D-loop Cycle

A CIRCULAR REACTION SEQUENCE WHICH COMPRISSES FORMATION AND DISSOCIATION OF D-LOOPS AND INACTIVATION AND REACTIVATION OF SUPERHELICAL CLOSED CIRCULAR DNA PROMOTED BY recA PROTEIN OF ESCHERICHIA COLI*

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Excess recA protein, a protein essential to general genetic recombination in Escherichia coli, promotes a sequence of formation and dissociation of D-loops from negative superhelical closed circular double-stranded DNA (form I DNA) and homologous single-stranded fragments in the presence of excess ATP, resulting in inactivation of the form I DNA without apparent damage to the DNA. The dissociation of D-loops is accompanied by hydrolysis of ATP to ADP that apparently depends on homologous DNA molecules (homology-dependent ATP hydrolysis). However, at a lower concentration of ATP, we observed anomalous kinetics in the formation and dissociation of D-loops; as the concentration of ATP was decreased, there was a progressively smaller dissociation of D-loops and a faster re-synthesis in the second phase, without changing the rate of the first formation of D-loops. This anomaly might suggest that, as the increase in the amount of ADP relative to that of ATP, dissociation of D-loops is inhibited and reactivation of inactivated form I DNA is stimulated instead of dissociation. We found that addition of ADP inhibited competitively both formation and dissociation of D-loops and that the latter process was more sensitive to the inhibition than the former process. Addition of an ATP-regenerating system, recA protein, when present in excess, formed D-loops, subsequently dissociated them, and converted the form I DNA to an inactive substrate for reinitiation of D-loop formation without apparent damage to the DNA (Ref. 37; Fig. 1, steps I and II). RecA protein requires ATP as a cofactor for this sequence of reactions and catalyzes ATP hydrolysis that apparently depends on both homologous DNA molecules (homology-dependent ATP hydrolysis) (38). The homology-dependent ATP hydrolysis is related to the dissociation of D-loops and the inactive state of form I DNA rather than the formation of D-loops (38). In this paper, we will present evidence for a pathway by which the form I DNA inactivated through formation and dissociation of D-loops is reactivated and can be used as a substrate for a new sequence of formation and dissociation (Fig. 1, step III).

MATERIALS AND METHODS

DNA and recA Protein—Superhelical closed circular replicative form [H]DNA (form I DNA) of colE1 phage fd and fragments of single-stranded phage DNA of fd and øX174 were prepared as described previously (31). The average chain length of single-stranded fragments used in this study was 200 nucleotides. The amounts of DNA are expressed in moles of nucleotide residues.

RecA protein (Fraction V, DEAE-cellulose fraction) was purified by the control of inducible functions (11, 12), the same protein plays a direct role in genetic recombination (13).

Since the discovery of DNA-dependent hydrolysis of ATP (14, 15) and pairing of double-stranded DNA and homologous single-stranded fragments (formation of D-loops) (16, 17) promoted by recA protein, many aspects of the in vitro activities of this protein have been revealed and have provided enzymological insights into the molecular mechanism of general genetic recombination. recA protein promotes (i) pairing of various forms of homologous DNA molecules if at least one of them has a single-stranded region (16–23), (ii) unidirectional elongation of heteroduplex joints formed between homologous double-stranded DNA and single-stranded DNA (21, 24–27), (iii) concerted reciprocal strand exchange between two homologous duplex DNA to form Holliday junctions (28, 29), and (iv) single-stranded DNA- and ATPyS-dependent unwinding of double helix (30, 31).

We are currently investigating the significance of negative superhelical DNA as a substrate for homologous pairing promoted by recA protein. Negative superhelicity of DNA is supposed to play roles in genetic recombination in vivo (32–36). We have calculated that superhelicity of double-stranded DNA accelerates about 50-fold the formation of D-loops (37). Moreover, we found that, with form I DNA and homologous single-stranded fragments as substrates, recA protein, when present in excess, formed D-loops, subsequently dissociated them, and converted the form I DNA to an inactive substrate for reinitiation of D-loop formation without apparent damage to the DNA (Ref. 37; Fig. 1, steps I and II). RecA protein requires ATP as a cofactor for this sequence of reactions and catalyzes ATP hydrolysis that apparently depends on both homologous DNA molecules (homology-dependent ATP hydrolysis) (38). The homology-dependent ATP hydrolysis is related to the dissociation of D-loops and the inactive state of form I DNA rather than the formation of D-loops (38). In this paper, we will present evidence for a pathway by which the form I DNA inactivated through formation and dissociation of D-loops is reactivated and can be used as a substrate for a new sequence of formation and dissociation (Fig. 1, step III).

