

On the mechanism of renaturation of complementary DNA strands by the *recA* protein of *Escherichia coli*

(recombination/DNA sequences/ATP hydrolysis/D loops)

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ABSTRACT The renaturation of complementary DNA strands by the *recA* protein of *Escherichia coli* has been found to exhibit the following features. (i) Optimal renaturation occurs at *recA* protein levels below that required to saturate the DNA strands; saturating amounts of *recA* protein significantly reduce the rate of reaction. (ii) The reaction proceeds in the absence of a nucleotide cofactor but is markedly stimulated by ATP in the presence of 10 mM Mg²⁺. A similar stimulation occurs in the absence of ATP when the Mg²⁺ concentration is increased from 10 mM to 30–40 mM. (iii) Both the ATP-stimulated and the Mg²⁺-stimulated reactions follow apparent first-order kinetics. These results, taken together with the known effects of ATP and Mg²⁺ on the state of aggregation of *recA* protein, suggest that the association of *recA* monomers may play an important role in *recA* protein-promoted DNA renaturation.

The *recA* protein of *Escherichia coli* is essential for homologous recombination and for the postreplication repair of damage to DNA (1). *In vitro*, *recA* protein will promote a variety of ATP-dependent DNA-pairing reactions, including the renaturation of complementary single strands, assimilation of linear single strands into duplex DNA (D-loop formation), and the exchange of strands between linear duplex and homologous circular single-stranded DNAs (ss DNAs). Although these pairing activities illustrate the role of the *recA* protein in recombination, their mechanisms are not yet understood (1).

The renaturation of complementary DNA strands was the first pairing reaction of *recA* protein to be discovered; it is also the simplest pairing activity associated with the *recA* protein (2). As such, it provides an attractive model to study the mechanism of the *recA* protein-dependent alignment of complementary DNA sequences. As a first step, we recently reported studies of the interaction of *recA* protein with ϕ X174 ss DNA [(+)-strand] in the absence of its complementary partner (3). We report here a detailed analysis of *recA* protein-promoted renaturation of the complementary (+)- and (-)-strands of ϕ X174 DNA.

EXPERIMENTAL PROCEDURES

Materials. *recA* protein was purified to homogeneity as described (4). Its concentration was calculated from $A_{280}^{1\%} = 5.9$. S1 nuclease, ADP, ATP, and calf thymus DNA were from Sigma. ATP[γ S] was from Boehringer Mannheim, and [³H]ATP was from ICN. GF/C filters were from Whatman.

Unlabeled and ³H-labeled circular ϕ X174 ss DNA [(+)-strand] were prepared as described (5). ³H-labeled linear duplex ϕ X174 DNA prepared by *Pst* I cleavage of ³H-labeled circular duplex ϕ X174 DNA also has been described (5). DNA concentrations were calculated by using an A_{260} value

of 1 as equivalent to 36 μ g of ss DNA and 50 μ g of duplex DNA per ml. All DNA concentrations are expressed as nucleotides.

³H-labeled linear duplex ϕ X174 DNA was denatured by addition of NaOH to a concentration of 0.1 M. After incubation on ice for 10 min, the sample was dialyzed at 4°C against buffer containing 1 mM NaCl, 0.1 mM EDTA, and 0.1 mM Tris base. The denatured DNA was stored at 4°C.

Methods. Renaturation assay. All renaturation reactions were carried out in reaction buffer containing 25 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, and 5% glycerol unless otherwise indicated. *recA* protein, ³H-labeled DNA, and nucleotide cofactor concentrations are given in the figure legends. All reactions were carried out at 25°C unless otherwise indicated. For reactions containing *recA* protein, all components (except *recA* protein) were incubated for 5 min, and an aliquot was removed for the zero time points. Reactions were then initiated by the addition of *recA* protein.

