Differential ATP Requirements Distinguish the DNA Translocation and DNA Unwinding Activities of the Escherichia coli PRI A Protein*

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The Escherichia coli primosome was originally isolated based on its requirement for priming of φX174 complementary strand DNA replication in vitro (1, 2). Assembly of this multienzyme complex occurs at a specific site (3, 4) (termed a primosome assembly site (PAS)† (5)) on single-stranded DNA-binding protein-coated single-stranded DNA. The PRI A protein (factor Y, protein n') is a PAS sequence-specific (d)ATPase as well as a DNA helicase and is believed to direct the assembly of the primosome at a PAS. In this report, the PRI A DNA helicase reaction is dissected in vitro, by use of a strand displacement assay, into three steps with distinct ATP requirements. First, the PRI A protein gains entry to the DNA via an ATP-independent, PAS sequence-specific binding event. Second, the PRI A protein translocates along the single-stranded DNA in the 3' → 5' direction at a maximal rate of 90 nucleotides/s. DNA translocation requires ATP hydrolysis. The ATP concentration required to support half of the maximal translocation rate is 100 μM, which is identical to the Kₚ for ATP of the PRI A protein DNA-dependent ATPase activity. Finally, the PRI A protein unwinds duplex DNA. The ATP concentration required for duplex DNA unwinding depends upon the length of the duplex region to be unwound. Displacement of a 24-nucleotide long oligomer required no more ATP than that required for the translocation of the PRI A protein along single-stranded DNA, whereas displacement of a 390-nucleotide long DNA fragment required a 10-fold higher concentration of ATP than that required for oligomer displacement.

The Escherichia coli primosome is a mobile multi-protein DNA replication-priming apparatus that assembles at a specific site (termed a primosome assembly site (PAS)) on single-stranded DNA-binding protein-coated single-stranded DNA. The PRI A protein (factor Y, protein n') is a PAS sequence-specific (d)ATPase as well as a DNA helicase and is believed to direct the assembly of the primosome at a PAS. In this report, the PRI A DNA helicase reaction is dissected in vitro, by use of a strand displacement assay, into three steps with distinct ATP requirements. First, the PRI A protein gains entry to the DNA via an ATP-independent, PAS sequence-specific binding event. Second, the PRI A protein translocates along the single-stranded DNA in the 3' → 5' direction at a maximal rate of 90 nucleotides/s. DNA translocation requires ATP hydrolysis. The ATP concentration required to support half of the maximal translocation rate is 100 μM, which is identical to the Kₚ for ATP of the PRI A protein DNA-dependent ATPase activity. Finally, the PRI A protein unwinds duplex DNA. The ATP concentration required for duplex DNA unwinding depends upon the length of the duplex region to be unwound. Displacement of a 24-nucleotide long oligomer required no more ATP than that required for the translocation of the PRI A protein along single-stranded DNA, whereas displacement of a 390-nucleotide long DNA fragment required a 10-fold higher concentration of ATP than that required for oligomer displacement.

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The abbreviations used are: PAS, primosome assembly site; SSB, E. coli single-stranded DNA-binding protein; Ssc, single-stranded circular; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AMP-PNP, adeny1-5'-yl imidodiphosphate; ATPγS, adenosine 5'-O-(thio)triphasphate.

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RESULTS

Stable and Productive Binding of the PRI A Protein to a PAS Sequence Does Not Require ATP—The PRI A protein is a PAS sequence-specific DNA helicase (13, 14) and is known to recognize and bind specifically to a PAS (6, 7). Thus, it seemed likely that the DNA unwinding activity of the protein was a result of PRI A translocation subsequent to a specific DNA binding event. It should, therefore, be possible to exploit specific localization of the position at which PRI A gains entry to the single-stranded DNA as a means for separating kinetically the DNA translocation and DNA unwinding steps. In order to assess the role of ATP in these steps, the requirement for ATP in the initial binding of PRI A to the PAS was first examined.

The PAS sequence-specific PRI A protein interaction has been probed previously (12) by DNase I footprinting and methylation enhancement/protection studies. ATP was not required in those studies in order to observe PRI A contact with the DNA. However, since only DNA binding was examined in the previous work, the ability of the PRI A protein to form a stable and productive complex with PAS sequence-containing DNA was assessed using the DNA helicase assay to score the presence of PRI A on the DNA.