RecA protein, a polypeptide of 40,000 daltons, is coded by the recA gene of Escherichia coli (1, 2) and is essential to general genetic recombination (3, 4), DNA repair (3, 5–7), and other cellular functions which are induced by damaging DNA or inhibiting DNA synthesis (8, 9; Ref. 10 for review). Whereas recA protein plays an indirect role as a specific protease in

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1 Form I DNA denotes negative superhelical closed circular double-stranded DNA formed in vitro.
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D-loop formation and dissociation of D-loops. In step I, recA protein promotes the formation of D-loops from form I DNA, and homologous single-stranded fragments (S) through a pathway described elsewhere (21-23, 43). In step II, free recA protein cooperatively binds to form I DNA from the site of a D-loop and unwinds the double helix unidirectionally (indicated by the small triangles), resulting in migration of single-stranded fragments out of the D-loops. Even after D-loops are completely dissociated, the form I DNA still unwound by cooperative reaction of free recA protein (RAm) is in a topologically constrained state, which makes the form I DNA an inactive substrate for reinitiation of D-loop formation. Unidirectional unwinding by recA protein was incorporated in this model (37) to explain also the unidirectional growth of heteroduplex joints (21-24-27). Dissociation of D-loops by unidirectional unwinding is supported by the recent observation by Wu et al. (45) that the D-loops formed with the single-stranded fragments that have the heterologous sequence at the 5' end are dissociated, whereas the D-loops formed with the fragments that have the heterologous sequence at the 3' end are not dissociated. This observation indicates that the directionality of the unwinding is 5' to 3' of the D-loop at which the unwinding starts. In step III, the inactivated form I DNA (RAm) is reactivated by release of recA protein from the duplex. This step is appreciable when steps I and II are inhibited by ADP or by incubation at low temperature during or after the dissociation of D-loops.

RESULTS

Anomalous Kinetics in the Formation of D-loops by Limiting the Amount of ATP—When form I DNA and homologous single-stranded fragments are incubated with excess recA protein in the presence of ATP, D-loops are formed initially and then dissociate completely; in the presence of 0.40 μM single-stranded fragments and 1.3 mM ATP, formation of D-loops reaches its peak at about 4 min, and subsequent dissociation is complete by 30 min (Figs. 2A and 4B; see Ref. 37). Since we have observed that recA protein hydrolyzed ATP to ADP during this process (38) and that formation of D-loops was inhibited by ADP (22), we examined the effect of the concentration of ATP on these processes. When the initial amount of ATP was decreased, the kinetics of the formation and dissociation of D-loops became anomalous; as the concentration of ATP was decreased from 1.3 mM to 40 μM, there appeared a progressively earlier cessation of decrease in the amount of D-loops, followed by a faster increase in the amount (Fig. 2, A and B). At any concentration of ATP more than 40 μM, the D-loops were inactivated form I DNA (RAm) is reactivated by release of recA protein from the duplex. This step is appreciable when steps I and II are inhibited by ADP or by incubation at low temperature during or after the dissociation of D-loops.

