suggestions during the course of this work.

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Kinetics of DNA Renaturation Catalyzed by the *Rec*A Protein of *Escherichia coli*†

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ABSTRACT: The *rec*A enzyme of *Escherichia coli* catalyzes renaturation of DNA coupled to hydrolysis of ATP. The rate of enzymatic renaturation is linearly dependent on *rec*A protein concentration and shows saturation kinetics with respect to DNA concentration. The kinetic analysis of the reaction indicates that the K_m for DNA is 65 μM while the k_{cat} is approximately 48 pmol of duplex formed (pmol of *rec*A)$^{-1}$ (20 min)$^{-1}$. *Rec*A protein catalyzed renaturation has been characterized with respect to salt sensitivity, Mg^{2+} ion and pH optima, requirements for nucleoside triphosphates, and inhibition by nonhydrolyzable nucleoside triphosphates and analogues. These results are consistent with a Michaelis–Menten mechanism for DNA renaturation catalyzed by *rec*A protein. A model is described in which oligomers of *rec*A protein bind rapidly to single-stranded DNA, and in the presence of ATP, these nucleoprotein intermediates aggregate to bring complementary sequences into close proximity for homologous pairing. As with other DNA pairing reactions catalyzed by *rec*A protein, ongoing DNA hydrolysis is required for renaturation. However, unlike the strand assimilation or transfer reaction, renaturation is inhibited by *E. coli* helix-destabilizing protein.

Many single-stranded DNA binding (SSB) proteins (also termed helix-destabilizing proteins) from prokaryotes and eukaryotes can facilitate DNA renaturation under physiological conditions. The gene 32 protein of bacteriophage T_4 (gp32), the best studied example of these binding proteins, can accelerate the renaturation of denatured phage T_4 DNA more than 300-fold at 37 °C. The mechanism by which gp32 promotes renaturation involves its cooperative binding to single-stranded DNA. The saturation of the single-stranded DNA with binding protein results in the removal of intrastrand secondary structure which acts as a kinetic barrier to renaturation. Moreover, the DNA chains are extended upon binding gp32 which may further favor strand pairing (Alberts & Frey, 1970). Kinetic analysis of DNA binding protein driven renaturation demonstrates that, like spontaneous renaturation, the rate is second order in DNA concentration. These results indicate that in the presence of gp32, renaturation proceeds in two steps, nucleation of small complementary sequences between two DNA strands followed by a rapid "zippering" to yield long duplex regions. By removing secondary structure from single-stranded DNA, gp32 accelerates the rate-limiting nucleation event and permits zippering to proceed unhindered. Although gp32–single-stranded DNA complexes are thermodynamically favored, there is a kinetic block to binding duplex DNA by gp32. Thus, the protein drives reannealing to near-completion (Jensen et al., 1976).

The *rec*A protein of *Escherichia coli* is another example of a single-stranded DNA binding protein that can promote renaturation or annealing of complementary single strands of DNA (Weinstock et al., 1979). Analysis of this reaction indicates that unlike gp32, *rec*A protein is stimulated by ATP which is hydrolyzed during the pairing process. *Rec*A protein can promote renaturation of heat-denatured linear viral DNAs as well as annealing of short complementary sequences contained within circular molecules and flanked by heterologous DNA (Keener & McEntee, 1984). In this latter reaction, the joint formed between the paired DNA strands is called a paranemic joint to distinguish it from the normal plectonemic coiling in duplex DNA (Bianchi et al., 1983).

This report describes a kinetic analysis of *rec*A protein catalyzed renaturation. The results demonstrate that *rec*A protein catalyzes homologous pairing of single-stranded DNA by a novel mechanism distinct from those of other single-stranded DNA binding proteins.

Experimental Procedures

Enzymes. *Rec*A protein was purified to homogeneity by using ATP elution chromatography (Cox et al., 1981). The source of the enzyme was strain KM1842 which is described elsewhere (Weinstock et al., 1979). The concentration of *rec*A protein was calculated by using a value of $\epsilon^{1\%}_{280nm} = 5.16$ (Weinstock et al., 1981a).

The *E. coli* single-stranded DNA binding (SSB) protein was obtained from John Chase, Albert Einstein Medical School,

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and from Robert Fuller, Department of Biochemistry, Stanford University School of Medicine. The T_4 gp32 was obtained from Rae Lyn Burke, Department of Biochemistry, University of California, San Francisco. S_1 nuclease was purchased from Sigma Chemical Co.

DNA. Radiolabeled bacteriophage P22 DNA was prepared as described (Botstein, 1968). Bacteriophage ϕX174 DNA was prepared from virions as described (Yamamoto et al., 1970). Oligo- and polynucleotides were purchased from P-L Pharmacia Biochemical Co. Calf thymus DNA was purchased from Sigma Chemical Co. All DNA concentrations were determined spectrophotometrically and calculated by using 1 OD_{254} = 50 μg/mL for duplex P22 DNA and 1 OD_{254} = 35 μg/mL for heat-denatured P22 DNA or ϕX174 viral DNA. Values are expressed in terms of nucleotide residues.

