, Wonderling et al. (32) also observed a linear relationship between helicase activity and Rep concentration. Using the same nucleotide binding site mutation cited above, Owens and colleagues have shown that a 1:1 mixture of mutant and active Rep proteins dramatically reduces both endonuclease and helicase activities (16, 32). This was interpreted to mean that the mutant was dominant and exerted its effect via a multimeric Rep complex. Smith et al. (20) also provided evidence that Rep could form multimeric complexes in the presence of DNA substrates containing an RBE, although it was not clear whether these complexes had enzymatic function. These facts are difficult to reconcile with the linear relationship between enzyme concentration and helicase activity seen in this study, which would suggest that a monomer of Rep is sufficient for helicase activity. Thus, a simple model in which Rep dimers are active for helicase, endonuclease, and ATPase function may not be correct. Ultimately, more-detailed structure-function studies will be necessary before the active form of Rep is definitively established for each Rep function.

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ADDENDUM

During review of the manuscript, Smith and Kotin (20a) reported that a fusion protein of the maltose binding domain and Rep52, a related Rep family member, is also a DNA helicase with 3′-to-5′ polarity.

REFERENCES

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