Fig. 2. Effect of the concentration of ATP on kinetics of the formation and dissociation of D-loops. Fd form I [3H]DNA and homologous (closed symbols) or heterologous (open symbols) single-stranded fragments were incubated with 0.40 μM recA protein at 37°C in the presence of the indicated amount of ATP. A, samples were withdrawn at the indicated times and assayed for the amount of D-loops. The concentrations of ATP were 40 μM (○, ○), 160 μM (■, □), 300 μM (△, △), and 1.3 mM (▲, ▲). At 50 min (indicated by ▼), 300 μM ATP (total input ATP was 460 μM) was added to a reaction mixture that contained 160 μM ATP initially (Φ). B, the amount of D-loops was assayed at 4 min (○) or 30 min (▲) of incubation.
In the amount of D-loops was equally rapid (Fig. 2A, closed diamonds). The observations described above suggest that the dissociation of D-loops requires more ATP than does the formation or that the dissociation is more sensitive to the inhibition by ADP than is the formation. As shown in Fig. 3, A and B, the latter possibility was the case. ADP inhibits dissociation of D-loops at a concentration that is ¼ to ½ of that required to inhibit the formation in the presence of either 160 μM or 1.3 mM ATP. The results shown in Fig. 3, A and B, indicate also that the mode of inhibition of both formation and dissociation of D-loops by ADP is competitive, because the increase in the concentration of ATP from 160 μM to 1.3 mM increased correspondingly the amount of ADP needed to inhibit formation or dissociation.

Reactivation of the Form I DNA That Has Been Inactivated by Formation and Subsequent Dissociation of D-loops—The dissociation of D-loops is accompanied by homology-dependent hydrolysis of ATP (Ref. 38 and Fig. 4B). The hydrolysis apparently depends on the homologous combination of form I DNA and single-stranded fragments and appears to be initiated by D-loops (38). In the presence of a limited amount of ATP (300 μM), the amount of D-loops stopped decreasing and started to increase again when homology-dependent ATP hydrolysis ceased (compare Fig. 4A, closed circles, and Fig. 4C, closed circles). The second increase in the amount of D-loops might be due either to undepressed formation of D-loops from unreacted form I DNA or to reactivation of the form I DNA inactivated by a sequence of formation and dissociation of D-loops (37). In the presence of 1.3 mM ATP, D-loops were completely dissociated by 30 min (Fig. 4B, closed circles). The amount of D-loops started to increase again (Fig. 4B, closed circles) only after the homology-dependent hydrolysis of ATP ceased (Fig. 4C, triangles). Considering our earlier observation that, even after the completion of the dissociation of D-loops, the reaction mixture still contained both active recA protein and active single-stranded fragments (37), this result suggests that inactivated form I DNA is gradually reactivated after the homology-dependent hydrolysis is depressed, probably by the accumulation of ADP. In order to confirm this conclusion, we added an amount of ADP sufficient to inhibit both formation and dissociation of D-loops before the completion of the first decrease in the amount of D-loops, incubated for a while at 37 °C, and then added a sufficient amount of ATP which overcame the inhibition by the amount of ADP. As shown in Fig. 5, the addition of ATP reinitiated a sequence of formation and subsequent dissociation of D-loops. Furthermore, the velocity of increase and decrease in the amount of D-loops after the reinitiation was the same as that in the first sequence of the reactions (Fig. 5). This result clearly indicates that, during the

![Fig. 3. Inhibition of formation and dissociation of D-loops by ADP. Fd form I [3H]DNA and homologous single-stranded fragments were incubated with 0.40 μM recA protein at 37 °C in the presence of 160 μM (A) or 1.3 mM (B) ATP and an indicated amount of ADP. The amounts of D-loops were assayed at 4 min (●) and 10 min (▲). The former indicates the formation; the difference between the former and the latter indicates the dissociation.](image)

![Fig. 4. The kinetics of ATP hydrolysis and D-loop formation promoted by recA protein. A and B, [3H]ATP (300 μM (A) and 1.3 mM (B)) was incubated with fd form I [3H]DNA, single-stranded fragments, and recA protein at 37 °C. Open symbols and closed symbols indicate the amount of ADP and the amount of D-loops, respectively. □, complete reaction mixture with homologous single-stranded fragments; ○, with heterologous single-stranded fragments; △, without form I DNA; Δ, without single-stranded fragments; V, without recA protein. The concentrations of recA protein were 0.40 μM (A) and 1.0 μM (B). C, difference between the amount of ADP produced with homologous single-stranded fragments and that with heterologous ones was taken as the amount of the homology-dependent hydrolysis. ●, with 300 μM ATP; ▲, with 1.3 mM ATP.](image)

![Fig. 5. Reactivation of form I DNA by ADP. Fd form I [3H]DNA, homologous (closed symbols), or heterologous (open symbols) single-stranded fragments and 0.40 μM recA protein were incubated at 37 °C with 160 μM ATP. At 11 min, 400 μM ADP was added to the reaction mixture and incubation was continued at 37 °C (●, ○). Excess ATP (1.9 mM) was added to an aliquot of the reaction mixture 19 min after the addition of ADP, and the incubation was continued at 37 °C (▲, △).](image)
incubation with ADP, almost all of the inactivated form I DNA was reactivated.

