Role of Superhelicity in Homologous Pairing of DNA Molecules Promoted by Escherichia coli recA Protein*

(Received for publication, June 29, 1981)

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In the presence of ATP and an excess of recA protein, superhelical closed circular DNA (form I DNA) and homologous single-stranded fragments paired to form D-loops in the early stage of incubation and dissociated during subsequent incubation. RecA protein that was not bound to single-stranded DNA ("free recA protein") was shown to be responsible for the dissociation of D-loops. Larger amount of free recA protein gave a lower final yield of D-loops. When the concentration of form I DNA was increased in the presence of a fixed amount of single-stranded DNA, larger amounts of free recA protein were required to produce a certain extent of dissociation. When form I DNA, excess recA protein, and ATP were incubated without single-stranded DNA, or with heterologous single-stranded fragments before the addition of homologous single-stranded fragments, formation and subsequent dissociation of D-loops were observed as in the case when all components of the reaction were added from the start. Therefore, the dissociation of D-loops is a result of the stoichiometric interaction between free recA protein and form I DNA bearing D-loops. In the process of formation and dissociation of D-loops, form I DNA was converted to an inactive substrate without any apparent damage to the DNA. The concentration of free recA protein appeared to decrease during the reaction.

These observations revealed that formation and dissociation of D-loops are sequential reactions when form I DNA is the substrate and recA protein is present in excess. The dissociation of D-loops and the inactivation of form I DNA can be explained by a model in which recA protein cooperatively binds to form I DNA from the site of D-loop, resulting in stimulation of unidirectional unwinding of the double helix.

One of the fundamental questions in genetics is how two homologous chromosomes pair prior to crossing over. Many models have been derived from the observations on genetic recombination and gene conversion in fungi and bacteria (1-3). However, until recently, little physicochemical and enzymologic evidence was available to support or challenge the models.

Holloman et al. (4) and Liu and Wang (5) demonstrated that, in the absence of any protein, superhelical closed circular DNA (form I DNA) rapidly took up homologous single-stranded fragments to form D-loops at 75°C but did so very slowly at 37°C. Subsequently, recA protein, which is essential for homologous recombination in Escherichia coli (6-8), was found to promote the formation of D-loops in the presence of ATP (9,10). Studies on homologous pairing promoted by recA protein revealed that recA protein forms stable joint molecules of DNA at a high frequency from a pair of homologous molecules if one of them is single-stranded or partially single-stranded, and if either one has free end (11-14). However, a free end is not essential for the homologous pairing by recA protein since: 1) recA protein does not specifically recognize the end of DNA molecules (11,12,15); 2) circular single-stranded DNA and form I DNA pair at homologous sites to form unstable joint molecules (11); and 3) these joint molecules are stabilized by the action of E. coli topoisomerase I (16). Therefore, in the first stage of homologous pairing, recA protein promotes the formation of base-pairing between single-stranded DNA and the complementary strand in double-stranded DNA in a side-by-side fashion (11,12,16). This base pairing is formed through the following steps: 1) In the presence of ATP, recA protein is activated by the binding of single-stranded DNA, which stimulates the binding of double-stranded DNA to form a ternary complex. 2) In this ternary complex, the double-stranded DNA is locally unwound, which presumably facilitates homologous pairing between the single-stranded DNA and double-stranded DNA. 3) When these 2 DNA molecules are aligned in register at a homologous site, a heteroduplex joint is formed (15,17,18).

The observations described above extended the view about the roles of recA protein in homologous recombination. Any events which expose single strand with or without ends, such as strand displacement (19-24), internal unwinding of double-stranded DNA (25) or the formation of a gap, potentially initiate homologous pairing by recA protein. A high frequency (almost 100%) of recA+ dependent recombinational repair at the sites of gaps was observed in vitro by Rupp et al. in post-replication repair of UV-damaged DNA in E. coli (26).

In eukaryotes, DNA and histones form a structure called a nucleosome (see Ref. 27 for review). When the histone cores of the structure are removed artificially, or by some natural events, the DNA should exhibit superhelical structure. As pointed out by Holloman and Radding (28,29), superhelicity of the substrate DNA significantly affects the properties of homologous pairing promoted by recA protein in vitro, such as the requirements for the reaction, and the kinetics (12). We also observed that recA protein promotes the dissociation of D-loops (12). In the present study, we have further investigated homologous pairing of superhelical DNA and single-stranded fragments promoted by recA protein. We found that dissociation of D-loops by recA protein was not due to the back-reaction in the classical sense, but rather that formation and dissociation of D-loops were sequential reac-
tions promoted by recA protein when double-stranded DNA was superhelical.

