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# Interactions of Diastereomeric Tripeptides of Lysyl-5-fluorotryptophyllysine with DNA. 2. Optical, $^{19}F$ NMR, and Strand Cleavage Studies of Apurinic DNA Complexes†

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ABSTRACT: The interactions of the diastereomers lysyl-5-fluoro-L-tryptophyllysine and lysyl-5-fluoro-D-tryptophyllysine with apurinic DNA have been examined as a model for the action of DNA repair enzymes. The binding characteristics of the tripeptide diastereomers to DNA, modified to contain ~5% apurinic sites, were studied by measuring $^{19}F$ NMR parameters, fluorescence quenching, and activity in promoting single-strand cleavage of plasmid DNA. The affinities of each of the peptides to apurinic DNA are similar to those for native DNA. However, the $^{19}F$ NMR chemical shift and relaxation behavior indicates that both diastereomers form complexes with apurinic DNA that are distinct from those formed with native DNA. In addition, the $^{19}F$ NMR measurements differ for the L-Trp and D-Trp complexes with apurinic DNA. In spite of these differences, when either of the tripeptide diastereomers is incubated with plasmid DNA containing apurinic sites, no difference in the rate of single-strand cleavage of the DNA is detectable.

The results of a recent binding study using fluorescence spectroscopy (Behmoaras et al., 1981a) indicated that the affinity of the tripeptide Lys-L-Trp-Lys for double-stranded DNA increased after the introduction of apurinic sites. The binding affinity was shown to increase in proportion to the level of depurination. It was proposed that the aromatic amino acid, by substituting for the missing purine, was responsible for the selection and preferential binding at the apurinic sites. This interpretation suggests the possibility that aromatic amino acids could play an important role in DNA repair mechanisms. The in vivo repair of some forms of damaged DNA requires the selective recognition of apurinic sites [see review by Lindahl (1979)]. These sites are created by the enzymatic removal of damaged purines and are subsequently located by Ap endonucleases,[1] which catalyze the cleavage of the phosphate backbone at this site.

The potential of the interaction of this simple tripeptide with DNA as a model for the study of DNA repair enzymes is substantiated further by recent experimental evidence that this peptide is also capable of promoting strand breakage of apurinic DNA (Behmoaras et al., 1981b,c; Pierre & Laval, 1981). When apurinic sites were introduced into either plasmid pBR322 (Behmoaras et al., 1981b) or phage PM2 DNA (Behmoaras et al., 1981c; Pierre & Laval, 1981), conversion of the supercoiled form to the relaxed form upon incubation with the tripeptide provided evidence for nicking activity. Thus, this tripeptide not only appears to locate apurinic sites as is done by the apurinic endonucleases but also mimics the catalytic activity of these repair enzymes. That amines, lysine in particular, are capable of promoting cleavage of apurinic DNA (Lindahl & Andersson, 1972) suggests that the $\epsilon$-amino or the N-terminal amino group of this tripeptide may be responsible for chain breakage. However, there is also evidence suggesting that, in the case of this tripeptide, the aromatic residue may play an important role (Pierre & Laval, 1981; Behmoaras et al., 1981b,c).

In the preceding paper, the distinct complexes formed by the diastereomeric tripeptides lysyl-5-fluoro-L-tryptophyllysine and lysyl-5-fluoro-D-tryptophyllysine with native DNA were easily discriminated by using $^{19}F$ NMR. In order to determine whether the tryptophyl indole ring is bound in a unique manner at apurinic sites as compared to native sites, the $^{19}F$ NMR studies were extended to include the complexes formed with

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[1] Abbreviations: Ap endonucleases, endonucleases for apurinic/apyrimidinic sites; EDTA, ethylenediaminetetraacetic acid; $T_m$, temperature at which the helix-to-coil transition of DNA is 50% complete; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; FTrp, 5-fluoro-DL-tryptophan; Tri(L), lysyl-5-fluoro-L-tryptophyllysine; Tri(D), lysyl-5-fluoro-D-tryptophyllysine; Tris, tris(hydroxymethyl)aminomethane.

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DNA containing apurinic sites.