The inactivated form I DNA was fully reactivated also by just incubating the reaction mixture on ice-water for 15 min. After the formation and dissociation of D-loops were finished at 37 °C, we incubated the reaction mixture on ice-water for 15 min and then again shifted the temperature of the incubation to 37 °C. As shown in Fig. 6A, the temperature shift from 0 to 37 °C initiated the second sequence of formation and dissociation of D-loops at the same rate as that in the first sequence. This cycle, consisting of inactivation of form I DNA through formation and dissociation of D-loops and its reactivation by incubation at 0 °C, could be repeated at least twice (Fig. 6A). In this cycle, the third decrease in the amount of D-loops was not completed, probably because of the accumulation of ADP. The amounts of D-loops formed during 4 min of incubation after a temperature shift from 0 to 37 °C are plotted against the period of an incubation at 0 °C in Fig. 6B, and this result indicates that the incubation at low temperature for 15 min is required for full reactivation of the form I DNA.

Anomalous Kinetics in the Formation of D-loops by Limiting the Amount of recA Protein—We have described that dissociation of D-loops required a stoichiometric amount of recA protein and that limiting the amount of recA protein resulted in the anomalous kinetics in the formation and dissociation of D-loops (37). This anomaly resembles apparently that observed at a lower concentration of ATP described above (Figs. 2A and 4A); the amount of D-loops increased quickly in the first stage of incubation, then decreased and again increased slowly (37). Then, we tried to confirm that this anomaly is caused by limiting the amount of recA protein and not by the accumulation of ADP. We added an ATP-regenerating system to the reaction mixture and re-examined the kinetics of the formation of D-loops in the presence of a limited amount of recA protein. As shown in Fig. 7, net production of ADP was completely depressed (open circles), but the kinetics of D-loop formation still exhibited an anomaly (closed circles).

Therefore, there are at least two independent sets of conditions which result in anomalous kinetics in the formation of D-loops: limiting the initial concentration of ATP and limiting the amount of recA protein.

**DISCUSSION**

The observations described under “Results” reveal that ADP, a competitive inhibitor of DNA-dependent ATPase activity of recA protein (41), inhibits both formation of D-loops and their dissociation in an apparently competitive manner (Fig. 3). This indicates that both formation and dissociation are coupled with hydrolysis of ATP, although most of the homology-dependent hydrolysis is related to the dissociation and not to the formation (38). The hydrolysis of ATP is probably required for recycling of recA protein in these processes; by binding of ATP and either by binding of single-stranded DNA (in the case of the formation of D-loops) or by cooperative interaction with recA protein that already unwinds double-stranded DNA (in the case of the dissociation), each recA protein is stimulated to bind to double-stranded DNA, unwinds the double helix, and is then released from the duplex DNA upon hydrolysis of ATP (37, 42, 43).

We have shown that the form I DNA is inactivated without any damage to the DNA through a sequence of formation and dissociation of D-loops promoted by recA protein (37) and that homology-dependent hydrolysis of ATP is related to the dissociation of D-loops (38). This study showed that inactivated form I DNA was reactivated after hydrolysis-dependent hydrolysis ceased (Fig. 4) or by addition of sufficient ADP to inhibit both formation and dissociation of D-loops (Fig. 5). Therefore, it is likely that the homology-dependent hydrolysis of ATP is also related to keeping the form I DNA in an inactive state. The inactivated form I DNA could be reactivated by either of two pathways: (i) the true reversal of a sequence of formation and dissociation of D-loops or (ii) the independent reactivating process as shown in Fig. 1. The former possibility is not likely in the case of the reactivation by the addition of ADP or incubation at low temperature (Fig. 6) since both treatments did not stimulate the dissociation of D-loops, a partial step of the true reversal (Fig. 5); i.e. both

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2 Under the conditions which were used in our previous experiments to show the anomalous kinetics caused by limiting the amount of recA protein (Fig. 6B in Ref. 37), less than 0.12 μM of ATP was hydrolyzed to ADP during 90 min of incubation at 37 °C, and this amount of ADP is not sufficient to cause the anomaly (see the legend to Fig. 7, and Figs. 3 and 4).

