THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 260, No. 1, Issue of January 10, pp. 170-173, 1985
Printed in U.S.A.

The Formation of Plectonemic Joints by the recA Protein of *Escherichia coli*

REQUIREMENT FOR ATP HYDROLYSIS*

(Received for publication, June 20, 1984)

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Formation of D-loops during the exchange of strands between a circular single-stranded DNA and a completely homologous linear duplex proceeds optimally when the duplex DNA is added to the complex of recA protein and single-stranded DNA formed in the presence of single-stranded DNA-binding protein and ATP. D-loops are undetectable when 200 μM adenosine 5′-*O*-(thiotriphosphate) is substituted for ATP. D-loops can be formed in the presence of adenosine 5′-*O*-(thiotriphosphate) if recA protein is the last component added to the reaction. However, these D-loops, which depend upon homologous sequences, are unstable upon deproteinization and are formed to a more limited extent than the structures formed with ATP. This finding indicates that D-loops formed under these conditions may be largely nonintertwined paranemic structures rather than plectonemic structures in which two of the strands are interwoven. When adenosine 5′-*O*-(thiotriphosphate) is added to an ongoing reaction containing ATP, formation of plectonemic structures and ATP hydrolysis is inhibited to an equivalent extent. We, therefore, conclude that ATP hydrolysis is required for the formation of plectonemic structures.

The recA and single-stranded DNA-binding proteins of *Escherichia coli* together promote the efficient transfer of strands from linear duplex to circular single-stranded DNA (1–3). After formation of a stable complex between recA and ssDNA,[1] the reaction was found to proceed in two kinetically distinct steps, D-loop formation followed by directed branch migration (2, 4). Formation of D-loops could occur in the presence of ATPγS, a nonhydrolyzable analog of ATP, suggesting that D-loop formation was independent of branch migration, which had an absolute requirement for ATP hydrolysis (2). This observation further implied that D-loop formation which involves a search for homology, unwinding of duplex DNA, and pairing (even to a limited extent) could occur in the absence of ATP hydrolysis. ATPγS induces a tight binding of recA to ssDNA (6) and decreases markedly the ability of recA to transfer from one ssDNA to another. Consequently, formation of D-loops in the presence of ATPγS should involve a mechanism in which recA protein never leaves the ssDNA to which it is initially bound, whether it be (unwound) duplex or the circular ssDNA. In contrast, ATP destabilizes the recA protein-ssDNA complexes, since its hydrolysis allows the dissociation of recA protein from the ssDNA (6, 7). Thus, the mechanism of D-loop formation in the presence of ATPγS could be different from that with ATP. We have, therefore, undertaken a detailed examination of the role of ATP and ATPγS in D-loop formation and have found that ATP hydrolysis is required for formation of *plectonemic* structures in which the (−)-strand of the linear duplex is intertwined with the (+)-ssDNA. It does not proceed in the presence of ATPγS. We further demonstrate that an ATPγS-supported pairing reaction leads to the formation of D-loop structures which are topologically similar to the *paranemic joints* described by Bianchi *et al.* (5). However, these form only after a specific order of addition of reaction components.

MATERIALS AND METHODS

DNAs, recA protein, and SSB were prepared as described previously (2). The same recA protein preparation was used throughout except where indicated. ATPγS was purchased from Boehringer Mannheim. D-loops were measured using assays that detect formation of paranemic and plectonemic joints as described in the accompanying paper (15). A heterologous control was performed for every assay, and unless explicitly stated no reaction could be detected by the nitrocellulose filter-binding assay. Plectonemic joints persist after treatment with 5.2 M GHCl at 32 °C; paranemic joints are dissociated under these conditions but remain largely intact following treatment with 2 M NaCl at 0 °C. The general term "D-loop" will be used to signify duplex DNA structures that bind to nitrocellulose filters by virtue of their association with homologous ssDNA. SDS was used as a protein denaturant as described previously (2). Proteinase K was obtained from Merck.