This paper contains the $^{19}F$ NMR and fluorescence results obtained for the complexes formed with apurinic DNA. In addition, the ability of both of these tripeptides to cleave DNA at apurinic sites has been examined.

## Materials and Methods

### *Materials*

The synthesis and chromatographic separation of the diastereomeric tripeptides have been described in the preceding paper (Shine & James, 1985).

*Preparation of DNA Containing Apurinic Sites.* Quantitatively depurinated DNA was obtained by following the procedure of Lindahl & Nyberg (1972). They found that the rate of release of purines proceeded at an initial rate of $3.8 \times 10^{-7}$ $s^{-1}$ when [$^{14}C$]purine-labeled *Bacillus subtilis* DNA was incubated in 0.1 M NaCl, 0.01 M sodium phosphate, and 0.01 M sodium citrate, pH 5.0, at 70 °C.

*Introduction of Apurinic Sites into Sonicated DNA.* Calf thymus DNA was sonicated as described previously (Shine & James, 1985) except that the sonication was continued for 4 h at a power level of 6. A high-salt buffer (1.0 M NaCl, 10 mM sodium cacodylate, 1 mM EDTA, pH 7.0) was used in order to obtain smaller fragments with a more uniform size distribution. The average molecular weight of the fragments obtained was 135 base pairs as determined on 8% polyacrylamide gel electrophoresis using φX174–*Hae*III fragments (Bethesda Research Laboratories, Inc.) as markers.

The sonicated calf thymus DNA was initially dialyzed extensively against 0.1 M NaCl, 0.01 M sodium phosphate, and 0.01 M sodium citrate, pH 7.2, and the sample was adjusted to pH 5.0 immediately prior to depurination. In order to ensure that 70 °C was sufficiently below the $T_m$ of this molecular weight DNA, the melting profile of an aliquot was examined. The $T_m$ obtained was 79.5 °C, and no hyperchromicity was observed at 70 °C.

Capped Pyrex tubes containing 1.0 mL of DNA (1.1 mg/mL) were incubated at 70 °C for 29 h by using a Pierce Reacti-Therm heating module. The samples were then transferred to centrifuge tubes, the Pyrex tubes were washed with 500 μL of buffer (2 times), and the DNA was precipitated by addition of 3.0 mL of cold 95% ethanol. The samples were kept at 0 °C overnight and then centrifuged (Sorvall RC-5B, SS-34 rotor, 12000 rpm) at 2 °C for 1 h. The supernatant was transferred to a graduated conical tube, and the pellet was washed with an additional 2.0 mL of cold ethanol. The recovered DNA was dissolved in 10 mM sodium cacodylate, 10 mM NaCl, and 1.0 mM EDTA, pH 6.0, and stored frozen.

*Analysis of Depurination.* Lindahl & Nyberg (1972) found that >95% of the radioactively labeled purines were recovered in ethanol-soluble form after precipitation of the DNA. For this study, the composition of the supernatant solutions was examined by HPLC to check the amount of depurination that had occurred.

A Beckman liquid chromatograph (Model 334) equipped with a Model 421 CRT microprocessor–controller, a Model 110A pump, a Model 210 sample injector, and a Gilson variable wavelength detector operating at 250 nm was used. Separation of all four bases, adenine, guanine, cytosine, and thymine, was achieved on a Partisil 10 μM ODS (11) 250 × 5 mm column (HPLC Technology, Ltd.) by isocratic elution with 2.5% methanol and 0.1% TFA in $H_2O$ at a flow rate of 1.0 mL/min.

The supernatant solutions obtained as described above were evaporated under $N_2$ to approximately 1.0 mL, 200 μL of 1.0% TFA was added, and the volume was adjusted to 2.0 mL with water. TFA, added so that the final concentration equaled that of the eluant, was included to lower the pH and ensure that all the guanine, which is highly insoluble at neutral pH, would be recovered. Also, to ensure that all materials were in solution, these samples were sonicated in a bath sonicator (Bransonic 220) for 1.5 h.

The amount of guanine and adenine present in the supernatant was determined by comparing the intensities with a standard curve generated from a mixture of these two purines at known concentrations. The percent depurination was then determined from the relationship between this amount and that obtained from an equal aliquot of DNA (1.0 mL of 1.1 mg/mL) that had been fully depurinated by boiling for 1.0 h at pH 2.85. Three 1.0-mL aliquots were analyzed separately for the extent of depurination, and the results indicate that under the conditions used above an average of approximately 5% of the purines was removed.