S_1 Nuclease Protection Assay for DNA Renaturation. A modified version of an S_1 protection assay (Weinstock et al., 1979) was used to measure the amount of DNA renaturation. ^{3}H-Labeled duplex P22 DNA in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and 1 mM ethylenediaminetetraacetic acid (EDTA) (TE, pH 7.5) was heat denatured in a 100 °C water bath for 2.5 min and cooled quickly by incubating in an ice–water bath. Reaction mixtures (120 μL) in 1.5-mL plastic Eppendorf tubes contained 20 mM Tris-HCl (pH 7.5), 10 mM KCl, 30 mM $MgCl_2$ (unless stated otherwise), 1 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, and the indicated amounts of *rec*A protein and denatured ^{3}H-labeled P22 DNA. Other additions and modifications are indicated in the legends to figures and tables. The reactions were started by addition of ^{3}H-labeled DNA to reaction mixtures that had been preincubated (5 min at 30 °C) in the absence of DNA but were otherwise complete. Incubations were performed at 30 °C for 20 min unless stated otherwise. Sodium dodecyl sulfate (0.5% final concentration) was added to terminate the reaction, and excess single-stranded calf thymus DNA (167 μg/mL final concentration) was added. The mixtures were further diluted by addition of 0.6 mL of S_1 nuclease digestion buffer (150 mM NaCl, 50 mM sodium acetate, and 1 mM zinc acetate, pH 4.5) and 150 (Sigma) units of S_1 nuclease. Digestions were performed for 40 min at 37 °C and terminated by addition of 100 μg of calf thymus DNA and 0.1 volume of 100% (w/v) trichloroacetic acid (Cl_3CCOOH). After incubation on ice for 15 min, the mixtures were filtered through GF/C filters, washed with 10 volumes of 10% Cl_3CCOOH and 5 volumes of 95% ethanol, and dried, and the filter-bound radioactivity was measured.

A background of 4–6% of the total radioactivity in the ^{3}H-labeled P22 DNA was detected in control incubations in the absence of *rec*A protein. That this S_1-resistant material is likely to result from limited intrastrand pairing of the denatured P22 DNA is based on two observations. First, the relative amount of background radioactivity does not increase even when a 3–5-fold excess of unlabeled denatured P22 DNA is added to the reaction. Second, the background level remains unchanged during a 60-min incubation in renaturing buffer (see Figure 1A). Thus, the S_1-resistant DNA formed rapidly and was independent of the DNA concentration in the reaction. In all experiments other than that shown in Figure 1A, this background level has been subtracted from the experimentally determined values.

Results

Kinetics of RecA Protein Catalyzed Renaturation. The initial studies of *rec*A protein catalyzed renaturation employed high concentrations of DNA (240 μM) in reaction buffers containing bovine serum albumin (BSA) (Weinstock et al., 1979). In these experiments, relatively high levels of ATP-independent renaturation of denatured P22 DNA were observed. A considerable proportion of this S_1-resistant material was formed in the absence of *rec*A protein, indicating that spontaneous renaturation was a significant factor under these assay conditions. To reduce the contribution from spontaneous renaturation, the reactions were modified in three ways: the DNA concentration was lowered, BSA was omitted from the reaction mixture, and reaction volumes were increased to minimize surface effects. As shown in Figure 1A, these changes significantly reduced the levels of ATP-independent renaturation seen in earlier experiments. The extent of renaturation in the presence of *rec*A protein without ATP was only slightly higher than the background level of S_1 nuclease resistant radioactivity that likely resulted from hairpin structures and other intrastrand folding in the P22 single strands (see Experimental Procedures). The amount of DNA that renatured in the presence of *rec*A protein and ATP (1 mM) increased linearly with time for more than 60 min of incubation. For each *rec*A protein concentration, the extent of renaturation was approximately 60% of the input DNA (data not shown).

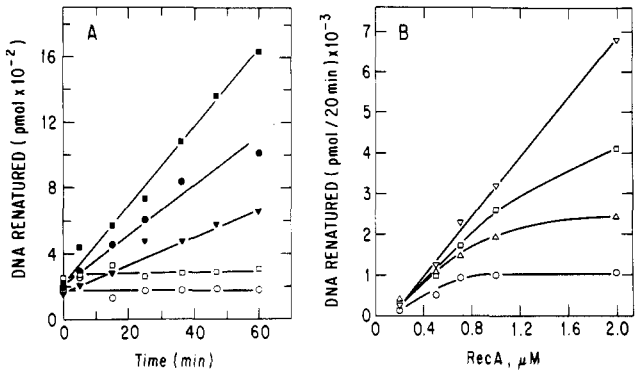


FIGURE 1: Time course and enzyme dependence of *rec*A protein catalyzed renaturation. (A) Kinetics of *rec*A protein catalyzed renaturation. Reaction mixtures (500 μL) contained 7.8 μM heat-denatured ^{3}H-labeled P22 DNA and the indicated concentration of *rec*A protein. The amount of duplex DNA formed was measured by using S_1 nuclease as described under Experimental Procedures. (○) No *rec*A protein, 1 mM ATP; (□) 0.35 μM *rec*A protein, no ATP; (▼) 0.17 μM *rec*A protein, 1 mM ATP; (●) 0.26 μM *rec*A protein, 1 mM ATP; (■) 0.35 μM *rec*A protein, 1 mM ATP. (B) *Rec*A protein concentration dependence of renaturation. *Rec*A protein and the indicated concentration of heat-denatured ^{3}H-labeled P22 DNA were incubated for 20 min at 30 °C, and the amount of duplex DNA was determined as described under Experimental Procedures. (○) 16.2 μM DNA; (△) 31.2 μM DNA; (□) 46.2 μM DNA; (▽) 76.2 μM DNA.