MATERIALS AND METHODS

Enzymes—RecA protein (fraction V) was prepared as described (30, 31). The concentration of recA protein is expressed in moles of polypeptide of M₀ = 40,000. Pancreatic DNase I was purchased from Worthington Biochemical Co.

DNA—Form I DNA¹ and fragments of single-stranded DNA of phages fd and ϕX174 were prepared as described or cited earlier (13). Form II DNA with a single nick was prepared by treatment with DNase I in the presence of ethidium bromide (31, see Ref. 30). Amounts of DNA are expressed in moles of nucleotide residues.

DNase I in the presence of ethidium bromide (31, see Ref. 30). The concentration of ethidium bromide in the presence of ϕX174 (heterologous) (average chain length, about 500 nucleotides), and various amounts of purified recA protein. A preliminary incubation in the presence of 1 mM MgCl₂ (30, 31) was omitted. After an incubation for 30 min at 37°C, we terminated the reaction by chilling it at 0°C and diluting the mixture three times with 25 mM EDTA (pH 9.4). Then, we treated the products with 0.5% Sarkosyl (NL97) at 0°C more than 20 min, and diluted the mixture about 10 times with cold 25 mM EDTA (pH 9.4). We took an aliquot of 50 μl to measure total radioactivity, and an aliquot of 200 μl to assay the fraction of nicked double-stranded DNA by the nicking assay of Kuhlkin et al. (34). The amount of nicked double-stranded DNA was determined by the nicking assay of Kuhlkin et al. (34). Then, we diluted 200 μl of the mixture six times with cold 1.5 M NaCl, 0.15 M Na citrate, incubated it at 41°C for 4 min, and immediately diluted seven times with cold NaCl/Na citrate at 0°C. We filtered the sample at about 4 ml/10 s through a nitrocellulose filter (Sartorius membrane filter, SM 11306, pore size 0.45 μm). The filter had been washed with 2 ml of NaCl/Na citrate. We washed the filter successively with 1.5, 1.0, and 0.5 ml of cold NaCl/Na citrate. Radioactivity retained on the filter was measured in a scintillation counter (Beckman LS8100) by using toluene scintillator.

RESULTS

Apparent Inhibition of the Formation of D-Loops by Excess recA Protein—Previously, we observed that no D-loops were formed until a certain concentration of recA proteins was present, after which the yield of D-loops rose sharply. The least amount of recA protein that was required for the formation of D-loops was not affected by the concentration of double-stranded DNA, but rather was determined by the concentration of single-stranded DNA and was 0.40 μM. The concentration of single-stranded DNA was decreased from 6.0 to 12 μM to 0.40 μM.

When form I DNA and homologous single-stranded fragments were incubated for 30 min at 37°C with recA protein in the presence of ATP, the minimal concentration of recA protein required for the formation of D-loops was proportional to the concentration of single-stranded DNA between 0.40 μM and 12 μM (Fig. 2B). In addition, when double-stranded DNA was superhelical, excess recA protein appeared to inhibit the formation of D-loops almost completely (Fig. 1). The concentration of recA protein required for the optimal yield of D-loops from form I DNA was plotted against the concentration of single-stranded DNA and was also shown to be proportional to the latter (Fig. 2A). The optimal concentration of recA protein is not significantly affected by the concentration of double-stranded DNA as shown in Figs. 1 and 2A; in the latter figure, the data from experiments using two different concentrations of double-stranded DNA (3.7 μM and 8.8 μM).

The terms form I DNA, form II DNA, and form III DNA denote, respectively, negatively superhelical closed circular DNA, nicked circular DNA, and linear double-stranded DNA.