At the indicated times, aliquots (25 μ l) were removed and quenched by mixing with 2.5 μ l of sodium dodecyl sulfate (10%). The mixtures were then diluted into 300 μ l of digestion buffer containing 150 mM NaCl, 50 mM sodium acetate (pH 4.6), and 1 mM zinc acetate; then 3.0 μ l of heat-denatured calf thymus DNA (2 mg/ml) and 40 units of S1 nuclease (Sigma) were added. The mixtures were kept at 37°C for 30 min, and the reaction was terminated by the addition of 15 μ l of heat-denatured calf thymus DNA (2 mg/ml) and 350 μ l of ice-cold trichloroacetic acid (10%). The mixtures were kept on ice for 30 min and then filtered on GF/C filters. The filters were washed with cold 10% trichloroacetic acid (three times with 1 ml) and 95% ethanol (one time with 1 ml) and then dried, and the radioactivity was measured.

Under these conditions, both ϕ X174 ss DNA (+)-strand and denatured duplex ϕ X174 DNA (30 μ M in original aliquot) were >98% degraded as judged by acid-precipitable radioactivity. An equivalent amount of linear duplex ϕ X174 DNA remained >95% resistant to digestion under the same conditions. The formation of S1 nuclease-resistant DNA was completely dependent on the presence of both complementary strands of ϕ X174 DNA. In all cases, control reactions with ³H-labeled ϕ X174 ss DNA (+)-strand produced no detectable S1 nuclease-resistant material (<1% under all conditions).

ss DNA-dependent ATPase assay. This assay was performed as described, with [³H]ATP as substrate (2).

RESULTS

Nonenzymatic Renaturation of Complementary Single Strands. Conversion of the S1 nuclease-susceptible separated (+)- and (-)-strands of *Pst* I-cleaved ϕ X174 form I DNA to an S1 nuclease-resistant form was used to measure renaturation (5). When the ³H-labeled separated strands (30 μ M)

were incubated in reaction buffer at 37°C, nonenzymatic renaturation occurred. The data gave a linear second-order plot, yielding a second-order rate constant of $6.7 \text{ M}^{-1}\text{s}^{-1}$. A similar value ($6.8 \text{ M}^{-1}\text{s}^{-1}$), obtained at $150 \mu\text{M}$ DNA, demonstrated that the rate constant was independent of concentration, thus confirming that the reaction followed second-order kinetics. At 25°C, the nonenzymatic renaturation was slower by a factor of 6, with a rate constant of $1.1 \text{ M}^{-1}\text{s}^{-1}$. The second-order rate constant measured with $30 \mu\text{M}$ DNA under optimal conditions (60°C, 1 M NaCl) was $1235 \text{ M}^{-1}\text{s}^{-1}$, about 1100 times greater than that measured in reaction buffer at 25°C.

recA Protein-Promoted Renaturation of Complementary Single Strands. When recA protein ($1 \mu\text{M}$) was added to the separated ϕX174 DNA (+)- and (-)-strands ($30 \mu\text{M}$) in the presence of ATP ($500 \mu\text{M}$) at 25°C, rapid renaturation occurred with $\approx 75\%$ of the DNA becoming S1 nuclease resistant (Fig. 1 *Left*) within 10 min. In the presence of recA protein but without ATP, renaturation occurred with an initial phase ≈ 2 - to 3-fold slower than the ATP-stimulated reaction; this was followed by a slow second phase (Fig. 1 *Left*). Addition of ATP to the reaction during the slow phase caused an immediate stimulation, with the reaction reaching the same plateau as that obtained with ATP present throughout. In the absence of recA protein, there was little renaturation during the time of the reaction ($< 2\%$).

Effect of recA Protein Concentration on the Rate of Renaturation. The initial rate of renaturation of ϕX174 DNA ($30 \mu\text{M}$) was measured as a function of recA protein concentration. Both the ATP-independent and ATP-stimulated reactions were examined. The rate of the ATP-stimulated reaction increased with increasing recA protein until a maximal rate was reached at a ratio of 1 recA monomer to 30 nucleotides of ss DNA (Fig. 2). A similar optimal ratio was found for the ATP-independent reaction. Based on a binding stoichiometry of 1 recA monomer to 4 nucleotides of ss DNA, this ratio corresponds to 10–15% coverage of the DNA by recA protein (3). Higher levels of recA protein markedly reduced the rate of renaturation.