A stable PRI A protein-PAS DNA complex should be resistant to competition by excess PAS sequence. Thus, a series of experiments were performed (Fig. 1) in which PRI A was first incubated with the DNA helicase substrate in the presence or absence of 5 μM ATP. Excess competitor DNA that had no partially duplex regions was then added. The ATP concentration was then raised to 2 mM and DNA helicase activity was scored. It was expected that 5 μM ATP would be sufficient if ATP was required for stable and productive binding of PRI A to the PAS, since this concentration of ATP was sufficient to allow complete and functional assembly of the preprimosome (8).

Only DNA containing a PAS site (flR229) was capable of acting as an efficient competitor of PRI A binding when the enzyme was incubated under standard assay conditions in the presence of both the substrate and excess competitor DNA (compare lanes 2 and 3 with lane 4, Fig. 1). A 5-fold excess of PAS-X containing competitor DNA inhibited PRI A DNA helicase activity by 75% (compare lanes 2 and 4, Fig. 1), whereas excess nonspecific DNA (i.e. no PAS sequence, flR229) had no effect on the rates of reaction (compare lanes 3 and 4, Fig. 1).

In order to determine whether ATP was required for a stable PRI A-DNA interaction, the protein was incubated for 2 min with the DNA helicase substrate in the presence or absence of 5 μM ATP. Competitor DNA was then added, the ATP concentration raised to 2 mM, and DNA helicase activity was measured. The addition of specific competitor DNA inhibited subsequent DNA helicase activity to the same extent (15%) whether ATP was present (compare lanes 5 and 6, Fig. 1) or absent (compare lanes 7 and 8, Fig. 1) during the initial stage of the incubation. Since the competitor DNA reduced the DNA fragment displacement activity by 75% when it was added first (lanes 2 and 4, Fig. 1), this indicated that PRI A could form a stable and productive complex with PAS-containing DNA in the absence of any ATP. This is consistent with the previous DNase I footprinting analyses (12).

PRI A-catalyzed Displacement of DNA Fragments of Various Lengths—The PRI A DNA helicase reaction can be dissected into three steps: (i) entry of the PRI A protein to the DNA mediated by the PAS sequence, as discussed above, (ii) translocation of PRI A along the single-stranded DNA, and (iii) unwinding of the duplex DNA. Kinetic analysis of the displacement of a 24-nucleotide long oligomer showed a burst in the accumulation of displaced DNA fragments, whereas displacement of a family of DNA fragments having an average length of 390 nucleotides was gradual, showing a linear pattern of product accumulation for at least 4 min (Fig. 2A). These data suggest that the first two steps of the reaction, as well as the unwinding of a few duplex helical turns, could occur very fast and in a relatively synchronous fashion. The rate-limiting step in the DNA helicase reaction, therefore, must be the unwinding of extended regions of duplex DNA.

The ATP requirement for the PRI A DNA helicase activity was examined. flR229 DNA substrates having either a 24-nucleotide long oligomer or a family of DNA fragments with an average length of 390 nucleotides were compared. The
DNA helicase substrates (data not shown). Displacement of DNA fragments as long as 1200 nucleotides was observed only in the presence of an ATP-regenerating system (data not shown).

It had been demonstrated previously that the PRI A protein could drive translocation of the preprimosome in the 3' to 5' direction at ATP concentrations in the range of 50-100 μM (8). Thus, the variation in ATP requirement with the size of the duplex region to be displaced seemed to indicate that the concentration of ATP required for oligomer displacement reflected in large part the ATP requirement for PRI A translocation along the DNA, whereas the concentration of ATP required for displacement of the 390-nucleotide long fragment reflected the actual energy requirement for the continuous disruption of hydrogen bonds.

An Assay for PRI A Translocation along Single-stranded DNA—The fast PRI A-catalyzed displacement of the oligomer at low ATP concentrations contrasts the requirements for unwinding of the longer fragment and suggests that PRI A undergoes a transition in its mode of action sometime during the unwinding of the first two or three turns of a duplex region. This is discussed in more detail later in this report.