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During the process of the D-loop assay itself, since the formation of a small number of base pairs between single-stranded DNA and double-stranded DNA is paired in a side-by-side fashion (11, 12, 16). It is possible that such an intermediate might be converted to a D-loop by the energy of supercoiling during the process of the D-loop assay itself, since the formation of a small number of base pairs between single-stranded DNA and the complementary strand of superhelical DNA is a rate-limiting step in uncatalyzed formation of D-loops (32). Therefore, we examined whether or not the product detected by the D-loop assay was an authentic D-loop. If all the double-stranded DNA were nicked prior to the assay, releasing thereby the energy of superhelix formation, one would not expect an unstable precursor to become a D-loop by the concentration of recA protein; higher concentrations of recA protein gave a lower plateau. This dissociation of D-loops was not due to the nicking of double-stranded DNA, since the fraction of nicked double-stranded DNA was constant (about 25%) during the whole period of the incubation (Fig. 3A, open circles).

When we added excess recA protein (one recA monomer per 1 nucleotide residue in single-stranded DNA) after the completion of the reaction with the optimal amount of recA protein, D-loops were also dissociated during subsequent incubation (Fig. 3C, closed circles). In a control experiment, addition of excess recA protein that had been boiled for 3 min did not promote dissociation of D-loops, indicating that native recA protein promoted dissociation rather than some heat-stable contaminant (data not shown).

Formation and dissociation of D-loops by excess recA protein were also observed by gel electrophoresis of the products of the reaction through 1% agarose (data not shown). D-Loops behave like relaxed closed circular double-stranded DNA in electrophoresis through an agarose gel in buffer E (10).7

The continuity of both strands of superhelical DNA appeared to be required for the dissociation of D-loops by excess recA protein, because no significant decrease of D-loops occurred during 160 min of incubation at 37 °C when form II DNA was nicked, and exactly the same heat sensitivity when all of the double stranded DNA was nicked (Fig. 5A). The fraction of D-loops that survived nicking and subsequent incubation at 41 °C for 4 min was measured during the formation of D-loops by the optimal concentration of recA protein. As shown in Fig. 5B and C, the fraction of surviving D-loops was constant during 30 min of incubation at 37 °C. These tests confirmed

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Fig. 3. Time course of the formation and dissociation of D-loops. A, form I DNA (3.7 μM), homologous (●, ▲), or heterologous (○, ■) single-stranded fragments (0.40 μM), and ATP were incubated at 37 °C with recA protein. Concentrations of form I DNA and recA protein were 3.7 μM and 0.40 μM, respectively (●), or 1.8 μM and 0.61 μM, respectively (▲). C, form I DNA (3.7 μM), homologous single-stranded fragments (0.40 μM) and ATP were incubated at 37 °C with 0.06 μM recA protein (●). At 30 min (●), 0.8 mM ATP plus (▲) or minus (■) recA protein (final concentration, 0.40 μM) were added. Controls: ○, heterologous single-stranded fragments instead of homologous ones; ●, without recA protein. Products were measured by D-loop assay (closed symbols) and nicking assay (open symbols).

Fig. 4. Time course of the formation of D-loops from form II DNA. Form II DNA with a single nick (3.7 μM, 95% form II, 2% form III, and 3% form I), homologous (●) or heterologous (▲) single-stranded fragments, and ATP were incubated at 37 °C with recA protein. The concentrations of single-stranded fragments and recA protein were 2.0 μM and 2.1 μM, respectively (●), or 0.40 μM (▲).
recA protein is not catalytic but might be a result of stoichiometry of D-loops or formation of D-loops. Single-stranded DNA itself did not support the formation of D-loops when recA protein was not in excess (Fig. 6). As shown in Fig. 6, added heterologous single-stranded DNA neither nicking nor heating was taken as 100%. B, form I DNA (8.8 μM), homologous single-stranded fragments (0.80 μM) and ATP were incubated in the presence of an optimal concentration (0.16 μM) of recA protein at 37 °C. Samples were withdrawn at the indicated times and divided into two aliquots. One aliquot was assayed directly by standard D-loop assay but incubation at 41 °C was omitting free recA protein. Another aliquot was treated with DNaase I (2 μg/ml) for 5 min at 0 °C, deproteinized, and then assayed by standard D-loop assay including incubation for 4 min at 41 °C (DVO, □). C, the fraction of D-loops that survived nicking and heating at 41 °C (DVO/DVO × 100 (%)) was plotted against the time of incubation with recA protein at 37 °C. Data were taken from the experiment shown in B.