Enzyme Dependence of Renaturation. The rate of renaturation increased with increasing protein concentration (Figure 1A). The dependence of renaturation rate on *rec*A protein concentration was investigated more thoroughly over a 10-fold range of enzyme concentrations and a 4.7-fold range of single-stranded DNA concentrations. As shown in Figure 1B, the rate of renaturation increased linearly from 0.2 to 0.6 μM *rec*A protein for all DNA concentrations examined. At the lower DNA concentrations (16.25 and 31.25 μM), approximately 50–70% of the DNA is renatured in 20 min when 1 μM *rec*A protein or greater is used, and these values represent the extent rather than the rate of renaturation. At higher DNA concentrations, the rate of renaturation increases linearly up to 2 μM *rec*A protein.

The data in Figure 1B also demonstrate that below 0.1 μM *rec*A protein there is little or no renaturation measurable above background. This result may reflect the fact that *rec*A protein

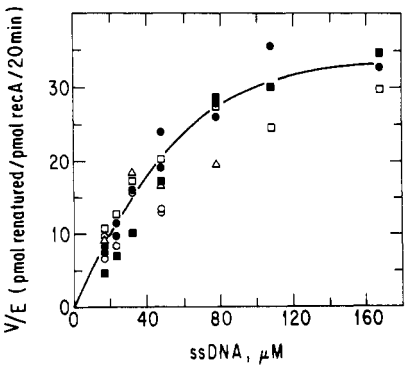


FIGURE 2: DNA concentration dependence of *rec*A protein catalyzed renaturation. The rate of renaturation was determined as described under Experimental Procedures for a range of *rec*A protein and heat-denatured P22 DNA concentrations. *Rec*A protein concentrations used were 0.2 (○), 0.5 (△), 0.7 (□), 1.0 (●), and 2.0 μM (■).

binds cooperatively to single-stranded DNA (Dunn et al., 1982) and that these protein–DNA complexes are prevented from forming at low enzyme concentration.

DNA Dependence of RecA Protein Catalyzed Renaturation. The rate of renaturation was measured at constant *rec*A protein concentration for several DNA concentrations between 15 and 45 μM. For three *rec*A protein concentrations examined (0.5, 0.7, and 1.0 μM), the rate of renaturation increased linearly with DNA concentration in this range (data not shown). A linear dependence of the rate of renaturation was also evident when 2 μM *rec*A protein and concentrations of DNA between 20 and 85 μM were used (data not shown). The data from these and other rate measurements were normalized to enzyme concentration and are presented in Figure 2. These results indicate that the rate (turnover number) of enzymatic renaturation showed saturation kinetics with respect to DNA concentration. An Eadie–Hofstee plot of these data (not shown) indicated that the K_m for DNA was approximately 65 μM and the k_{cat} (V_{max}/E) was 48 pmol of duplex (pmol of *rec*A)$^{-1}$ (20 min)$^{-1}$. The molar ratio of *rec*A protein to DNA nucleotides varied from approximately 1:10 to 1:80 for the different rate measurements used for determining the data shown in Figure 2. *Rec*A protein saturates single-stranded DNA at a molar ratio of 1 *rec*A monomer to 4 or 5 nucleotides (McEntee et al., 1979). Thus, renaturation is efficiently catalyzed by *rec*A protein when the enzyme is present at levels that are not sufficient to saturate the DNA, a result that is consistent with other reports (Weinstock et al., 1979).

Mg^{2+} Concentration Dependence of Renaturation. The dependence of *rec*A protein catalyzed renaturation on $MgCl_2$ concentration was determined as shown in Figure 3A. A maximum rate was achieved when 30 mM $MgCl_2$ was present in the reaction mix. At 10 mM $MgCl_2$, a concentration used in earlier studies, the rate was determined to be 65–70% of the rate measured at 30 mM $MgCl_2$. No renaturation was detected in the absence of divalent cation and above 100 mM $MgCl_2$. The amount of renaturation seen in the absence of ATP showed a slight increase at approximately 10 mM, but this rate was less than 10% of the rate in the presence of ATP (400 μM or 1 mM). A $MgCl_2$ concentration of 30 mM was chosen for kinetic measurements shown in Figures 1 and 2 and for subsequent experiments designed to characterize additional features of *rec*A protein catalyzed renaturation.

Effects of pH on Enzymatic Renaturation. The pH optimum for the renaturation reaction was between pH 7.0 and pH 7.8 (Figure 3B). The rate of the reaction declined sharply below pH 6.8, and at pH 6.5, the rate was approximately 60% of the rate at pH 7.0. Above approximately pH 8.0, the rate