Two regions of the recA protein concentration-dependence curve are of particular interest. One is at the optimal ratio of recA protein to ss DNA. At this level the ATP-stimulated reaction proceeded at the maximal rate; however, there

was a substantial ATP-independent reaction (Fig. 1 *Left*). The ATP-stimulated reaction was > 500 -fold faster than the nonenzymatic reaction under these conditions. The other region of interest was at a ratio of ≈ 3 recA monomers to 30 nucleotides of ss DNA. At this level, the ATP-stimulated reaction was reduced to $\approx 40\%$ of the maximal rate; however, the ATP-independent reaction was completely eliminated (Fig. 1 *Right*). Thus, at the higher level of recA protein, the renaturation reaction is completely ATP dependent.

Determination of Reaction Order of Renaturation. To determine the kinetic order of the ATP-stimulated renaturation reaction, the ratio of recA protein to ss DNA was kept at 1 recA monomer to 30 nucleotides, and the DNA concentration was varied over a 10-fold range. Full-time courses of renaturation were measured at each DNA concentration and gave linear first-order plots; furthermore, the first-order rate constants were essentially independent of DNA concentration, providing further evidence that the ATP-stimulated reaction follows first-order kinetics (Table 1). The rate constants also were independent of the ATP concentration over the range of 0.2 to 2.0 mM.

The kinetic order of the ATP-independent renaturation reaction was not analyzed because it appeared to follow biphasic kinetics.

ATP Hydrolysis During Renaturation. The time course of ATP hydrolysis to ADP and P_i during the ATP-stimulated renaturation reaction was identical to the ATPase activity measured when the mixture of the (+)- and (-)-strands of ϕX174 DNA was replaced by ϕX174 DNA (+)-strand, a substrate that does not undergo renaturation, as determined by S1 nuclease analysis (data not shown).

Little ATP hydrolysis occurred during renaturation (catalytic constant $k_{\text{cat}} = 6 \text{ min}^{-1}$), and the inclusion of an ATP-regeneration system had no effect on the renaturation kinetics. These results suggest that accumulation of ADP in solution is not important for the ATP-stimulated reaction.

ADP ($200 \mu\text{M}$) at the optimal recA protein-to-DNA ratio produced a stimulation in the rate of renaturation relative to the nucleotide-independent reaction, although not quite as great as that with ATP (Fig. 1 *Left*). Also, addition of ADP to a nucleotide-independent reaction that had entered the slow phase resulted in a burst of renaturation similar to that seen upon addition of ATP. Stimulation of the renaturation reac-