ATP hydrolysis was measured using a previously described procedure (8).

RESULTS

Effect of Order of Addition on Formation of Plectonemic Joints—The effect of order of addition of reactants on the rate of plectonemic joint formation is shown in Fig. 1. When recA protein was added after preincubation of the remaining components, plectonemic joint formation occurred only after a considerable lag (approximately 5 min) and then increased linearly with time. Presumably, recA protein must compete with the already-bound SSB for the ssDNA, and the lag is consistent with the prerequisite formation of sufficient complex to permit pairing.

* This work was supported by Grant GM 06196 from the National Institutes of Health and Grant PCM-7904638 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Commonwealth Scientific and Industrial Research Organization, Division of Tropical Animal Science and the Australian Meat Research Committee. On leave from Commonwealth Scientific and Industrial Research Organization, Division of Tropical Animal Science, Long Pocket Laboratories, Private Bag No. 3, Indooroopilly, Queensland 4068, Australia.

[1] The abbreviations used are: ssDNA, single-stranded DNA; ATPγS, adenosine 5′-*O*-(thiotriphospahte); AMP-PNP, adenyl-5′-yl imidodiphosphate; SSB, *E. coli* single-stranded DNA-binding protein; GHCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate.

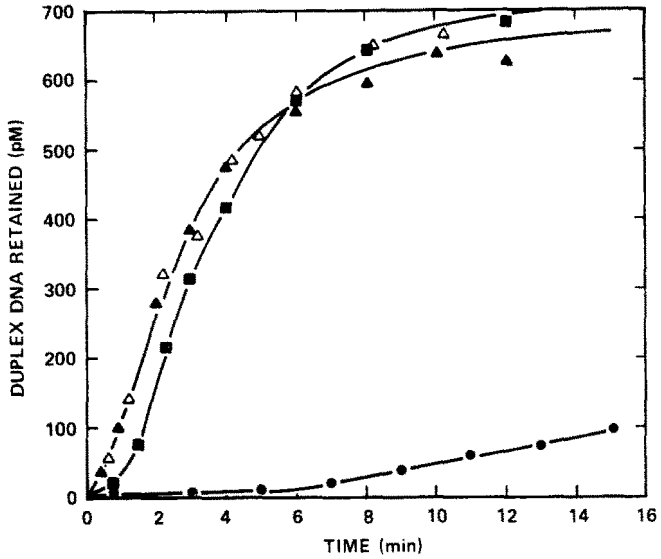

FIG. 1. **The effect of the order of addition of reaction components on D-loop formation.** Reactions were performed as described under "Materials and Methods." Deproteinization was by treatment with 5.2 M GHCl at 25 °C. The order of addition for recA protein added last (●): M13*mp8* ssDNA, M13*mp8* linear duplex DNA, ATP, SSB, recA protein; SSB/ATPγS added last (■): recA protein, M13*mp8* ssDNA, M13*mp8* linear duplex, SSB/ATP; M13*mp8* linear duplex added last (△, ▲): recA protein, M13*mp8* ssDNA, SSB/ATP, M13*mp8* linear duplex.