*Introduction of Apurinic Sites into Plasmid pBR322 DNA.* Lindahl & Andersson (1972) have also shown that the rate of depurination of double-stranded DNA can be applied to phage PM2 DNA (9800 base pairs). Under the same conditions as described above, approximately one apurinic site is introduced per plasmid molecule after 4 min of heating at 70 °C. Longer incubation times result in more depurination.

For the present study, pBR322 DNA (4362 base pairs) was purchased from International Biotechnologies, Inc. The sample was shown to contain >95% supercoiled molecules on 1% agarose gels. To introduce approximately one apurinic site per molecule, an appropriate amount was first precipitated on dry ice for 30 min after addition of 2.5 volumes of cold 95% ethanol. The sample was spun at 13750 rpm for 10 min in a microcentrifuge (Fisher Model 235A), the supernatant decanted, and 100 μL of ethanol used to wash the pellet. After the second spin (5 min) the pellet was dried under vacuum and subsequently dissolved in 0.1 M NaCl, 0.01 M sodium phosphate, and 0.01 M sodium citrate, pH 5.0, that had been preequilibrated at 70 °C. After incubation for 8 min, the plasmid was recovered by precipitation as above. The pellet was dissolved in 1.0 mM sodium cacodylate, 1.0 mM NaCl, and 0.2 mM EDTA, pH 6.0, and used immediately.

*Analysis of Nicking Activity of Both Diastereomeric Tripeptides.* The plasmid pBR322 DNA (200 μM in 10 μL), prepared to contain an average of one apurinic site per molecule as described above, was incubated at 37 °C with 10 μL of 100 μM Tri(L) or Tri(D). The control was run with 10 μL of added buffer. Aliquots (2 μL, 0.13 μg) were removed at 10-min intervals from 0 to 60 min and placed on ice. The samples were loaded on 1% agarose gels, which were subsequently stained with ethidium bromide and photographed. The negatives were scanned, and the relative amounts of nicked and supercoiled plasmid molecules were determined from the area under the appropriate peaks.

### *Methods*

The methods used in this investigation have been described in the preceding paper (Shine & James, 1985). The 1% agarose gels (5 × 7.5 × 0.35 cm) were run on a horizontal gel electrophoresis unit (IBI Model QSH) with Tris–borate–EDTA (TBE) (0.09 M Tris borate, 2.5 mM EDTA, pH 8.3) as the running buffer. The electrophoresis was carried out at 100 V for approximately 50 min. The gels were stained with ethidium bromide and photographed by using Polaroid positive/negative 4 × 5 Land Film, Type 55. The negatives were scanned with a soft laser scanning densitometer (Biomed Instruments, Inc., Model SL-504).