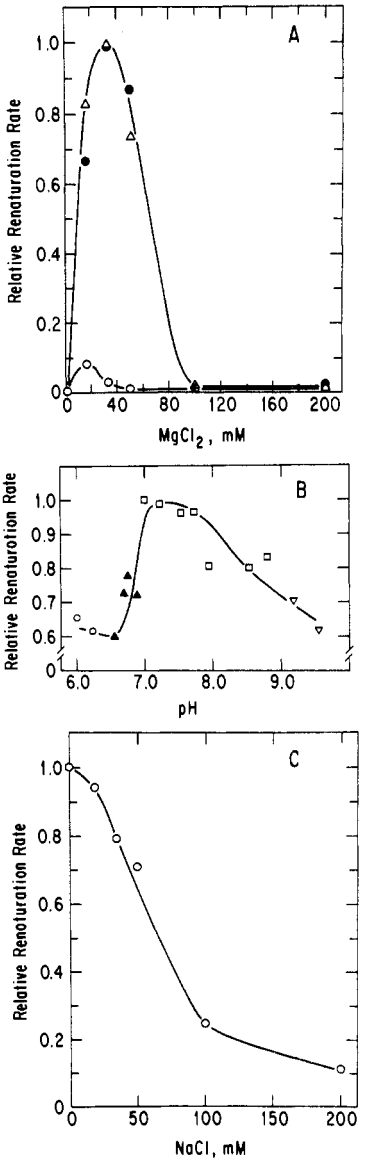


FIGURE 3: Characterization of the $MgCl_2$ and pH dependence and NaCl sensitivity of *rec*A protein catalyzed renaturation. (A) Reaction mixtures contained 0.5 μM *rec*A protein, 16.2 μM heat-denatured ^{3}H-labeled P22 DNA, the indicated concentration of $MgCl_2$, and 0 (○), 0.48 (△), or 1 mM (●) ATP. The amount of duplex P22 DNA was determined after a 20-min incubation at 30 °C as described under Experimental Procedures. (B) pH dependence of *rec*A protein catalyzed renaturation was determined by using 0.5 μM *rec*A protein, 16.2 μM heat-denatured ^{3}H-labeled P22 DNA, and 2 mM ATP. Reaction mixtures were maintained at the indicated pH by using the following buffers (at 20 mM): sodium maleate (○); potassium phosphate (▲); Tris-HCl (□); glycine (▽). The amount of duplex P22 DNA was measured after a 20-min incubation as described under Experimental Procedures. (C) Inhibition of renaturation by NaCl was determined after a 20-min incubation of reaction mixtures containing 0.5 μM *rec*A protein, 16.2 μM heat-denatured ^{3}H-labeled P22 DNA, 0.5 mM ATP, and NaCl as indicated.

declined more slowly than at low pH values, and at pH 9.0, the rate was 70% of the maximal rate of renaturation.

Salt Inhibition of RecA Protein Catalyzed Renaturation. The effects of increasing salt concentration on renaturation are shown in Figure 3C. The extent of renaturation was markedly inhibited by NaCl and was reduced to 50% of the maximal value at 60 mM NaCl. Both *rec*A protein binding to single-stranded DNA (McEntee et al., 1981) and its single-stranded DNA dependent nucleoside triphosphatase activity (Weinstock et al., 1981a) are considerably less sensitive to NaCl. This result suggests that a step other than DNA or

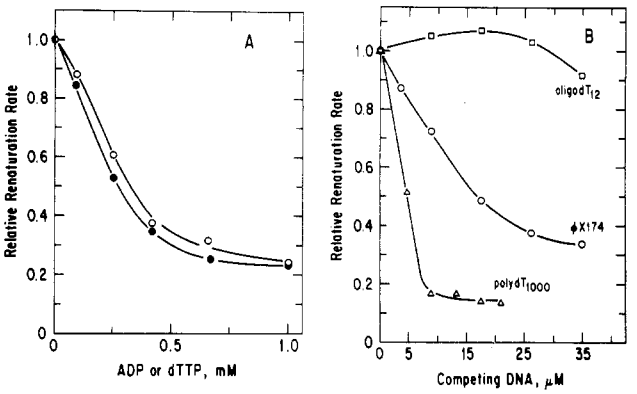


FIGURE 4: Inhibition of enzymatic renaturation by ADP, dTTP and heterologous DNAs. (A) Reaction mixtures contained 0.5 μM *rec*A protein, 16.2 μM heat-denatured, ^{3}H-labeled P22 DNA, 1 mM ATP, and the indicated concentration of ADP (●) or dTTP (○). The amount of duplex P22 DNA was measured after a 20-min incubation as described under Experimental Procedures. (B) Reaction mixtures contained 1 μM *rec*A protein, 16.2 μM heat-denatured ^{3}H-labeled P22 DNA, 1 mM ATP, and the indicated amount of $(dT)_{12}$, $(dT)_{1000}$, or φX174 viral DNA. The amount of duplex P22 DNA formed after a 20-min incubation was determined as above.

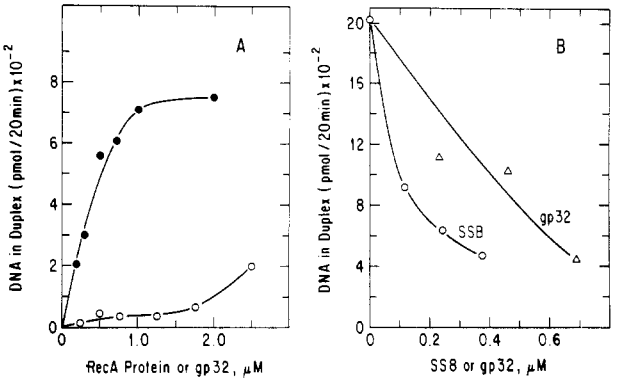


FIGURE 5: Effects of *E. coli* SSB protein and phage T_4 gp32 on *rec*A protein catalyzed renaturation. (A) Reaction mixtures contained the indicated concentration of *rec*A protein (●) or gp32 (○), 16.2 μM heat-denatured ^{3}H-labeled P22 DNA, and 1 mM ATP. Duplex DNA was measured after a 20-min incubation as described under Experimental Procedures. (B) Reaction mixtures contained 1 μM *rec*A protein, 76.2 μM heat-denatured ^{3}H-labeled P22 DNA, 1 mM ATP, and the indicated concentration of *E. coli* SSB protein (○) or phage T_4 gp32 (△). The amount of duplex DNA was determined after a 20-min incubation as described above.