It was clear, although, that the effectively instantaneous displacement of the oligomer could serve to mark the position of the PRI A protein on the DNA. Since the point of entry of PRI A to the single strand is fixed by the position of the PAS, determination of the variation in the lag in time before oligomer displacement is observed as a function of the distance between the PAS and the oligomer should serve to provide a direct analysis of the translocation of the PRI A protein along the single-stranded DNA.

In order to investigate this possibility, the kinetics of oligomer displacement was examined using three different DNA helicase substrates with oligomers of the same length placed at different distances from the PAS (Fig. 3). In these studies PRI A was prebound to the PAS in the absence of ATP and the reaction was initiated by the addition of ATP to 1 mM. The time lag before the appearance of the f1-HaeIII, f1-HaeII, and f1-ClaI oligomers, which are 336, 3043, and 6114 nucleotides, respectively, 5' of the PAS, was 5, 35, and 75 s, respectively (Fig. 3). Given the sensitivity of the DNA helicase assay and the 10-s intervals in sampling time, the estimated error in these measurements is ±5 s.

Oligomer displacement reached a plateau value in each case very quickly after the lag. However, the extent of displacement decreased with increasing distance from the PAS. About two-thirds of the PRI A molecules that acted to displace the f1-HaeIII oligomer seemed capable of translocating roughly 2700 nucleotides downstream to displace the f1-HaeII oligomer. Thereafter, the rate of dissociation of PRI A from the single-stranded DNA increased dramatically, yielding a much lower efficiency of displacement at the most distal site (f1-ClaI). Similar results have been obtained with a DNA helicase substrate carrying simultaneously all three oligomers (data not shown).

The data described thus far indicates that PRI A interacts in an ATP-independent fashion with the PAS and then starts moving along the DNA with an average processivity of 3600 nucleotides (based on the apparent dissociation rate of PRI A from the substrate as described above) after the addition of ATP.

The observed lag is the sum of: (i) the time required for activation of PRI A after the addition of ATP (ta), (ii) the time required for translocation of PRI A from the PAS along the single strand to the oligomer (tb), and (iii) the time required for unwinding of the duplex region (tc). ta and tb are

![Image of DNA helicase reactions](https://example.com/dna-helicase-reactions.png)
The PRI A DNA helicase reaction has been dissected into three steps. (i) PRI A specifically and stably binds to a PAS sequence in the absence of ATP. (ii) PRI A then translocates along the single-stranded DNA in the 3' → 5' direction at the expense of ATP hydrolysis. (iii) PRI A unwinds the duplex DNA. The ATP concentration required for duplex unwinding increases with the length of the duplex DNA.

Examination of the amino acid sequence derived from the open reading frame of the priA gene revealed two consensus nucleotide-binding sites (19, 20). If these two sites are the sole nucleotide-binding regions in PRI A, then at least one of these sites must be occupied (at an [ATP]₀ = 100 μM) by either ATP or dATP in order to support PRI A translocation along single-stranded DNA. The role of the second nucleotide-binding site remains to be determined. Several possibilities exist.

Occupyance of the second nucleotide-binding site by ATP

<table>
<thead>
<tr>
<th>Additions during first stage incubation</th>
<th>Concentration</th>
<th>Lag before oligomer is displaced in second stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0 μM</td>
<td>35 s</td>
</tr>
<tr>
<td>ATP</td>
<td>10 μM</td>
<td>35 s</td>
</tr>
<tr>
<td>ATP</td>
<td>20 μM</td>
<td>35 s</td>
</tr>
<tr>
<td>ATP</td>
<td>50 μM</td>
<td>16 s</td>
</tr>
<tr>
<td>ATP</td>
<td>100 μM</td>
<td>5 s</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>100 μM</td>
<td>30 s</td>
</tr>
<tr>
<td>ATPγS</td>
<td>100 μM</td>
<td>55 s</td>
</tr>
</tbody>
</table>
As an allosteric effector might be required for the series of protein-protein interactions necessary for primosome assembly. Lee and Marians (8) showed that functional primosome assembly absolutely required ATP, however, the ATP$_{0.5}$ of this reaction was 0.8 μM. Under these conditions, DNA translocation in the 5' → 3' direction (via the DNA B protein) could be supported by GTP, while DNA translocation in the 3' → 5' direction (via PRI A) could be supported by dATP. Thus the very low concentration of ATP required for primosome assembly probably reflects occupancy by ATP of a high affinity nucleotide-binding site on PRI A.