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The inactivation and reactivation of form I DNA and their correlation with homology-dependent hydrolysis of ATP described above are consistent with a conclusion of our previous papers (37, 38) that a common mechanism causes dissociation of D-loops from form I DNA and inactivation of the form I DNA; after the completion of the dissociation of D-loops, the form I DNA is still unwound unidirectionally by cooperative binding of recA protein and is in a relaxed or topologically constrained state which makes the DNA an inactive substrate for reinitiation of D-loop formation. We recently demonstrated (44) that the form I DNA in an inactivated state is extensively unwound. Unidirectional unwinding by recA protein is supported by the directionality in the dissociation of D-loops observed by Wu et al. (45; see Fig. 1). Recycling of recA protein promoted by the hydrolysis of ATP is also required for keeping the form I DNA in an inactive state. If the cooperative binding of recA protein is inhibited by ADP or by incubation at low temperature, the form I DNA regains negative superhelicity and becomes an active substrate again as recA protein is released from the duplex (see Fig. 1).

We also showed that dissociation of D-loops is inhibited by ADP at a lower concentration than formation of D-loops. Therefore, at some lower concentration of ATP, homology-dependent hydrolysis of ATP and dissociation of D-loops stop and reactivation of an inactivated form I DNA starts by accumulation of ADP. But there is still enough ATP to support a second cycle of formation of D-loops. Observed anomalous kinetics in the formation of D-loops in the presence of excess recA protein is the results of these events (Figs. 2A and 4A). At a higher concentration of ATP, the dissociation of D-loops was completed and the homology-dependent hydrolysis continued even after the completion. Even in this case, after the homology-dependent hydrolysis ceased, the second phase of the formation of D-loops started (Fig. 4B).

We described previously that, in the presence of excess ATP, limiting the amount of recA protein also causes anomalous kinetics in the formation of D-loops. In this study, we showed that limiting the amount of recA protein caused the anomaly even in the presence of an ATP-regenerating system which efficiently converted ADP to ATP throughout the incubation (Fig. 7). Therefore, there are at least two independent sets of conditions which result in anomalous kinetics in the formation of D-loops. We gave a possible mechanism of the anomaly in the latter case in our previous paper (37). Since this study reveals that there is a pathway by which inactivated form I DNA is reactivated, the following is also a possible mechanism of the anomaly; as the amount of D-loops increases, the amount of (active) form I DNA decreases. D-loops are then dissociated and the amount of inactive form I DNA increases. As the amount of inactive form I DNA increases, the amount of active form I DNA increases through step III in Fig. 1, resulting again in the increase in the amount of D-loops (see Fig. 1). Finally, a D-loop cycle might be in a steady state if enough ATP is supplied. Accordingly, formation and dissociation of heteroduplex DNA or the amount of heteroduplex DNA in genetic recombination can be controlled by either the ratio of ATP to ADP or the amount of recA protein.

A D-loop cycle promoted by a protein like the E. coli recA protein might have a critical role in the segregation of homologous chromosomes in meiosis in eukaryotes. Pairing of homologous chromosomes is thought to assure the accurate segregation of them. Related or identical sequences may appear on nonhomologous chromosomes or on the same chromosome; e.g. a gene and its pseudogenes (46), long repeated sequences (see Ref. 47 for review), and so on. Therefore, the pairing of homologous chromosomes seems to require the completion of the whole length of the sequences between them. If a D-loop cycle starts between local homologous sequences, these sequences might pair once but soon dissociate. If the chromosomes are truly homologous, successive D-loop cycles at many sites might result in stable pairing and in precise alignment. Recently, a recA-like protein was purified from the cells of a simple eukaryote, Ustilago maydis, by Kmiec and Holloman (48).

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