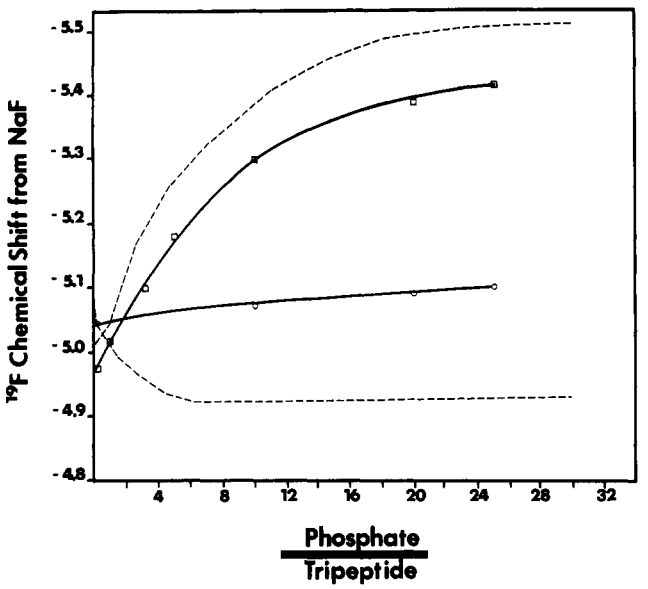


FIGURE 1: Plot of $^{19}$F NMR chemical shifts for Tri(L) (□) and Tri(D) (○) as a function of the phosphate-to-tripeptide ratio. Solid curves were obtained by using DNA modified to contain ~5% apurinic sites. For comparison, the dashed curves obtained with native DNA are included (Shine & James, 1985). The curves were generated titrating a solution containing 3 mM peptide with a solution 75 mM in DNA phosphates and 3 mM in peptide. Both solutions were in 10 mM sodium cacodylate, 10 mM sodium chloride, 1 mM EDTA, pH 6.0, and 10% (v/v) $D_2O$. The titrations were done at 37 °C.

## RESULTS

### *$^{19}$F NMR Studies*

The $^{19}$F NMR studies were done at 37 °C in order to be able to compare these data with those obtained with native DNA (cf. preceding paper). Although the L-Trp peptide has been shown to cleave supercoiled plasmid DNA containing apurinic sites, cleavage of linear apurinic DNA has not been reported.

*Titration of Tripeptides with Apurinic DNA.* The D-tryptophan- and L-tryptophan-containing diastereomers of Lys-FTrp-Lys were titrated with DNA modified to contain ~5% apurinic sites. The changes in the $^{19}$F chemical shift observed as a function of the phosphate-to-tripeptide ratio are shown in Figure 1. The fluorine resonance position of the L-Trp peptide shifts upfield as the concentration of apurinic DNA increases. At a ratio of 25:1, the magnitude of the upfield shift is −0.41 ppm. The direction of the fluorine chemical shift of the D-Trp peptide complex is also upfield. However, at the same ratio (25:1) a shift of only −0.06 ppm is observed.

*Temperature Dependence of the Line Width.* The observed line width for both of these tripeptides increases as the concentration of depurinated (~5%) DNA is raised. The increase in line width for the L-Trp peptide complex (16 Hz; phosphate-to-peptide ratio = 25:1) is greater than that for its diastereomer (4 Hz; 25:1). In order to determine possible contributions from chemical exchange processes, the line width was monitored as a function of temperature for both tripeptide–apurinic DNA complexes. The signal for the L-Trp peptide complex is broadened to 40 Hz and that of the D-Trp peptide complex increases to 11 Hz as the temperature is decreased from 37 to 20 °C.

### *Fluorescence Data*

The affinities of both of these tripeptides for apurinic DNA were determined by using fluorescence spectroscopy. The extent of quenching observed as a function of tripeptide concentration is shown in Figure 2. The data were obtained under the same experimental conditions and analyzed by the same methods as used for the native DNA complexes. These procedures are described in the preceding paper (Shine & James, 1985). The $I_b/I_f$ ratios obtained were 0.21 and 0.35 for the L-Trp and D-Trp peptides, respectively. Association constants of $5.3 \times 10^4$ $M^{-1}$ and $7.0 \times 10^4$ $M^{-1}$ for the L-Trp and D-Trp peptides, respectively, were obtained by fitting the data to the