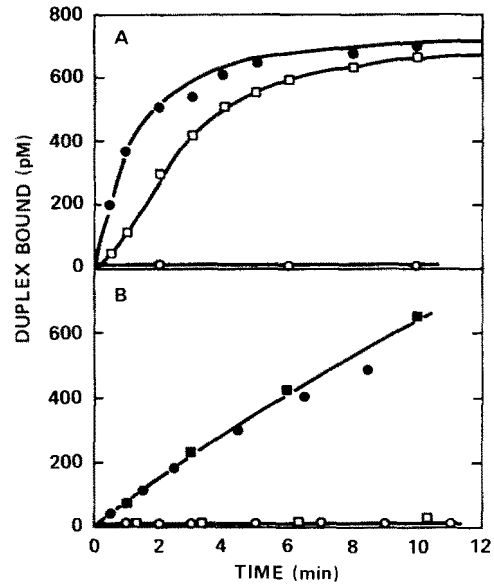

FIG. 2. **D-loop formation in the presence of ATP (*A*) and ATPγS (*B*) (linear duplex DNA added last).** Reactions performed as described under "Materials and Methods" contained M13*mp8* ssDNA and M13*mp8* duplex DNA linearized with *Pst*I. Treatment was with 2 M NaCl, 22 mM EDTA, 0 °C for 10 min (●) or 5.2 M GHCl, 22 mM EDTA, 32 °C for 10 min (□). φX174 ssDNA and M13*mp8* duplex: DNA deproteinization with 2 M NaCl, 22 mM EDTA, 0 °C for 10 min (■) or 5.2 M GHCl, 22 mM EDTA, 32 °C for 10 min (○). recA protein was present at 1 monomer/3.0 nucleotides of ssDNA; SSB at 1 monomer/8.2 nucleotides; ATP was added at 4 mM and ATPγS at 200 μM. *A*, ATP; *B*, ATPγS.

When a mixture of SSB and ATP was used to initiate the reaction, there was a shorter lag (approximately 1.5 min, measured from zero time to the point of inflection) before a relatively rapid increase in plectonemic joint formation occurred. Part of the lag may be due to the time required to complete complex formation. However, it is apparent that the resulting curves (recA added last, or SSB and ATP added last) are the products of several effects (including complex formation and plectonemic joint formation); the kinetics are, therefore, not amenable to simple interpretation.

When duplex DNA was added to the preformed recA protein-ssDNA complex a shorter lag occurred before the rapid increase in plectonemic joint formation. This lag was independent of the length of preincubation (15) and is, therefore, unlikely to be due to time required for complex formation.

Comparison of ATP- and ATPγS-dependent D-loop Formation—In the presence of ATP and under conditions where the order of addition was optimized (duplex DNA the last component added), there was a rapid formation of both paranemic and plectonemic joints between M13*mp8* ss- and duplex DNAs (Fig. 2*A*). The nonhomologous control reaction between φX ssDNA and M13*mp8* duplex DNA was negligible (<1%). A different result was obtained when ATPγS was substituted for ATP (Fig. 2*B*). Using conditions that detect paranemic joints (15), an identical rate of reaction was observed whether or not the DNAs were homologous. Furthermore, the initial rate of these reactions was about 5-fold lower than that for the ATP-dependent formation of paranemic joints. When treatment that detects only plectonemic joints was used to monitor pairing in the presence of ATPγS (15), there was no measurable reaction (heterologous or homologous).

When recA protein (1.7 recA protein monomers/nucleotide) was added following preincubation of the remaining components of the reaction, ATPγS supported some D-loop formation (4.4% of the total possible reaction) (Fig. 3). These D-loops are unlikely to be plectonemic joints since they were

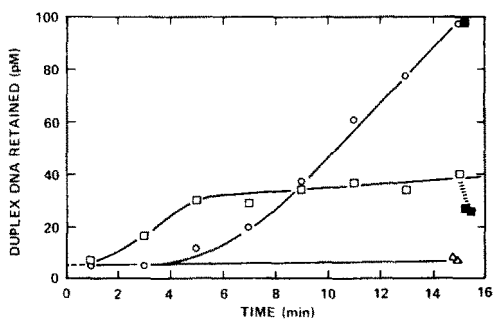

FIG. 3. **The effect of ATPγS on D-loop formation (recA protein added last).** Reactions were performed as described under "Materials and Methods." ○, 4 mM ATP, deproteinization with GHCl at 25 °C; □, 200 μM ATPγS, deproteinization with GHCl at 25 °C; △, φX174 ssDNA substituted for M13*mp8* ssDNA (■) treatment was with GHCl at 37 °C. recA protein was present at 1 monomer/1.7 nucleotides of ssDNA.

sensitive to further heating (Fig. 3). Furthermore, the conditions of treatment with GHCl[2] do not deproteinize completely the recA protein-ssDNA complexes formed in the presence of ATPγS (not shown). In contrast, the D-loops formed in the presence of ATP, presumably plectonemic joints, were not affected by further heating and showed different kinetics of formation. It is likely that the ATPγS-dependent pairing depends on protein for stability and may occur by a mechanism different from that with ATP.