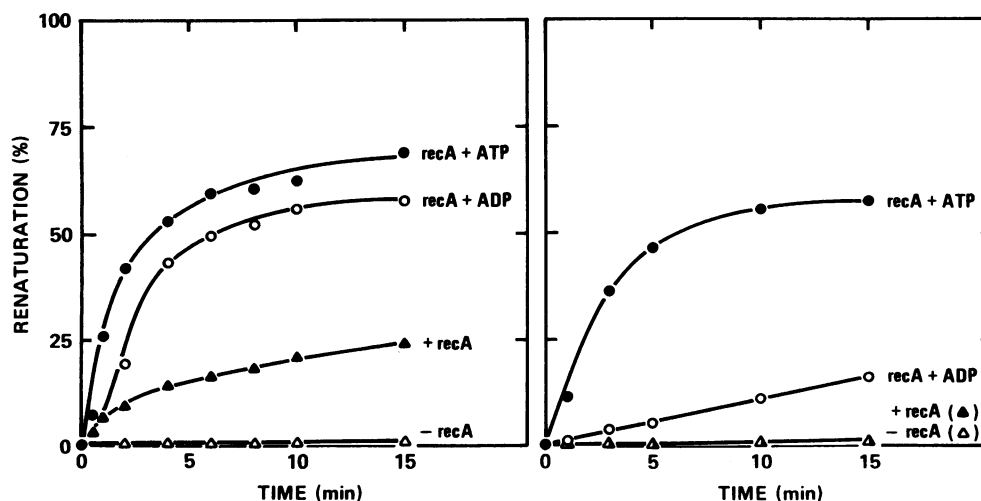


FIG. 1. Renaturation of alkali-denatured ϕX174 [^3H]DNA by recA protein. Reactions were carried out as described. (*Left*) Reaction solutions contained 25 mM Tris-HCl (pH 7.2), 10 mM MgCl_2 , and $30 \mu\text{M}$ denatured ϕX174 [^3H]DNA with no additions (Δ) or with $1.0 \mu\text{M}$ recA protein (\blacktriangle), $1.0 \mu\text{M}$ recA protein/ $200 \mu\text{M}$ ADP (\circ), or $1.0 \mu\text{M}$ recA protein/ $200 \mu\text{M}$ ATP (\bullet). (*Right*) Reaction solutions contained 25 mM Tris (pH 7.2), 10 mM MgCl_2 , and $30 \mu\text{M}$ denatured ϕX174 [^3H]DNA with no additions (Δ), $3.0 \mu\text{M}$ recA protein (\blacktriangle), $3.0 \mu\text{M}$ recA protein/ 1.0 mM ADP (\circ), or $3.0 \mu\text{M}$ recA protein/ 1.0 mM ATP (\bullet).

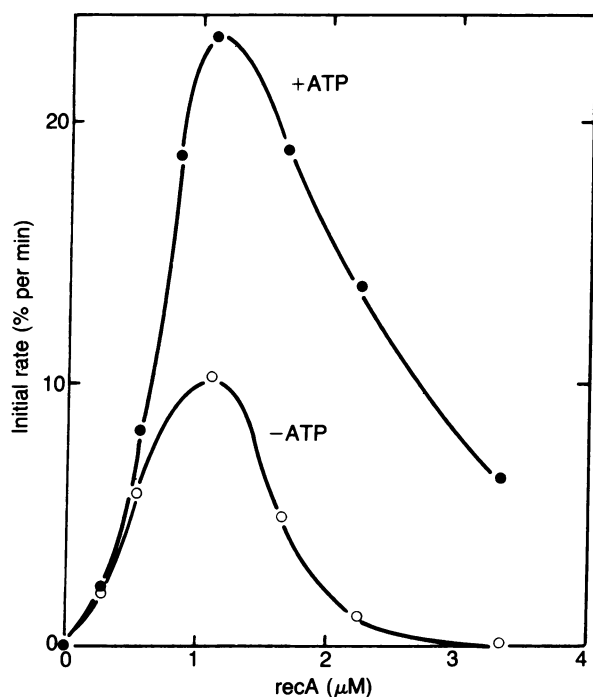


FIG. 2. Dependence of rate of DNA renaturation on recA protein concentration. Reactions were carried out as described. Reaction solutions contained 25 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, 30 μM denatured ϕ X174 [³H]DNA, and the indicated amount of recA protein without (○) or with (●) ATP (1.0 mM). The points represent the initial rates of renaturation expressed as percentage of the ϕ X174 [³H]DNA becoming resistant to S1 nuclease digestion per min.

tion by ADP also occurred at the higher recA protein-to-DNA ratio, where there is no nucleotide-independent reaction. Again, the stimulation was not as great as that with ATP (Fig. 1 Right).

A limited amount of renaturation ($\approx 10\%$) occurred under optimal conditions when ATP[γ S] (200 μM) was added as a nucleotide cofactor. This reaction could not be further stimulated by the addition of ATP. When the reaction was repeated with ϕ X174 ss DNA (+)-strand, there was no reaction, demonstrating that the limited amount of S1 nuclease-

Table 1. Rate of ATP-stimulated renaturation as a function of recA protein-DNA concentration

DNA, μM	0.2 mM ATP k_{obs} , min ⁻¹	2.0 mM ATP k_{obs} , min ⁻¹
15	0.35	0.35
30	0.43	0.40
45	0.43	0.43
75	0.43	0.39
150	—	0.45

Reaction solutions contained 25 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, and the indicated concentrations of ATP and denatured ϕ X174 [³H]DNA. recA protein was added to each reaction in a ratio of 1 recA monomer to 30 nucleotides of DNA.

resistant material produced from ϕ X174 DNA (+)- and (-)-strands was due to renaturation and not to nuclease protection by tightly bound recA protein (unpublished data).