ATP binding and ATP hydrolysis is sensitive to NaCl in the renaturation reaction.

Effects of ADP and dTTP on Enzymatic Renaturation. The amount of renaturation was measured in the presence of increasing concentrations of ADP, a product of the ATP hydrolysis reaction. The results shown in Figure 4A indicated that renaturation was half-inhibited at an ADP concentration of 0.25 mM. This concentration of ADP is equivalent to the amount of diphosphate generated by the ATPase of *rec*A protein after approximately 30 min. Thus, *rec*A protein catalyzed renaturation is inhibited by at least one product of the reaction.

Renaturation was also inhibited by dTTP, which has been shown to effectively compete with ATP for binding to *rec*A protein but is not a substrate for hydrolysis (Weinstock et al., 1981b). The concentration of dTTP which inhibited renaturation by 50% was approximately 0.3 mM (Figure 4A).

Effects of Competing DNAs on Renaturation. The ability of different polynucleotides and natural DNAs to inhibit *rec*A protein catalyzed renaturation was examined (Figure 4B). Short oligonucleotides $[(dT)_{12}]$ were ineffective in inhibiting the renaturation of denatured P22 DNA. This result is consistent with the notion that *rec*A protein binds poorly to short oligonucleotides which do not act as cofactors for *rec*A protein catalyzed ATP hydrolysis (Weinstock et al., 1981a). Long polymers of thymidylic acid $[(dT)_{1000}]$ were unusually good inhibitors of renaturation and inhibited the rate 50% at a concentration of approximately 5 μM when the single-stranded P22 DNA in the reaction was present at a concentration of 16 μM. This result is consistent with binding competition experiments indicating that *rec*A protein shows a strong binding preference for polypyrimidines such as poly(dT) and poly(dC) (McEntee et al., 1981).

Inhibition of *rec*A protein catalyzed renaturation was also observed with φX174 viral DNA lacking a complementary strand. The concentration of φX174 single-stranded DNA necessary to inhibit renaturation by 50% was approximately 16 μM, which was the concentration of denatured P22 in the reaction (Figure 4B).

The effect of duplex P22 DNA on *rec*A protein catalyzed renaturation was also examined. In reactions containing 10 μM single-stranded P22 DNA and 8.5 μM duplex P22 DNA, the rate of renaturation in the presence of 0.5 μM *rec*A protein was 80% of the rate measured in the absence of duplex DNA. Because the amount of *rec*A protein was below that needed to saturate the denatured DNA, little or no strand uptake or assimilation occurred under these conditions (data not shown). These results are consistent with the observed binding preference of *rec*A protein for single-stranded DNA relative to duplex DNA (McEntee et al., 1981) and argue that the duplex DNA formed during the renaturation reaction does not significantly inhibit *rec*A protein.

Comparison of Renaturation Promoted by RecA Protein and Other Single-Stranded Binding Proteins. In Figure 5A, the protein dependence of renaturation is shown for *rec*A protein and for the T_4 gp32, under the same reaction conditions. Below 1 μM *rec*A protein, the rate of renaturation increases approximately linearly. The "saturation" effect seen above 1 μM *rec*A protein was due to the renaturation reaching an extent of approximately 50% reannealing. Under identical conditions, the T_4 gp32 promoted a low level of renaturation at low protein concentrations as measured by the S_1 nuclease assay. Above 2 μM, gp32 stimulated the rate of renaturation further, as expected, since this concentration was close to saturation of the single-stranded P22 DNA with this protein. The rate showed an additional stimulation at higher gp32 concentrations (data not shown).

Spontaneous renaturation was measured under the conditions shown in Figure 5A and resulted in 15–20 pmol of duplex DNA formed in 20 min of incubation. Thus, gp32 stimulated renaturation 10–15-fold under these conditions, and *rec*A protein (1 μM) accelerated renaturation 50–70-fold.

Inhibition of RecA Protein Catalyzed Renaturation by SSB Protein and gp32. Since *rec*A protein and gp32 individually promote renaturation, it was of interest to determine the effects of a combination of the two proteins on renaturation. In reaction mixtures containing *rec*A protein (1 μM) and subsaturating concentrations of gp32, the renaturation rate *decreased* with increasing gp32 (Figure 5B). This same inhibitory effect was observed when SSB protein of *E. coli* was added together with *rec*A protein in a separate reaction mixture [under these conditions of pH, SSB protein does not promote DNA renaturation (Christiansen & Baldwin, 1977)]. At the highest gp32 concentration examined (0.68 μM), it was estimated that less than 8% of the denatured P22 DNA was bound by gp32. The extent of inhibition by *E. coli* SSB protein