Use of SDS as Protein Denaturant—The measurement of D-loops formed in the presence of ATP or ATPγS using SDS at 0 °C for 20 s in place of GHCl as a protein denaturant is shown in Fig. 4*A*. The reaction with ATPγS reached a plateau

[2] P. W. Riddles and I. R. Lehman, unpublished observation.

TABLE I

Effect of SDS treatment on stability of D-loops

Experiment	Treatment	Duplex DNA bound to nitrocellulose			
		recA protein added last[a]		Duplex DNA added last[b]	
		4 mM ATP	200 μM ATPγS	4 mM ATP	200 μM ATPγS
		%			
1	Homologous reaction[c]	12.9	13.7	40.2	2.1
1a	Heterologous reaction	0.49	0.84	0.53	
2	SDS/37 °C/proteinase K/30 min	9.58	1.40		
3	SDS/37 °C/30 min	11.50	2.13		
4	SDS/37 °C/3 min			37.2	
	SDS/37 °C/9 min			46.5	
	SDS/37 °C/12 min			44.9	

[a] The reaction was stopped at 8 min by the addition of SDS and EDTA at 0 °C. Aliquots were immediately withdrawn and subjected to the treatments indicated.

[b] The reaction was stopped at 3 min by the addition of EDTA at 0 °C, and aliquots were treated as indicated.

[c] M13*mp*8 ssDNA and M13*mp*8 duplex linearized with *Pst*I; recA protein was at 1 monomer/1.7 nucleotides of ssDNA. All other conditions were as described under "Materials and Methods."

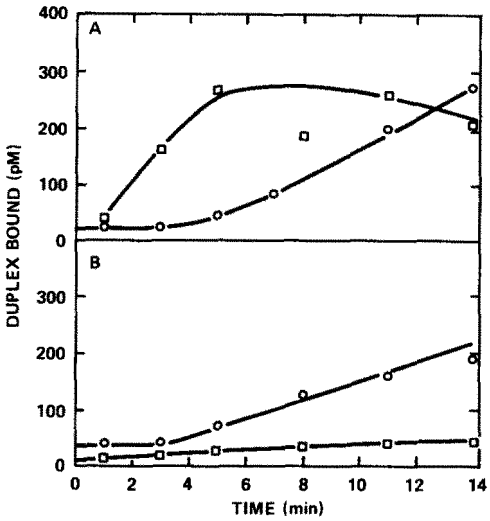

FIG. 4. **Effect of temperature of SDS treatment on measurement of D-loop formation with M13*Gori*1 ssDNA and M13*Gori*1 duplex DNA linearized with *Xho*I in the presence of ATP and ATPγS.** Reactions were performed as described under "Materials and Methods." *A*, SDS treatment at 0 °C for 20 s. *B*, SDS treatment at 37 °C for 10 min in the presence of 4 mM ATP (○) or 200 μM ATPγS (□). recA protein was added last at 1 monomer/1.5 nucleotides; SSB at 1 monomer/8.2 nucleotides.

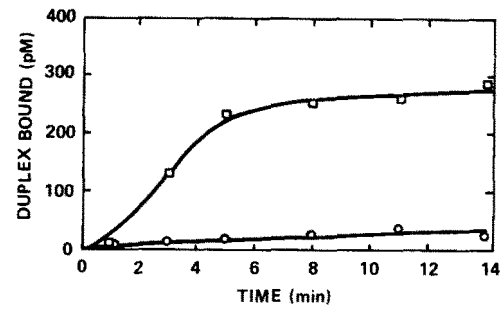

FIG. 5. **Effect of temperature of SDS treatment on measurement of D-loop formation with M13 wild-type ssDNA and M13*Gori*1 duplex DNA linearized with *Xho*I.** Reactions were performed as described under "Materials and Methods." SDS treatment at 0 °C for 20 sec (□); SDS treatment at 37 °C for 10 min (○). recA protein was added last at 1 monomer/1.5 nucleotides of ssDNA.