Dependence of Renaturation on Mg²⁺ Concentration. The Mg²⁺ concentration dependence of the ATP-stimulated and the ATP-independent renaturation reactions under optimal conditions is shown in Fig. 3. There was no ATP-stimulated renaturation in the absence of added Mg²⁺. Increasing levels of Mg²⁺ resulted in increasing rates of renaturation until saturation was achieved at ≈ 10 mM. Higher levels of Mg²⁺ (up to 50 mM) did not cause any significant increase in rate. In all ATP-stimulated reactions described thus far, 10 mM was the standard Mg²⁺ concentration.

Nucleotide-independent renaturation also required added Mg²⁺ (Fig. 3 Right). Increasing levels of Mg²⁺ resulted in increasing rates and extents of reaction. In contrast to the ATP-stimulated reaction, however, 10 mM Mg²⁺ was not saturating; instead, the optimal Mg²⁺ concentration was ≈ 40 mM. At this Mg²⁺ concentration, the nucleotide-independent reaction proceeded at the same rate and to the same extent as the ATP-stimulated reaction. Mg²⁺ concentrations ≥ 50 mM resulted in complete loss of the nucleotide-independent reaction. The stimulation at 40 mM Mg²⁺ relative to 10 mM Mg²⁺ does not appear to be due simply to the increase in ionic strength, since inclusion of various concentrations of NaCl (15–90 mM) in addition to 10 mM Mg²⁺ resulted in inhibition of the nucleotide-independent reaction (data not shown).

When the Mg²⁺ concentration dependence of the nucleotide-independent reaction was examined at the higher recA-

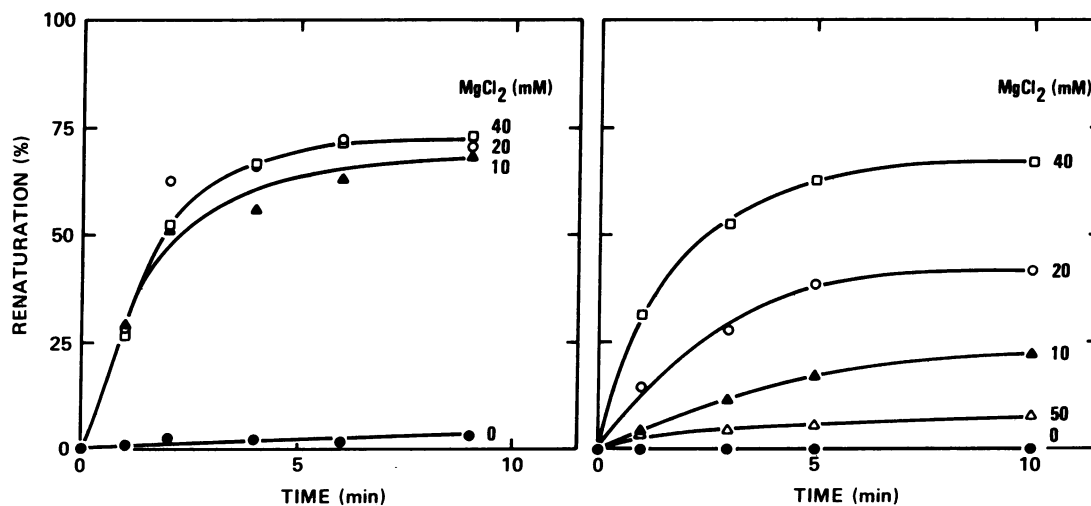


FIG. 3. Dependence of DNA renaturation on Mg²⁺ concentration at low recA protein. Reactions were carried out as described. (Left) Reaction solutions contained 25 mM Tris-HCl (pH 7.2), 30 μM denatured ϕ X174 [³H]DNA, 1.0 μM recA protein, 200 μM ATP without MgCl₂ (●) or with MgCl₂ at 10 mM (▲), 20 mM (○), and 40 mM (□). (Right) Reaction solutions contained 25 mM Tris-HCl (pH 7.2), 30 μM denatured ϕ X174 [³H]DNA, and 1 μM recA protein without MgCl₂ (●) or with MgCl₂ at 10 mM (▲), 20 mM (○), 40 mM (□), or 50 mM (Δ).

