Enzyme-catalyzed DNA Unwinding

THE ROLE OF ATP IN HELICASE III ACTIVITY*

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The enzyme helicase III catalyzes ATP-dependent unwinding of double-stranded DNA (Yarranton, G. T., Das, R. H., and Gefter, M. L. (1979) J. Biol. Chem. 254, 11997-12001). The free enzyme is able to bind to double- and single-stranded DNA. In the presence of ATP the enzyme can bind single- but not double-stranded DNA. The enzyme catalyzes an ADP-ATP exchange reaction in the absence of DNA. It is suggested that there is an enzyme-phosphate complex that discriminates between the two forms of DNA. These results are discussed in relation to a model that accounts for catalytic unwinding of DNA coupled to ATP hydrolysis.

We have described previously the isolation and characterization of the enzyme helicase III (1, 2). The enzyme is a single-stranded DNA-dependent ATPase and is but one of many such activities that have been described in normal cells (3-10) as well as bacteriophage-infected Escherichia coli (11-14). The physiological role of several of these proteins has been elucidated including the rep protein of E. coli (15, 16), the recBC enzyme (17), the gene 45 and 44/62 proteins of bacteriophage T4, the T7 encoded gene 4 protein, and helicases I and II (18, 19).

In addition to the DNA-dependent ATPase activity, we have shown previously that helicase III as well as rep protein catalyzes strand separation of DNA double helices in the presence of ATP- and DNA-binding protein. The rep protein and helicase III protein are distinct entities in that they catalyze strand separation by moving along DNA with opposite polarity. The rep protein invades and unwinds a duplex by moving in a 3' to 5' direction, whereas the helicase III protein moves in a 5' to 3' direction (2).

We have postulated previously that energy obtained from ATP hydrolysis is utilized both for movement as well as unwinding, the latter being brought about by an ATP-induced conformational change of rep or helicase III protein while they are bound to DNA (16). In this report, we demonstrate that ATP binding does alter the physical state of helicase III and that the enzyme will catalyze an ADP-ATP exchange reaction.

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EXPERIMENTAL PROCEDURES

Bacteria and Bacteriophages—E. coli HF4704 rep3 was obtained from Dr. D. Denhardt, McGill University, Montreal, Canada. The bacterial strain HF4704 carrying a ColEI plasmid containing the rep gene (pLC 4-77) was obtained from Dr. J. Carbon, University of California, Santa Barbara, California. Other E. coli strains and bacteriophages are as described earlier (16, 20).

Cell Growth and Storage—Cells for enzyme isolation were grown to a density of 6 x 10⁹ cells/ml in superbroth and collected at 4°C by centrifugation. The cell pellet was immediately suspended in 30 mm Tris-HCl, pH 7.5, 10% sucrose (w/v) to a concentration of 1 g/ml and stored in liquid nitrogen.

Chemicals and Enzymes—Bovine serum albumin, lysozyme, and other reagents were as described in previous reports (1, 2). Radiochemicals were purchased from New England Nuclear, Boston, MA, and ICN Corp., Irvine, CA, and used at specific activities as described. Nitrocellulose filter type HA of pore size 0.45 μm was purchased from Millipore Corp., Bedford, MA 01730. Chromatographic media and nucleases S1 were from Whatman and Boehringer Mannheim, respectively. Bio-Gel A-5m, 100 to 200 mesh, was the product of Bio-Rad Laboratories. ATPyS was a gift from Dr. Richard Robert, Cornell University, Ithaca.

E. coli DNA-binding protein was purified to homogeneity as previously described (21). This preparation does not contain detectable RNase H or exonuclease I activity. E. coli rep protein (5 x 10⁶ ATPase units/mg of protein) was purified to the DNA cellulose stage and was bound to DNA as described previously (10). Helicase III (1 x 10⁸ ATPase units/mg of protein) was purified following the procedure of Yarranton et al. (1). Bacteriophage T4-induced helix-destabilizing protein (gene 32 protein) was a generous gift of Dr. B. Alberts. Neither the gene 32 protein nor the DNA-binding protein preparation contained detectable DNA-dependent or DNA-independent ATPase activity.