Alternatively, occupancy of the second nucleotide-binding site could play a role in the activation of the PRI A DNA helicase activity. The distinctly different ATP requirements for DNA translocation and DNA unwinding suggest that the PRI A protein can undergo several distinct cycles of conformational changes.

Both the kinetic analysis and ATP requirement showed distinct differences between the PRI A DNA helicase reactions performed on the DNA substrate carrying a 24-nucleotide long oligomer and on the substrate carrying an elongated DNA fragment. These data suggest that the translocation of PRI A along the single-stranded DNA, as well as the unwinding of a few duplex DNA helix turns, could occur rapidly and in a relatively synchronous fashion at low concentrations of ATP. On the other hand, the displacement of an elongated DNA fragment was slower, requiring at least a 10-fold higher concentration of ATP than that required for oligomer displacement. If translocation along the single-stranded DNA is a result of the same conformational rearrangements as those required for DNA unwinding, as proposed by Duguet et al. (21) and Hill and Tsuchiya (22), then these discrepancies may be explained by the presence of a free energy barrier for the displacement of an elongated DNA fragment. Elimination of this barrier requires activation of PRI A, which in turn apparently requires the presence of higher concentrations of ATP. Alternatively, extensive duplex DNA unwinding might simply require more energy consumption than translocation along a single strand of DNA.

What triggers the required activation of PRI A for duplex DNA unwinding? The ATP requirement for unwinding a 24-nucleotide long oligomer 3380 nucleotides away from the PAS was no different than that required for DNA translocation to that location, while displacement of a 70-nucleotide long fragment in the identical position required an 8-fold higher ATP concentration. For this discussion, let us assume the transition (from low to high ATP requirement) is triggered at a length of displaced DNA half-way in between, at about 45 nucleotides. Conceivably, contact of this displaced strand with PRI A (perhaps in a specific manner) induces a conformational change that alters the affinity of the ATP-binding site to one requiring higher concentrations of ATP for occupancy. This would suggest that the ATP requirements for the displacement of DNA carrying 5' tails might be different than those observed here. This remains to be investigated. Alternatively, the activation step may be related to the energy required to maintain a certain fixed velocity of PRI A translocation across the DNA. When no duplex region is involved, low ATP concentrations are sufficient. A PRI A molecule translocating along the DNA might acquire a certain momentum that is dissipated after the unwinding of two or three turns of the helix. Subsequent movement at the same velocity through duplex DNA may now require higher concentrations of ATP.

Either case suggests that the designation of enzymes capable of displacing short oligomers from partial heteroduplexes as DNA helicas may be misleading. A bona fide DNA helicase, as opposed to an enzyme capable of DNA translocation, should be capable of unwinding extended duplex DNA regions.

The primosome is thought to be positioned on the lagging strand DNA template at a DNA replication fork by virtue of its possible role in priming of Okazaki fragment synthesis (23). It has been proposed that the DNA B protein functions at the front of the replication fork, primarily as a DNA helicase, unwinding the parental duplex in the 5' → 3' direction along the lagging strand DNA template, whereas PRI A acts as a DNA translocase behind the DNA B protein (8).
Association of these two DNA translocation activities of opposite directionality could possibly generate a loop in the lagging strand DNA template that allows a dimeric DNA polymerase to synthesize both nascent DNA strands in the same direction. In this report, PRI A alone was shown to be capable of translocating along single-stranded DNA at a maximal rate of 90 nucleotides/s at 30 °C. This does not appear to be fast enough for the proposed role of PRI A at the replication fork as a single-stranded DNA translocase. The addition of other primosomal proteins or the DNA polymerase III holoenzyme, both independently and in combination, to a PRI A DNA translocation assay had no effect on the observed rate (data not shown). However, missing here is the architecture of the replication fork that could possibly make PRI A translocation more highly efficient.

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