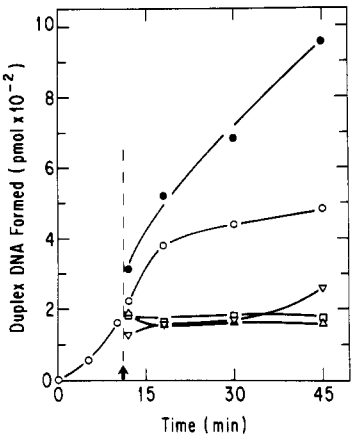


FIGURE 6: Kinetics of inhibition of renaturation by SSB protein, ATPγS, and heterologous DNA. A reaction mixture (1200 μL) containing 1 μM *rec*A protein, 16.2 μM heat-denatured ^{3}H-labeled P22 DNA, and 1 mM ATP was incubated for 12 min at 30 °C. Aliquots (200 μL) were taken and added to Eppendorf tubes containing ATPγS (□) (final concentration 1.2 mM), *E. coli* SSB protein (△) (final concentration 1.5 μM), φX174 viral DNA (▽) (final concentration 70 μM), no addition (○), and heat-denatured P22 DNA (●) (final concentration 33.2 μM). At the indicated times, aliquots (50 μL) were taken, and the amount of duplex DNA was measured as described under Experimental Procedures.

was even more effective than gp32 with respect to its concentration: the *E. coli* SSB protein inhibited the reaction rate by 50% at a concentration of 0.1 μM whereas 0.42 μM gp32 was required for the same degree of inhibition (Figure 5B). These results suggest that these DNA binding proteins may act by sequestering *rec*A protein on the DNA (see Discussion).

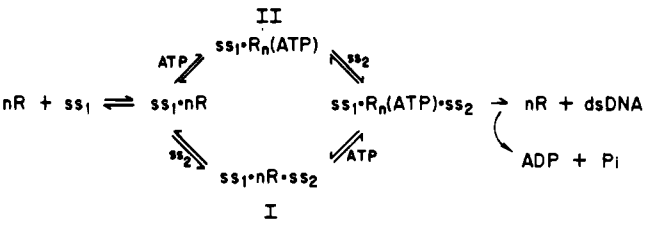
Inhibition of Ongoing Renaturation by SSB Protein, ATPγS, and Heterologous DNA. The inhibitory effects of adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), heterologous DNA, and SSB protein (or gp32) on renaturation had been determined when these components were present prior to or at the same time as the addition of *rec*A protein. It was of interest to determine whether these same factors would inhibit enzymatic renaturation that had already been initiated. Renaturation was initiated by addition of DNA and allowed to proceed in the absence of any inhibitor for 12 min. At this time, either ATPγS (1.2 mM final concentration), heterologous φX174 viral DNA (70 μM final concentration), or SSB protein (1.5 μM final concentration) was added to separate reactions. In two control reactions, either no addition was made or additional denatured P22 DNA was added. As shown in Figure 6, the addition of ATPγS, heterologous φX174 DNA, or SSB protein resulted in a rapid and complete inhibition of *rec*A protein catalyzed renaturation. Only in the reaction containing the heterologous single-stranded φX174 DNA was additional renaturation detected, but this occurred only after a lag of approximately 25 min. Both SSB protein and ATPγS blocked renaturation for the duration of the incubations (more than 30 min). The control experiments indicated that renaturation continued in the absence of these inhibitors and the rate of pairing could be increased by adding more homologous single-stranded P22 DNA.

These results suggest that *rec*A protein dependent renaturation requires ongong ATP hydrolysis (since the reaction was inhibited by ATPγS). The rapid inhibition observed upon addition of heterologous DNA suggests that the *rec*A protein dissociates readily from renaturation complexes (see Discussion).

Discussion

*Rec*A protein catalyzed renaturation of DNA is unlike renaturation promoted by other single-stranded DNA binding proteins in at least three respects. First, *rec*A protein is strongly stimulated by ATP as a cofactor for efficient renaturation. During renaturation, ATP is hydrolyzed to ADP. Second, *rec*A protein acts enzymatically in catalyzing renaturation and is not required in amounts needed to saturate the single-stranded DNA. Renaturation driven by single-stranded DNA binding (SSB) proteins requires saturation or near-saturation of DNA with the SSB protein for optimal annealing. The mechanism by which these SSB proteins stimulate renaturation involves the removal of folds and intrastrand hairpin loops in the DNA which act as a kinetic barrier to interstrand pairing. Thus, the mechanism by which *rec*A pairs complementary sequences of DNA does not proceed by a similar mechanism. A third unique feature of *rec*A protein catalyzed renaturation is the saturability of the reaction rate with respect to DNA substrate concentration. At the lowest DNA concentrations used in this study, the renaturation rate shows an apparent first-order dependence on DNA concentration. At high DNA concentrations, the rate becomes independent of DNA concentration as would be expected if *rec*A protein catalyzed renaturation proceeded via a Michaelis–Menten mechanism in which the pairing steps occurred on *rec*A protein–DNA complexes. Analysis of the kinetic data shown in Figure 2 indicates that the K_m for DNA is approximately 65 μM. This value is 15–30 times higher than the K_m value for DNA in the ATP hydrolysis reaction (unpublished results). A kinetic scheme accounting for these observations and other features of *rec*A protein–DNA interactions is shown in Scheme I.