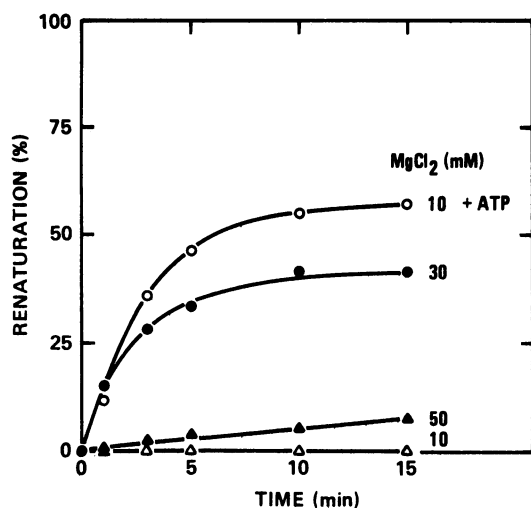


FIG. 4. Dependence of DNA renaturation on Mg^{2+} concentration at high recA protein. Reactions were carried out as described. Reaction solutions contained 25 mM Tris-HCl (pH 7.2), 30 μ M denatured ϕ X174 [3 H]DNA, and 3.0 μ M recA protein with $MgCl_2$ at 10 mM (Δ), 30 mM (\bullet), or 50 mM (\blacktriangle), or with 10 mM $MgCl_2$ /1.0 mM ATP (\circ).

to-DNA ratio (3 recA monomers to 30 nucleotides), the stimulatory effect was even more striking (Fig. 4). At 10 mM Mg^{2+} there was no detectable renaturation in 15 min. However, at 30 mM Mg^{2+} there was a rapid renaturation reaction approaching that seen at 10 mM Mg^{2+} in the presence of ATP. Again, at 50 mM Mg^{2+} there was little detectable renaturation. It should be noted that the reaction at 30 mM Mg^{2+} is reduced relative to that measured at the lower recA protein-to-DNA ratio, indicating that the general pattern of recA protein concentration dependence holds, with higher levels of recA protein being inhibitory.

To determine the kinetic order of the Mg^{2+} -stimulated renaturation reaction, the ratio of recA protein to ss DNA was kept at 1 recA monomer to 30 nucleotides, and the DNA concentration was varied over a 10-fold range. The Mg^{2+} -stimulated reaction, like the ATP-stimulated reaction, was found to follow apparent first order-kinetics (data not shown).

Products of the Renaturation Reaction. Linear duplex ϕ X174 DNA and alkali-denatured duplex ϕ X174 DNA have distinct mobilities during electrophoresis in a 0.8% agarose gel; the denatured DNA migrates identically to ϕ X174 ss DNA (+)-strand. As observed previously with P22 DNA (2), ϕ X174 DNA that was renatured in the presence of recA protein and ATP appeared as a high molecular weight product that did not migrate out of the gel well. The same result was found for ϕ X174 DNA that was renatured in the presence of recA protein and 30 mM Mg^{2+} .

The products of renaturation also were observed by electron microscopy. ϕ X174 DNA that was renatured in the presence of recA protein and ATP appeared as complex networks of multipaired DNA molecules. Some single-stranded regions were visible, corresponding to imperfect pairing, providing an explanation for the finding that renaturation proceeds to only $\approx 75\%$ completion as judged by the S1 nuclease assay. Similar networks were found for ϕ X174 DNA renatured in the presence of recA protein and 30 mM Mg^{2+} .

DISCUSSION

The ss DNA binding proteins, *E. coli* SSB and T4 gene 32 protein, have been shown to promote the renaturation of complementary single strands of DNA (6, 7). These reac-

tions require saturating amounts of protein relative to the DNA. In both cases, the reaction follows apparent second-order kinetics, as does nonenzymatic renaturation. In the case of nonenzymatic renaturation, the second-order kinetics have been interpreted in terms of a two-step mechanism involving an initial bimolecular nucleation step joining homologous sites on two strands, followed by a rapid zippering up of the DNA helix (8). It has been proposed that ss DNA binding proteins may accelerate renaturation by holding the single strands in a favorable unfolded conformation that leaves the bases available for pairing during chance collisions between complementary sites (7). In the case of *E. coli* SSB, however, the removal of intrastrand folding does not appear to explain fully the catalysis of renaturation (6).