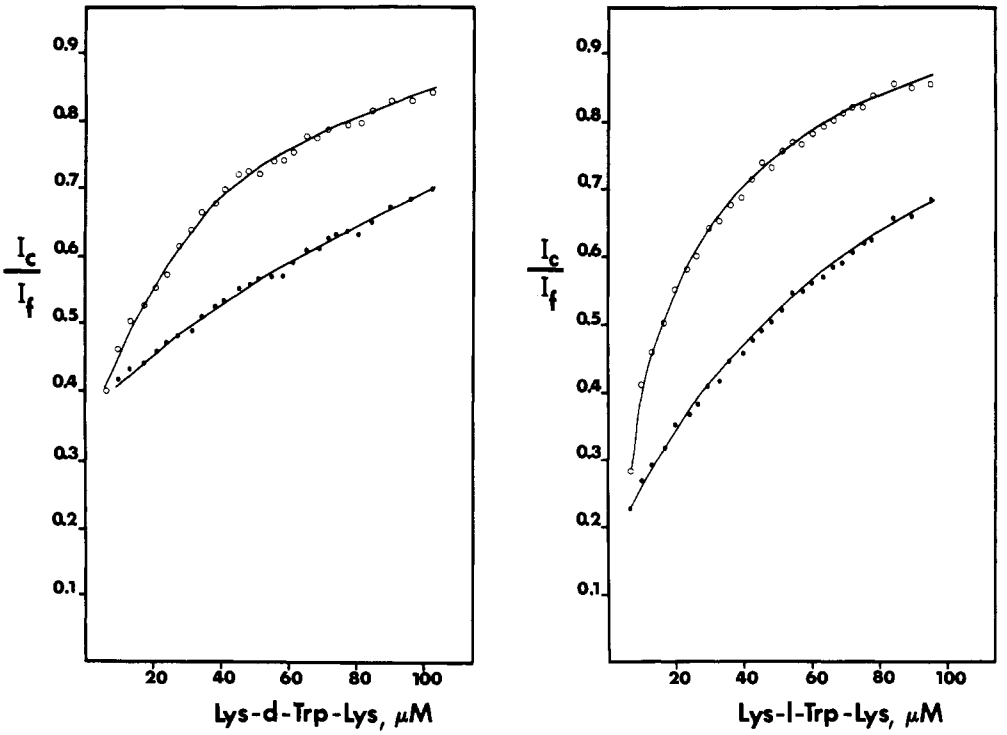


FIGURE 2: Fluorescence titration of 5% depurinated DNA with Tri(D) and Tri(L), left and right, respectively. The relative fluorescence change, $I_c/I_f$, as a function of tripeptide concentration at two different DNA concentrations, 197 (●) and 99 μM (○), is shown for each peptide, where $I_c$ and $I_f$ are the intensities measured in the presence and absence of apurinic DNA, respectively.

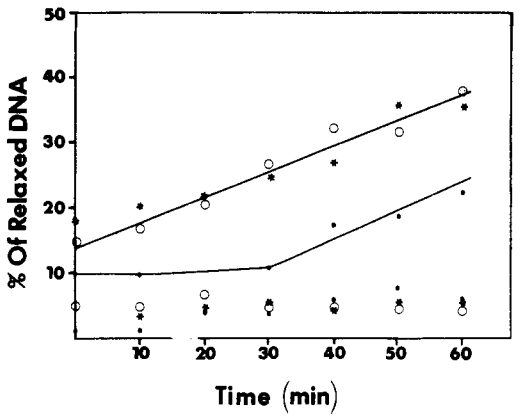


FIGURE 3: Chain nicking of pBR322 DNA containing an average of one apurinic site per molecule on incubation alone (●) and on incubation with Tri(L) (○) and Tri(D) (*). Data points for native pBR322 alone (●) and native pBR322 in the presence of Tri(L) (○) and Tri(D) (*) under the same conditions were obtained as controls. The conversion of supercoiled plasmid to the relaxed form was monitored on 1% agarose gels. Negatives of the ethidium bromide stained gels were scanned. The peaks were cut out and weighed; the percent relaxed was determined from the area of relaxed/(area of relaxed + area of supercoiled).

McGhee and von Hippel equation (cf. preceding paper).

*Nicking Activity of Both Diastereomeric Tripeptides*

The ability of these tripeptides to cleave DNA at apurinic sites was examined by using agarose gel electrophoresis. Single-strand cleavage of plasmid DNA converts the supercoiled to the relaxed form. For plasmid pBR322 DNA, these two forms are well separated on 1% agarose gels; the supercoiled form migrates faster than the nicked form. As shown in Figure 3, neither tripeptide introduces nicks to native pBR322 DNA. Both tripeptides promote cleavage of pBR322 DNA that contains apurinic sites. The rate at which nicks are introduced is the same for both diastereomers.