within 3 min and remained at that level for greater than 30 min (not shown). On the other hand, in the presence of ATP there was a considerable lag before the onset of a slow linear rate. When identical experiments are performed, but the temperature of SDS treatment raised to 37 °C (10 min), the products of the reaction with ATP showed moderate sensitivity to this treatment, whereas the products formed in the presence of ATPγS were promptly dissipated (Fig. 4*B*). The same results were obtained in an independent experiment using a different preparation of recA protein (Table I, Experiments 1–3). The addition of proteinase K had little effect indicating that deproteinization in the presence of SDS alone is complete at 37 °C (Experiment 2, Table I). Inasmuch as SDS is not as effective a protein denaturant at 0 °C as it is at 37 °C (or 5.2 M GHCl at 32 °C), a plausible inference from this experiment is that the D-loops formed in the presence of ATPγS require protein for their stabilization.

When linear duplex DNA containing heterologous ends was incubated with ssDNA in the presence of ATPγS, a reaction profile identical to that observed in Fig. 4*A* was observed (Fig. 5). The products of this reaction were virtually all destroyed by treatment with SDS at 37 °C. Thus, the ATPγS-dependent reaction does not require a free homologous end for an efficient reaction. The properties of D-loops formed under these conditions are consistent with those of a paranemic joint (5).

When SDS was used as a protein denaturant under conditions in which duplex DNA was the last component added, little homology-dependent reaction (<2% total) was observed.

We have noted some variability among preparations of recA protein in their ability to support the ATPγS-dependent D-loop formation, ranging from 5 to 60% of the maximum extent possible, despite the fact that all other parameters measured *viz.* branch migration[3] and ssDNA-dependent ATPase activity[2] were identical.[4]

Requirement of ATP Hydrolysis for Plectonemic Joint Formation—ATPγS causes recA protein to bind tightly to ssDNA without measurable dissociation at 37 °C (9). It is also a strong inhibitor of the ssDNA-dependent ATPase activity of recA protein (9). To examine directly the effects of ATPγS on plectonemic joint formation, ATPγS was added to a reaction performed in the presence of ATP. As shown in Fig. 6, when 200 μM ATPγS was added 1.5 min after initiation of the reaction, there was a rapid decline in the rate of plectonemic joint formation (Fig. 6*A*) corresponding to an instantaneous inhibition of ATP hydrolysis (Fig. 6*B*). When lower concen-

[3] D. A. Soltis and I. R. Lehman, unpublished observation.

[4] We do not understand the reason for this variability; however, the results in the present work were obtained with a single batch of recA protein that showed a "good" reaction with ATPγS, except for experiments 1, 2, and 3 of Table I which showed a "poor" reaction.

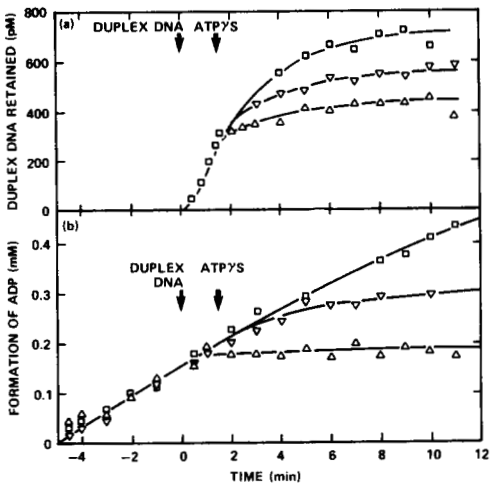

FIG. 6. **The effect of addition of ATPγS to an ongoing reaction.** *A*, D-loop formation; *B*, ATP hydrolysis. The reaction mixture contained 4 mM ATP and was started by the addition of linear duplex DNA. ATPγS: 200 μM (△), 45 μM (▽), and 0 μM (□) were added 1.5 min after the addition of linear duplex DNA, shown by *arrows*. ATP hydrolysis was measured from the time of addition of ATP/SSB at −5 min.