Scheme I



According to Scheme I, the binding of the first single-stranded DNA molecule (ss_1) to *rec*A protein (nR) is rapid and is characterized by a low K_d. The *rec*A protein–DNA complex that results (ss_1•nR) is capable of binding a second DNA strand (ss_2) to form a ternary complex (ss_1•nR•ss_2), or it may bind ATP which induces a conformational change in the *rec*A protein [ss_1•R_n(ATP)]. It is assumed that this latter complex is capable of hydrolyzing ATP and is identical with an intermediate in the single-stranded DNA-dependent ATP hydrolysis reaction. The binding of the second DNA strand (ss_2) to ss_1•R_n(ATP) (or to the ss_1•nR intermediate) is characterized by a significantly higher K_d than that for ss_1 binding. When ss_1 and ss_2 contain complementary sequences, then renaturation occurs on the ss_1•R_n(ATP)•ss_2 complex, and it is assumed that ATP is hydrolyzed to ADP and P_i.

This model explains several features of the renaturation reaction. First, the reaction shows an apparent first-order DNA dependence because the second DNA molecule (ss_2) binds with considerably lower affinity than the first (ss_1). The low K_m for DNA in the ATP hydrolysis reaction (1–2.5 μM) is consistent with this assumption and reflects a partial reaction in the renaturation pathway. All of the experiments reported here were performed at DNA concentrations that are likely to be much larger than the K_d for ss_1, and therefore, the *rec*A protein is always complexed with the first DNA strand (ss_1•nR). Preliminary renaturation experiments performed

with DNA concentrations near the K_m for ATP hydrolysis indicate that under these conditions the renaturation rate displays a second-order dependence on DNA concentration (unpublished results).

According to this model, the binding of ATP to *rec*A protein–DNA complexes induces a conformational change in the enzyme. It is known that ATP binding increases the sedimentation velocity of *rec*A protein and stimulates protein oligomerization (Ogawa et al., 1978; McEntee et al., 1981). Recently, we have demonstrated that ATP binding to *rec*A protein renders specific regions of the protein more sensitive to trypsin cleavage (K. Knight et al., unpublished results). Although the nature of this conformational change is not known, it is assumed that it provides more favorable base pairing opportunities for the bound DNA single strands. The formation of the $ss_1 \cdot R_n(ATP) \cdot ss_2$ complex greatly increases the local concentration of DNA and brings complementary sequences contained on the bound strands into close proximity for base pairing. Since nucleation of short complementary regions occurs on these complexes and because many DNA strands are bound in these structures, it is not unreasonable that multiple nucleation events can occur on one strand. Structural analysis of the renatured products indicates that an individual DNA chain can be paired over short regions with several other complementary strands.

The kinetic scheme indicates that there are two sites for ATP hydrolysis: the $ss_1 \cdot R_n(ATP)$ intermediate may hydrolyze ATP and rele .se *rec*A protein, and the $ss_1 \cdot R_n(ATP) \cdot ss_2$ complex may hydrolyze ATP and release *rec*A protein and renatured DNA. The hydrolysis of ATP by the $ss_1 \cdot R_n(ATP)$ intermediate accounts for uncoupling of ATP hydrolysis and renaturation since it can occur without the binding of ss_2. Thus, base pairing is not a prerequisite for ATP hydrolysis, a conclusion which is consistent with experiments using homopolymers and viral ϕX174 DNA (Weinstock et al., 1979, 1981a). It is presumed that hydrolysis of ATP to ADP causes dissociation of nucleoprotein complexes. Cox et al. (1983) demonstrated that ADP stimulates dissociation of complexes of *rec*A protein and single-stranded DNA. Following its release, *rec*A protein becomes available for additional rounds of renaturation. In this respect, the role of ATP hydrolysis is analogous to its proposed role in the mechanism of DNA gyrase catalyzed supercoiling of duplex DNA (Peebles et al., 1978). It is likely that the rate-limiting step in this renaturation scheme is the ATP hydrolytic step which drives recycling of *rec*A protein.

The data presented here do not indicate whether there is more than one DNA binding site per *rec*A protein monomer. Since it is likely that *rec*A protein is acting as a multimer, it is possible that there is a single binding site per monomer and that DNA binding displays negative cooperativity. Experiments are currently under way to determine the number and nature of the DNA binding regions of *rec*A protein.

*Rec*A protein catalyzed renaturation, like its strand transferase activity, is inhibited by ADP but not by duplex DNA. The accumulation of ADP during renaturation could account for reduction in the rate of renaturation at later times of incubation. It is unlikely, however, that ADP buildup accounts for the limited extent of renaturation (50–70% of the input DNA) since regenerating ATP during the reaction does not affect this limit (data not shown). The extent of renaturation could be determined by the structures of the renatured products, which are highly aggregated and branched DNA complexes (Weinstock et al., 1979). It seems likely that complete pairing of strands in these complexes is blocked by steric hindrance rather than product inhibition. These aggregates contain nearly all of the DNA in the reaction (>90%), but only 50–70% of this DNA is resistant to S_1 nuclease digestion (Weinstock et al., 1979; unpublished results). Since duplex DNA is not an inhibitor of *rec*A protein catalyzed renaturation, the ability of complementary single-stranded regions to pair is likely to be restricted in these aggregates.