DNA Substrates—Single-stranded circular fd DNA and duplex linear P22 phage DNA were prepared as described in our previous reports (1, 2). Single-stranded region of P22 phage DNA was removed by digesting with S1 nuclease.

DNA-dependent ATPase Assay—Standard reaction mixtures contained 30 nM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, 4 mM dithiothreitol, 0.024 μg of single-stranded fd DNA or P22 phage DNA, 5% glycerol, and [³²P]ATP of specific activity in a total volume of 21 μl. Incubations were carried out either at 30°C or 37°C for time as indicated. After incubation an aliquot of 3 μl was removed and spotted onto a polyethyleneimine cellulose thin layer strip and developed in 1 M formic acid containing 0.5 M LiCl at room temperature. After drying, the spots were localized under an ultraviolet lamp, cut out, and counted in a liquid scintillation spectrophotometer. One unit of ATPase activity is defined as 1 nmol of ADP formed at 37°C in 30 min.

Rep3 Binding Assay—This was carried out essentially by the method of Abdel-Monem et al. (7). Heat-denatured (12 min at 100°C) or double-stranded P22[³²P]DNA (10 to 15 x 10⁶ cpn) was incubated with protein at a specified concentration in a total volume of 30 μl containing 30 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, 4 mM dithiothreitol, 5% glycerol. After the incubation at 37°C for 2 min, the reaction mixture was chilled and diluted to 1 ml by ice-cold buffer containing 2 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 4 mM dithiothreitol, 10% glycerol (filtration buffer), and filtered on Millipore filters which were soaked in the filtration buffer. The filters were dried and counted in a liquid scintillation counter.

ADP-ATP Exchange Reactions—Helicase III (3.0 μg) was incubated in a total volume of 2 μl containing 30 mM Tris-HCl, pH 7.5, 2.5
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Fig. 1. Binding of rep protein, helicase III, and E. coli DNA-binding protein to native and denatured P22 DNA. The binding experiments were carried out as described under "Experimental Procedures." Retention of 0.2 pg of DNA on the filter was taken as 100% binding. A, helicase III binding with native (●●●) and denatured (○○○) DNAs; B, rep protein binding with native and denatured DNAs; C, binding of E. coli DNA-binding protein with native and denatured DNAs.

RESULTS

DNA-binding Properties of Helicase III—We have shown previously that the DNA-unwinding proteins, rep protein and helicase III, could bind to single-stranded DNA and that such binding was prevented if the single-stranded DNA was first complexed with DNA-binding proteins (2). This result was in accord with our hypothesis that the unwinding protein and the DNA-binding proteins (helix-stabilizing proteins) were able to discriminate between single- and double-stranded DNAs via the same interactions with DNA and as such might compete for the same sites on DNA. An additional property suggested by the similarities between the unwinding and binding proteins was that neither would bind to double-stranded DNA. It is shown below, however, that this is not the case.

Binding of various proteins to DNA was measured by the

Fig. 2. ATPase activity of helicase III in the presence of native and denatured P22 DNAs. Incubations were carried out at 37°C for 10 min. Standard reaction mixture contained 7 nmol of [14C]ATP (2 × 10^5 cpm/nmol). ●●●, denatured DNA; ▲▲▲, native DNA.
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Employing the same preparations of single- and double-stranded DNAs as above, it is clear that although helicase III can bind to double-stranded DNA, this binding is not effective in promoting ATPase activity. In accord with previous results, binding to single-stranded DNA does promote ATPase activity. These results are shown in Fig. 2.

The Effect of ATP on DNA Binding—We postulated that ATP binding to, or hydrolysis by, helicase III would induce a conformational change in the protein that enabled it to alternately recognize double- and single-stranded DNA (16). As helicase III is able to bind both forms of DNA it was of interest to determine the effect of ATP on DNA binding.

The results in Fig. 3 demonstrate that in the presence of ATP, helicase III apparently loses its ability to bind to double-stranded DNA. This is in agreement with our observation that double-stranded DNA does not inhibit single-stranded DNA-dependent ATPase activity of helicase III (results are not shown). In contrast, helicase III binds to single-stranded DNA in the presence of ATP. Under the conditions employed (2 min at 37°C) the ATPase activity promoted by the presence of single-stranded DNA is not sufficiently high (<1 nmol of ATP hydrolyzed) to alter the ATP concentrations used.