trations of ATPγS were used, the result was the same except that there was a lag before maximal inhibition of both ATPase and plectonemic joint formation occurred. Clearly, there is a strong correlation between the ability to form plectonemic joints and the hydrolysis of ATP. As indicated in Fig. 6*B*, there was little difference in the rate of ATP hydrolysis after the addition of linear duplex DNA, suggesting that ATP hydrolysis occurs independently of the pairing reaction.

DISCUSSION

ATP and its hydrolysis are required for the continuing formation of plectonemic joints. In the presence of ATP, plectonemic joint formation occurs at different rates and to varying extents depending on the order of addition of components. Maximal rates are achieved when duplex DNA is added last, and this is consistent with the concept of preformation of a reactive stable complex composed of ssDNA and recA protein. An analogous observation in the absence of SSB has been made by Gonda and Radding (10) except that a longer preincubation time (20 min) and an ATP regeneration system were required. Presumably, SSB facilitates formation of stable recA protein-ssDNA complexes and minimizes the requirement for ATP (7).

In the presence of ATPγS, significant D-loop formation is observed only when recA protein is added last and when SDS at 0 °C is the protein denaturant. The kinetics of this reaction are different from the equivalent ATP-dependent reaction and unlike the latter, the products are unstable to heating in SDS at 37 °C. In further contrast to the ATP-dependent reaction, a linear duplex DNA containing heterologous terminal sequences reacts as efficiently as one with homologous ends and shows the same stability to heating in SDS at 37 °C. We have, therefore, concluded that the pairing that occurs in the presence of ATPγS is predominantly paranemic (5). This assertion is supported by the observation that only a residual level (<4%) of plectonemic joint formation occurs when recA protein is the component added last.

The role of ATPγS in the formation of these paranemic joints is not clear; however, it is possible that the irreversible binding of recA protein to ssDNA in the presence of ATPγS is a crucial factor that enables otherwise unstable structures to be detected. An important condition for plectonemic joint formation and branch migration might be an equilibrium between recA protein and ssDNA that does not favor completely recA protein-ssDNA complex formation. This equilibrium is presumably ideally approached with ATP.

When duplex DNA is added to the preformed recA protein-ssDNA complex, conditions that are optimal for the ATP-dependent reaction, no D-loop formation (<2%) occurs in the presence of ATPγS. A similar observation was made with the rec1 protein from *Ustilago maydis* and AMP-PNP (12).

In the light of these findings, our previous view of the strand exchange reaction requires some modification (1, 2, 13). The conclusion that D-loop (plectonemic joint) is a kinetically distinct phase that precedes branch migration was based primarily on the observation that D-loops could form in the presence of ATPγS, whereas heteroduplex extension by branch migration required continual ATP hydrolysis.

The evidence presented here shows that plectonemic joints do not form without ATP hydrolysis and cannot proceed in the presence of ATPγS. We cannot, therefore, separate strand exchange into the two steps, plectonemic joint formation and branch migration as suggested previously (1, 2, 13). It is, however, possible that paranemic joints may form without ATP hydrolysis and may represent a kinetically distinct phase in strand exchange. It is relevant to note that paranemic joints (formed in the absence of SSB) dissociate upon accumulation of ADP (5). Thus, hydrolysis of ATP might allow formation of plectonemic joints after dissociation of paranemic joints. That is, ATP hydrolysis may be required for the conversion of paranemic to plectonemic joints. Finally, ATPγS-dependent pairing reactions have only been observed in the presence of SSB (1, 2, 14); our observation that D-loops formed in the presence of ATPγS are paranemic is consistent with the stabilization of paranemic joints by SSB noted previously (15).

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