## DISCUSSION

The $^{19}F$ NMR data presented here suggest that both peptides, Lys-L-Trp-Lys and Lys-D-Trp-Lys, form complexes with depurinated DNA that are distinct from those formed with native DNA. With depurinated DNA, an additional type of binding site (apurinic site) has been introduced. The differences observed in the $^{19}F$ NMR parameters when these peptides are in the presence of apurinic vs. native DNA could be due to molecules bound exclusively to apurinic sites or molecules bound at both apurinic and native sites. Since a single peak is observed in the presence of depurinated DNA, these peptides would have to rapidly exchange between the various sites if the measurements reflect an average of molecules bound on both sites. However, the relative binding affinities of the L-Trp peptide to apurinic and native sites have been reported (Behmoaras et al., 1981a). On the basis of fluorescence titration data obtained at various levels of depurination, Helene and co-workers have deduced that the affinity of this tripeptide for an apurinic site is approximately 2 orders of magnitude higher than that for a native site (Behmoaras et al., 1981a). Given that Lys-Trp-Lys binds strongly and preferentially to apurinic sites, it is reasonable to assume that the $^{19}F$ NMR results can be attributed to distinct modes of binding at the apurinic site.

When 5% of the purine bases are removed from native DNA, differences in both the $^{19}F$ NMR chemical shift and the line width measurements are observed. There is a deshielding of the L-Trp peptide (0.08 ppm downfield shift) when bound to apurinic DNA vs. native DNA while the D-Trp peptide is more shielded (0.19 ppm upfield shift). However, as reflected in the line width measurements, only the L-Trp peptide complex experiences a change in its dynamic state between the native (70 Hz) and apurinic sites (16 Hz). The temperature dependence of the line width observed for both peptides is essentially the same for depurinated DNA as with native DNA, suggesting that all line widths contain the same contribution from exchange. The data indicate that the indole ring of the L-Trp peptide is more constrained than that of its diastereomer in the presence of apurinic DNA. In addition, the mobility of the L-Trp ring is less restricted at the apurinic site, where a cavity has been created by removal of a purine, than at the native site.

The overall binding affinities of these tripeptides for apurinic DNA, calculated from the fluorescence data, are not significantly different from those obtained with native DNA. In addition, no significant difference between the binding affinities of these two diastereomers is observed before or after depurination. Hélène and co-workers, also using the results of fluorescence data, have reported overall association constants for the L-Trp peptide of $1.6 \times 10^4$ $M^{-1}$ and $1.0 \times 10^5$ $M^{-1}$ for native and apurinic DNA (5%) at 4 °C, respectively (Behmoaras et al., 1981a). The difference in affinities is less than 1 order of magnitude, which is not necessarily within the accuracy of the analysis used in this study of the fluorinated analogue. In their study, the stronger binding of the tripeptide at apurinic sites vs. native sites was inferred from the analysis of data obtained at different levels of depurination (0.3–5%). In our study only 5% depurinated DNA was used.

The degree of quenching of the tryptophan fluorescence observed for the D-Trp peptide is greater in the presence of apurinic DNA than in the presence of native DNA at low degrees of saturation (65% vs. 52%). This could be due to a different mode of binding at the apurinic site and is consistent with the $^{19}F$ NMR data. Any differences between the modes of binding of the L-Trp peptide are not reflected in the percent of quenching; the fluorescence is quenched to the same extent with both DNAs (76% vs. 79%).

When either of these tripeptides is incubated with plasmid DNA containing apurinic sites, no measurable difference in the rate of cleavage of the DNA backbone is observed. Previous studies comparing the nicking ability of several tripeptides have shown that those containing either tryptophan or tyrosine between two lysines are most active (Behmoaras et al., 1981b,c; Pierre & Laval, 1981). It has been suggested that the aromatic amino acid is important to both the recognition of and cleavage at apurinic sites.

Our $^{19}F$ NMR results conclusively show that each diastereomer has a distinct mode of binding to depurinated DNA. The fluorine chemical shift for the complexes indicates that the fluorine nuclei reside in different local environments, and the differential line broadening demonstrates that the motional properties of these peptides within or between their binding sites on apurinic DNA are significantly different. However, neither the change in local environment nor the change in mobility affects the rate of cleavage, suggesting that neither the precise positioning of the indole ring nor significant changes in its mobility are crucial to the nicking activity. Both the $^{19}F$ NMR parameters and the relative binding affinities should be followed as a function of the percent depurination. This would allow a more precise characterization and separation of the effects due to binding at apurinic vs. native sites in DNA.

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**Registry No.** Tri(L), 96914-16-8; Tri(D), 96914-17-9.

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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 $\mu$M 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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