In order to further study the role of ATP in enabling helicase III to discriminate between the two forms of DNA, we studied the effect of the nonhydrolyzable ATP analogue, AMP-P(NH)P, on the activities of helicase III. As expected from previous results (2) increasing concentrations of the analogue progressively inhibit the DNA-dependent ATPase activity of the enzyme. In keeping with the effect of ATP on double-stranded DNA binding described above, the analogue, likewise, prevents binding to this DNA. Surprisingly, the

ability to cause radioactive DNA to bind to a nitrocellulose filter. As is seen in Fig. 1, A and B, respectively, both single- and double-stranded DNA could be bound by helicase III and rep protein. Under conditions of limiting protein, single- and double-stranded DNA are bound with equal efficiency. As expected, E. coli DNA-binding protein is able to cause only single-stranded DNA to bind (Fig. 1C).

![Fig. 3 (top). Binding of helicase III to DNA in the presence of ATP. Reaction conditions were as described under "Experimental Procedures" and Fig. 1. Binding with denatured DNA (solid line) and with native DNA (dashed line) was done in the presence of 1 µg of helicase III.](image)

![Fig. 4 (bottom). Effect of AMP-P(NH)P on DNA binding and ATPase activity of helicase III. DNA-binding assays were performed as described under "Experimental Procedures" using 1 µg of helicase III, and 0.2 µg of native or denatured P22 DNA. ATPase activity was measured at 37°C for 30 min in a 30-µl reaction volume containing 10 nmol of [3H]ATP (2 × 10⁶ cpm/nmol) and 0.04 µg of denatured P22 DNA in addition to the components mentioned under "Experimental Procedures." Hydrolysis of 7 nmol of ATP and binding of 0.2 µg of DNA were taken as 100%.](image)

![Fig. 5. ADP-ATP exchange activity of helicase III in the presence and absence of ATP analogues. Incubations were carried out in a total volume of 21 µl at 30°C as described under "Experimental Procedures" using 3 µg of helicase III in the presence of 0.33 mM ATP (solid line), 0.33 mM ATP plus 0.66 mM AMP-P(NH)P (dashed line), or 0.33 mM ATP plus 0.33 mM ATP(γS) (dotted line).](image)

### Table 1

Comparison of ATPase and ADP-ATP exchange activities of helicase III under different conditions

| Condition          | ATP hydrolyzed | ADP exchanged |
|--------------------|----------------|---------------|
| Complete           | 37.7           | 21.0          |
| Minus enzyme       | <1             | <1            |
| Minus ATP          |               | 0.3           |
| Minus Mg²⁺         | 2.0            | 2.0           |

Incubations were carried out at 30°C for 1 h. Other unspecified conditions are as mentioned under "Experimental Procedures."
one of them, ADP or PO₄, would have to remain bound to the can catalyze both the unwinding of double-stranded DNA and stranded DNA. These results are depicted in Fig. 4. Accordingly, we have shown that radioactive ADP can be with ATP was taken for with ATP was taken in the presence of for ATPase and ADP-ATP exchange activities at 30°C for 30 min as described under “Experimental Procedures.” ATPase activity was measured in the presence of 0.024 μg of single-stranded fd DNA using [α-³²P]ATP (specific activity, 1.5 × 10⁶ cpm/mmol). Hydrolysis of 10 nmol of ATP and 10 pmol of ADP exchange activity with ATP was taken as 100% activity. ○--○, ATPase activity; ○--○, ADP-ATP exchange activity.

analogue also inhibits the binding of helicase III to single-stranded DNA. These results are depicted in Fig. 4. Since ATP can in principle be hydrolyzed when bound to the enzyme, whereas the analogue cannot, we explored the possibility that the difference between ATP and the analogue in terms of affecting single-stranded DNA binding is due to the hydrolysis of ATP. The hydrolysis products, or at least of one of them, ADP or PO₄, would have to remain bound to the enzyme since the enzyme is not an ATPase in the absence of DNA. As is shown below, this appears to be the case.