The $MgCl_2$ optimum for *rec*A protein catalyzed renaturation is approximately 30 mM, and at 10 mM $MgCl_2$, the reaction rate is reduced approximately 30%. This $MgCl_2$ optimum does not reflect a requirement for ATP hydrolysis (which has an optimum between 5 and 10 mM) or for DNA binding, but likely reflects a requirement for a step subsequent to binding of DNA by *rec*A protein. Recently, Bryant & Lehman (1985) have reported that ATP-*independent* renaturation of linear ϕX174 DNA strands by *rec*A protein is greatly stimulated by $MgCl_2$ between 10 and 50 mM with an optimal rate near 30 mM. The results reported here are not in agreement with their finding. As shown in Figure 3A, $MgCl_2$ stimulation of renaturation is almost completely ATP dependent. The basis for this difference may be found in the results of Cotterill & Fersht (1983) which demonstrate that the aggregation state of *rec*A protein (measured by light scattering) is unusually sensitive to monovalent ion concentrations between 10 and 20 mM NaCl. Additionally, scattering by *rec*A protein solutions can be further reduced by increasing the concentration of $MgCl_2$. These results suggest that under the conditions used by Bryant and Lehman for *rec*A protein catalyzed renaturation (no monovalent ions) the enzyme is highly aggregated. These aggregates presumably bind DNA and stimulate reannealing by sequestering the DNA in *rec*A protein–DNA aggregates. Such a "static" pathway eliminates the need for ATP binding to drive *rec*A protein oligomerization (pathway I of Scheme I) and for ATP hydrolysis to drive recycling of the protein. A prediction of this model is that low concentrations of NaCl would disrupt *rec*A protein aggregates and inhibit ATP-independent renaturation. Indeed, such a result is reported by Bryant and Lehman. The assay conditions employed in this study (10 mM KCl and 30 mM $MgCl_2$) were chosen because little or no ATP-independent reannealing could be detected (see Results). It is likely that the *rec*A protein is disaggregated in these reaction conditions with the result that renaturation is almost completely ATP dependent.

*Rec*A protein catalyzed renaturation is inhibited by other single-stranded DNA binding proteins at concentrations of these binding proteins that are not sufficient to saturate the DNA. Since less than 8% of the DNA is covered by SSB protein or gp32 under these reaction conditions, the inhibition of renaturation cannot easily be explained by a simple binding site competition model. These results suggest that *rec*A protein and SSB protein (or gp32) are sequestered together on the DNA. This sequestration could result from favorable protein–protein interactions between *rec*A protein and SSB protein (or gp32) or from a more favorable binding of *rec*A protein to a DNA sequence adjoining or in close proximity to the cluster of SSB protein (gp32). Although *rec*A protein and SSB protein do not interact in solution (Cohen et al., 1983; unpublished results), electron microscopic data suggest that these proteins may interact when bound to DNA (Flory & Radding, 1982). It is interesting to note that SSB protein (or gp32) *stimulates rec*A protein catalyzed strand transfer and exchange when it is present at concentrations approaching saturation of the single-stranded component. Although it has been suggested that this stimulatory effect is the consequence of removing secondary structure from the single-stranded DNA

(Muniyappa et al., 1984), this mechanism cannot explain its stimulatory effects with homopolymers where secondary structure is minimized (K. McEntee, unpublished results). Further studies will be required to elucidate the role of other SSB-like proteins in *rec*A protein catalyzed pairing reactions.

The immediate inhibition (within 3 min or less) of an ongoing renaturation reaction following addition of heterologous DNA suggests that *rec*A protein can readily dissociate from the nucleoprotein complexes that serve as intermediates in the renaturation reaction. Certainly, if renaturation proceeds with frequent binding and dissociation of DNA from the *rec*A protein complexes, then the inhibition by heterologous DNA would be due to simple competition for binding the enzyme complexes. Although these results are compatible with a distributive mechanism for *rec*A protein catalyzed renaturation, they do not rule out mechanisms that would involve one or more processive pairing steps. Nevertheless, the competition experiments argue against mechanisms that involve stable *rec*A protein–DNA complexes as intermediates in renaturation. Other types of experiments have indicated that *rec*A protein acts processively in promoting strand exchange between duplex and homologous single strands (Gonda & Radding, 1983). In this case, the processive feature of the exchange reaction may be a consequence of the high *rec*A protein concentrations needed in the reaction. Inhibition by heterologous DNA further supports the notion that binding to and dissociation from DNA is coupled to binding and hydrolysis of the ATP cofactor.

Although a circularly permuted viral DNA was used for analyzing the kinetics of *rec*A protein catalyzed renaturation, similar results have been obtained with nonpermuted viral DNAs from phages λ (K. McEntee, unpublished results) and φX174 (Bryant & Lehman, 1985). Significantly, the structures of the renaturation products with P22 DNA are similar to those formed with the nonpermuted substrates, complex aggregates that are the result of multiple nucleation events and limited extension of the duplex joints. Undoubtedly, these short duplex regions are a consequence of frequent hairpins and secondary structure in the single strands which block their extensive interwinding of the annealed partners. Similar structures have been reported for the products of spontaneous renaturation under conditions that do not disrupt intrastrand folding and hairpin formation (Studier, 1969). Taken together, these results support the conclusion that *rec*A protein acts efficiently as a synaptic enzyme to bring complementary DNA sequences into register and to nucleate duplex joint formation during renaturation. The subsequent limited extension of these duplex regions may occur spontaneously, or *rec*A protein may catalyze their extension.

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Registry No. ATP, 56-65-5; Mg, 7439-95-4; ADP, 58-64-0; dTTP, 365-08-2; ATPγS, 35094-46-3.

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