Superhelicity and Homologous Pairing by recA Protein

Stoichiometry between recA Protein and Form I DNA in the Dissociation of D-Loops—As shown in Fig. 3A, the final extent of dissociation of D-loops is determined by the concentration of recA protein, indicating that the dissociation by recA protein is not catalytic but might be a result of stoichiometric interaction of free recA protein and form I DNA bearing a D-loop or form I DNA without a D-loop. Therefore, we expected that if a larger amount of form I DNA were added to the reaction mixture, the extent of the dissociation would be decreased. We found that was the case. As shown in Fig. 1, in the presence of 0.40 μM homologous single-stranded fragments and 3.7 μM, 8.0 μM, or 18 μM form I DNA, about 0.09 μM recA protein gave the optimal yield of D-loops. In the presence of 0.40 μM recA protein, almost all D-loops were dissociated after 30 min of incubation when the initial concentration of form I DNA was 3.7 μM (Figs. 1 and 3, A and B), but in the presence of 0.40 μM or 0.60 μM recA protein, one-third to a half of D-loops remained when the initial concentration of form I DNA was 8.0 μM or 18 μM (Figs. 1 and 3B). The amount of free recA protein giving half-maximal yield of D-loops was plotted against the concentration of form I DNA (Fig. 1, inset), and shown to be roughly proportional to the latter. The amount of free recA protein required for the complete dissociation of D-loops is estimated to be more than one recA monomer per every 5 base pairs in form I DNA, and appears to be more than enough to cover all form I DNA. These observations confirm that the dissociation of D-loops is a result of a stoichiometric interaction between free recA protein and form I DNA either with or without D-loops.

The Effect of the Preliminary Incubation of Form I DNA with Excess recA Protein before Addition of Homologous Single-stranded Fragments—We tested two possibilities: 1 recA protein quickly forms D-loops in form I DNA in an early stage of the reaction. At the same time, free recA protein slowly inactivates the substrate form I DNA which does not bear D-loops yet, resulting in reversal of the formation of D-loops, or 2 free recA protein interacts only with form I DNA bearing a D-loop, resulting in the dissociation of D-loops. If

\[ \text{Fraction of surviving D-loops} = \frac{[\text{D-loops after heating}] - [\text{D-loops before heating}]}{[\text{D-loops before heating}]} \times 100\% \]

that we measured only mature D-loops by the D-loop assay.

Heterologous Single-stranded DNA Inhibits Dissociation of D-Loops by Excess recA Protein—From the observations described in the preceding sections, it is likely that the recA protein which is not bound to single-stranded DNA ("free recA protein") stimulates the dissociation of D-loops, since single-stranded DNA is saturated by recA protein at one recA monomer to every 4 nucleotide residues (35). In order to confirm this hypothesis, we added heterologous single-stranded DNA to the reaction mixture which contained form I DNA, homologous single-stranded fragments, ATP, and an excess of recA protein (one recA monomer per nucleotide residue in single-stranded DNA), since heterologous single-stranded fragments as well as homologous ones absorbed free recA protein. As shown in Fig. 6, added heterologous single-stranded DNA overcame the effect of excess recA protein almost as well as added homologous single-stranded DNA when the ratio of single-stranded DNA to recA protein was about 10 nucleotide residues/monomer of protein. Larger amounts of heterologous or homologous single-stranded DNA again reduced the yield of D-loops. As expected, heterologous single-stranded DNA itself did not support the formation of D-loops (Fig. 6, open squares), but rather inhibited the formation of D-loops when recA protein was not in excess (Fig. 6, closed triangles), as described previously (9, 10, 12, 17). Therefore, free recA protein is responsible for the dissociation of D-loops.

\[ \text{Stoichiometry between recA Protein and Form I DNA in the Dissociation of D-Loops} = \frac{[\text{D-loops after heating}] - [\text{D-loops before heating}]}{[\text{D-loops before heating}]} \times 100\% \]

3 Unpublished observation.
the first possibility were true, the formation of D-loops would not occur when homologous single-stranded DNA was added after a preliminary incubation of form I DNA, ATP, and excess recA protein with or without heterologous single-stranded DNA. Heterologous single-stranded DNA might stimulate the inactivation of form I DNA, since either homologous or heterologous single-stranded DNA have been shown to stimulate binding of recA protein to double-stranded DNA in the presence of an analogue of ATP, ATPyS (15,17). If the second possibility were true, D-loops would be formed soon after the addition of homologous single-stranded DNA and then dissociated as was observed when all components of the reaction were added from the start. The results of the experiments shown in Fig. 7A, compared with those shown in Fig. 3A or Fig. 7C, indicate that the latter is the case.