Helicase III-catalyzed ADP-ATP Exchange—If ATP was hydrolyzed when bound to the enzyme but the product(s) remained bound, the possibility existed that one of the bound product(s) can react with the other product to form ATP. Accordingly, we have shown that radioactive ADP can be converted into ATP by the action of helicase III and ATP. The requirements of this exchange reaction are given in Table 1 and Fig. 5.

The production of radioactive ATP from radioactive ADP requires enzyme, ATP, and magnesium ion. The rate of exchange is approximately 2000-fold less than the rate of ATP hydrolysis when the latter reaction is measured under the same condition but in the presence of single-stranded DNA. These results are summarized in Table 1. As shown in Fig. 5, the extent of exchange is dependent on the time of incubation and can be inhibited by the presence of AMP-P(NH)P and ATP(γS).

Although the helicase III preparation employed is essentially homogeneous and free of DNA-independent ATPase activity, it was of interest to obtain additional evidence that the DNA-dependent ATPase activity and the ADP-ATP exchange activity resided in the same molecule. As shown in Fig. 6, both activities show parallel rates of heat inactivation.

DISCUSSION

Recently two proteins have been isolated from E. coli which can catalyze both the unwinding of double-stranded DNA and the single-stranded DNA-dependent hydrolysis of ATP. We proposed a model which accounts for these activities and suggested that ATP hydrolysis and DNA unwinding were coupled (16, 20). The essence of the model is the ability of the unwinding proteins to bind to double-stranded DNA and then alter their conformation while remaining bound to that DNA utilizing the energy released from ATP hydrolysis. The new conformation of the protein is incompatible with the DNA remaining double-stranded, and thus the DNA becomes unwound. In the present report we present evidence that several forms of one of these proteins, i.e. the enzyme helicase III, exist. Under appropriate conditions, it is able to bind to either single- or double-stranded DNA, or both simultaneously, only to single-stranded DNA, or to neither form. These properties of the protein depend upon the presence of ATP and its hydrolysis.

We have shown that in the absence of ATP the free enzyme can bind either single- or double-stranded DNA. In the presence of an ATP analogue, AMP-P(NH)₅P, which cannot be hydrolyzed, the enzyme binds neither form of DNA. In the presence of ATP, we postulate that an enzyme-phosphate complex is formed which can bind single-, but not double-stranded DNA. This postulate is based on the fact that ADP can be converted to ATP by helicase III in an exchange reaction, a result which suggests the formation of an enzyme-phosphate complex. Until now, however, we have been unable to isolate such a complex by gel filtration. This may be due to rapid breakdown of the complex. Further work will be necessary to prove that the complex exists.

That the enzyme can exist in three different states is in keeping with our hypothesis which can now be stated more clearly. Enzyme bound to a single-stranded leader (or replication fork) at the junction of single- and double-stranded DNA, in the absence of ATP, can bind the double-stranded DNA ahead of it (for helicase III, if the enzyme is bound to a leader, "ahead" means moving toward the 3'-end of the strand it is bound to). On binding and hydrolyzing ATP, the enzyme (presumably enzyme-phosphate) changes its conformation and with it, the DNA to which it is bound such that the DNA "unwinds." This enzyme-phosphate form is converted to free enzyme again when the DNA to which the enzyme is bound becomes single-stranded (i.e. the full ATPase activity is realized). The cycle then repeats, leading to the stepwise unwinding of duplex DNA. The enzyme must also advance along DNA during this process as well. Perhaps this is achieved during the transient enzyme-ATP form of the enzyme, i.e. the enzyme loses its tight DNA binding but remains bound via interaction with DNA-binding protein or via other weak interactions. This paradoxical state of binding tightly to DNA and yet being able to advance along it transiently is a characteristic of many DNA and RNA polymerases. The physical basis for these processes is not yet understood, but it is obviously a property needed by accurate and processive polymerases.

It is clear that further work will be necessary to establish a true physical basis for the proposed model. In principle it should be possible to demonstrate the altered conformation of helicase III by studying the free enzyme, the form bound to ATP analogues, and the form bound to DNA by direct physical measurement.

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