The renaturation reaction promoted by the recA protein has several characteristics that distinguish it from renaturation promoted by the ss DNA binding proteins. First, the recA protein-promoted reaction proceeds optimally at levels of recA protein sufficient to cover 10–15% of the DNA as judged by nuclease protection analysis. Electron microscopy of recA protein- ϕ X174 ss DNA (+)-strand complexes formed at the optimal recA protein-to-DNA ratio reveal that virtually all of the ss DNA molecules are partially covered with tracts of contiguous recA monomers (3). RecA protein levels that are sufficient to approach saturation of the DNA strands produce a marked decrease in the efficiency of renaturation. Second, recA protein-promoted renaturation in the presence of ATP follows apparent first-order rather than second-order kinetics, suggesting that it may involve a mechanism in which an intermediate composed of recA protein-ss DNA complexes is formed prior to complete renaturation. Rad-ding *et al.* reached a similar conclusion on the basis of their analysis of recA protein-promoted D-loop formation (9).

A third characteristic concerns the effects of various nucleotide cofactors. In low Mg^{2+} (10 mM) buffer, maximal renaturation rates are obtained when ATP is included as a cofactor; at high recA protein levels, ATP is absolutely required for catalysis of renaturation. A lesser stimulation is seen with ADP as the cofactor, whereas ATP[γ S], an analog that induces the irreversible binding of recA protein to ss DNA, permits only low levels of recA protein-promoted renaturation.

The basis for the similar effects of ATP (at 10 mM Mg^{2+}) and 30 mM Mg^{2+} alone on recA protein-promoted renaturation is not clear. As reported previously, ATP and ADP stimulate the transfer of recA protein between ss DNA molecules in the presence of 10 mM Mg^{2+} . Preliminary experiments indicate that an increase of the Mg^{2+} concentration from 10 to 30 mM does not result in a similar stimulation of recA protein transfer in the absence of nucleotide cofactor. Also, there is little effect on the protection of ss DNA from digestion by recA protein at 30 mM Mg^{2+} relative to that seen in 10 mM Mg^{2+} (3). Thus, the similar effects of 30 mM Mg^{2+} and ATP may not be at the level of recA protein-ss DNA interactions, but rather the two conditions may affect recA protein-recA protein interactions. recA protein has been shown to aggregate (in the absence of DNA) in the presence of low concentrations of Mg^{2+} (10 mM), whereas higher levels of Mg^{2+} (50 mM) cause complete disruption of aggregates (10). The midpoint for this transition is about 30 mM, close to the optimal level we find for the ATP-independent renaturation reaction. This range of Mg^{2+} concentrations clearly affects recA protein-recA protein interaction. Thus, aggregation of recA protein may be a critical feature of the ATP-independent renaturation reaction.

We speculate that in the nucleotide-independent reaction, aggregation of recA-ss DNA complexes may serve to increase the effective concentration of DNA molecules, thereby increasing the rate of renaturation. Since the initial aggregation would presumably not bring all of the molecules into

productive proximity, renaturation would be facilitated by a reiterative cycle of disruption and reformation of the aggregates. This would account for the Mg^{2+} optimum; lower Mg^{2+} concentrations would result in complete aggregation (and a limited extent of renaturation) and higher Mg^{2+} concentrations would result in complete disaggregation (and no renaturation). Such a reaction would be expected to show first- rather than second-order kinetics. Also, subsaturating levels of recA protein would promote optimal renaturation, since there would be sufficient bound recA protein to cause all of the DNA to enter the aggregates but not enough to appreciably mask the bases and inhibit pairing.

ATP and ADP cause complete disruption of recA protein aggregates at concentrations that favor renaturation (unpublished data). This finding indicates that aggregation of recA protein monomers may not account for the ATP-stimulated renaturation reaction. Since ATP and ADP also affect the interaction of recA protein with ss DNA, the mechanism of the ATP-stimulated reaction may be different from the nucleotide-independent reaction. A more definitive evaluation of possible reaction mechanisms will require structural characterization of the recA protein aggregates in the presence and absence of ss DNA.

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