The publication DNA duplex from Sigma. strand [3H]ATP and (+)-recA protein-promoted renaturation of DNA mechanism of the (2). understood (1). though these protein homologous and renaturation damage to variety of ATP-dependent 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 φX174 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 φX174 DNA.

EXPERIMENTAL PROCEDURES

Materials. recA protein was purified to homogeneity as described (4). Its concentration was calculated from $A_{260}^{\text{abs}} = 5.9$. S1 nuclease, ADP, ATP, and calf thymus DNA were from Sigma. ATP[yS] was from Boehringer Mannheim, and [3H]ATP was from ICN. GF/C filters were from Whatman.

Unlabeled and [3H]-labeled circular φX174 ss DNA [(+)-strand] were prepared as described (5). [3H]-labeled linear duplex φX174 DNA prepared by Pst I cleavage of [3H]-labeled circular duplex φX174 DNA also has been described (5). DNA concentrations were calculated by using an $A_{260}$ value of 1 as equivalent to 36 μg of ss DNA and 50 μg of duplex DNA per ml. All DNA concentrations are expressed as nucleotides.

[3H]-labeled linear duplex φX174 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 MgCl$_2$, 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 μl) were removed and quenched by mixing with 2.5 μl of sodium dodecyl sulfate (10%). The mixtures were then diluted into 300 μl of digestion buffer containing 150 mM NaCl, 50 mM sodium acetate (pH 4.6), and 1 mM zinc acetate; then 3.0 μl 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 μl of heat-denatured calf thymus DNA (2 mg/ml) and 350 μl 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 φX174 ss DNA (+)-strand and denatured duplex φX174 DNA (30 μM in original aliquot) were >98% degraded as judged by acid-precipitable radioactivity. An equivalent amount of linear duplex φX174 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 φX174 DNA. In all cases, control reactions with [3H]-labeled φX174 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 φX174 form I DNA to an S1 nuclease-resistant form was used to measure renaturation (5). When the [3H]-labeled separated strands (30 μM)

Abbreviations: ss DNA, single-stranded DNA; ATP[yS], adenosine 5'-γ-thiotriphosphate.

297
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.

**rcA Protein-Promoted Renaturation of Complementary Single Strands.** When rcA protein (1 \(\mu\text{M}\)) was added to the separated \(\phi X 174\) DNA (+)- and (−)-strands (30 \(\mu\text{M}\)) in the presence of ATP (500 \(\mu\text{M}\)) at 25°C, rapid renaturation occurred with \(\approx75\%\) of the DNA becoming S1 nuclease resistant (Fig. 1 Left) within 10 min. In the presence of rcA protein but without ATP, renaturation occurred with an initial phase \(\approx2\)-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 rcA protein, there was little renaturation during the time of the reaction (\(<2\%\)).

**Effect of rcA Protein Concentration on the Rate of Renaturation.** The initial rate of renaturation of \(\phi X 174\) DNA (30 \(\mu\text{M}\)) was measured as a function of rcA protein concentration. Both the ATP-independent and ATP-stimulated reactions were examined. The rate of the ATP-stimulated reaction increased with increasing rcA protein until a maximal rate was reached at a ratio of 1 rcA 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 rcA monomer to 4 nucleotides of ss DNA, this ratio corresponds to 10–15% coverage of the DNA by rcA protein (3). Higher levels of rcA protein markedly reduced the rate of renaturation.

Two regions of the rcA protein concentration-dependence curve are of particular interest. One is at the optimal ratio of rcA 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 rcA monomers to 30 nucleotides of ss DNA. At this level, the ATP-stimulated reaction was reduced to \(\approx40\%\) of the maximal rate; however, the ATP-independent reaction was completely eliminated (Fig. 1 Right). Thus, at the higher level of rcA 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 rcA protein to ss DNA was kept at 1 rcA 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 \(\phi X 174\) DNA was replaced by \(\phi X 174\) 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 \(k_{cat} = 6 \text{ min}^{-1}\)), 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 rcA 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.](image-url) Renaturation of alkali-denatured \(\phi X 174\) [3H]DNA by rcA protein. Reactions were carried out as described. (Left) Reaction solutions contained 25 mM Tris-HCl (pH 7.2), 10 mM MgCl2, and 30 \(\mu\text{M}\) denatured \(\phi X 174\) [3H]DNA with no additions (o) or with 1.0 \(\mu\text{M}\) rcA protein (a), 1.0 \(\mu\text{M}\) rcA protein/200 \(\mu\text{M}\) ADP (c), or 1.0 \(\mu\text{M}\) rcA protein/200 \(\mu\text{M}\) ATP (o). (Right) Reaction solutions contained 25 mM Tris (pH 7.2), 10 mM MgCl2, and 30 \(\mu\text{M}\) denatured \(\phi X 174\) [3H]DNA with no additions (o), 3.0 \(\mu\text{M}\) rcA protein (a), 3.0 \(\mu\text{M}\) rcA protein/1.0 mM ADP (c), or 3.0 \(\mu\text{M}\) rcA protein/1.0 mM ATP (o).
Dependence of Renaturation on Mg\(^{2+}\) Concentration. The Mg\(^{2+}\) 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 Mg\(^{2+}\). Increasing levels of Mg\(^{2+}\) resulted in increasing rates of renaturation until saturation was achieved at ≈10 mM. Higher levels of Mg\(^{2+}\) (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 Mg\(^{2+}\) concentration.

Nucleotide-independent renaturation also required added Mg\(^{2+}\) (Fig. 3 Right). Increasing levels of Mg\(^{2+}\) resulted in increasing rates and extents of reaction. In contrast to the ATP-stimulated reaction, however, 10 mM Mg\(^{2+}\) was not saturating; instead, the optimal Mg\(^{2+}\) concentration was ≈40 mM. At this Mg\(^{2+}\) concentration, the nucleotide-independent reaction proceeded at the same rate and to the same extent as the ATP-stimulated reaction. Mg\(^{2+}\) concentrations ≥50 mM resulted in complete loss of the nucleotide-independent reaction. The stimulation at 40 mM Mg\(^{2+}\) relative to 10 mM Mg\(^{2+}\) 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 Mg\(^{2+}\) resulted in inhibition of the nucleotide-independent reaction (data not shown).

When the Mg\(^{2+}\) concentration dependence of the nucleotide-independent reaction was examined at the higher recA-

![Graph](image_url)
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 \(\phi X174\) DNA and alkali-denatured duplex \(\phi X174\) DNA have distinct mobilities during electrophoresis in a 0.8% agarose gel; the denatured DNA migrates identically to \(\phi X174\) ss DNA (+)-strand. As observed previously with P22 DNA (2), \(\phi X174\) 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 \(\phi X174\) 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. \(\phi X174\) 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 ~75% completion as judged by the S1 nuclease assay. Similar networks were found for \(\phi X174\) 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 reactions 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-\(\phi X174\) 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. Radding *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[\(\gamma\)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 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.