The following observations indicate that in the case of the formation and subsequent dissociation of D-loops, form I DNA was converted to an inactive substrate. When form I DNA, homologous single-stranded fragments, and ATP were incubated with excess recA protein at 37 °C for 30 min, a period during which D-loops were formed and then dissociated, addition of homologous single-stranded fragments (Fig. 7B, closed circles and closed squares) did not initiate a new increase in the amount of D-loops. This is not due to the inactivation of recA protein after one round of formation and dissociation of D-loops, because of the results obtained by the following experiments. If form I [3H]DNA was added after the incubation of unlabeled form I DNA, homologous single-stranded fragments, ATP, and excess recA protein for 1 min at 37 °C, the added form I [3H]DNA was converted to D-loops which then was dissociated as observed in the standard conditions (Fig. 7D, see Fig. 3A as a control experiment). This observation also shows that after dissociation of D-loops, the reaction mixture still contained active single-stranded fragments.

Form I DNA, once inactivated by the formation and the subsequent dissociation of D-loops, was found to be fully reactivated by the termination of the series of the reaction (see "Materials and Methods"). This observation and the absence of nicking of form I DNA during the reaction (Fig. 3A) indicate that the cause of the inactivation is not damaging of form I DNA, but rather, binding of free recA protein to form I DNA. As described in the following section, free recA protein appears to be sequestered during the reactions.

Anomalous Kinetics in the Formation of D-loops from Form I DNA—When we tried to make D-loops from form I DNA in the presence of much higher concentrations of single-stranded fragments (12 μM) and recA protein (2.4 μM, not a large excess for 12 μM single-stranded fragments), we observed anomalous kinetics in the formation; D-loops were formed very quickly in the first 2 min, then decreased, and again increased slowly (Ref. 12 and Fig. 8A, closed circles). This anomalous kinetics can be explained if we assume that recA protein binds cooperatively to form I DNA bearing D-loops (see "Discussion"), i.e. in an early stage of incubation, D-loops are formed and dissociated by recA protein, and at the same time, the amount of free recA protein is decreased by binding to form I DNA. After all free recA protein binds to form I

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**Fig. 6.** Effect of added homologous or heterologous single-stranded fragments on the reaction. The basic reaction mixture contained 37 μM form I [3H]DNA, 0 μM recA protein (A), 0.40 μM recA protein (B), 0 μM recA protein, 0.40 μM homologous single-stranded fragments (C), and 0.1 μM (D) or 0.40 μM (E) recA protein. Added single-stranded fragments (ssDNA): ○, homologous; △, □, heterologous.

**Fig. 7.** Effects of preincubation on the formation and dissociation of D-loops by excess recA protein. A, form I [3H]DNA (3.7 μM), 0.40 μM (○), or 1.6 μM (△) recA protein and ATP were incubated in the absence of single-stranded DNA (○) or in the presence of 0.72 μM heterologous single-stranded fragments (△) at 37 °C. At 30 min (○), 0.40 μM homologous (○) or heterologous (△) single-stranded fragments and ATP (1.5 mM) were added to the reaction mixture without single-stranded DNA, and 0.30 μM homologous single-stranded fragments (△) and ATP were added to the reaction mixture containing heterologous single-stranded fragments. B, form I [3H]DNA (3.7 μM), 0.72 μM (○), or 0.40 μM (△) homologous single-stranded fragments and ATP were added to the reaction mixture containing heterologous single-stranded fragments. C, form I [3H]DNA (3.7 μM), 1.6 μM recA protein and ATP were incubated with 0.40 μM homologous single-stranded fragments at 37 °C. D, unlabeled form I DNA (3.7 μM), 0.80 μM recA protein and ATP were incubated with 0.40 μM homologous single-stranded fragments at 37 °C. At 30 min (△), [3H]labeled form I DNA (3.7 μM) was added.
The observations described above show that 1) D-loops are transient intermediates of a series of reactions of form I DNA, homologous single-stranded DNA, and excess recA protein in the presence of ATP; that 2) a stoichiometric amount of free recA protein interacts with form I DNA bearing D-loops, resulting in the dissociation of D-loops; and that 3) through formation and subsequent dissociation of D-loops, form I DNA is converted to an inactive substrate for the formation of D-loops, and free recA protein is sequestered. However, form I DNA is not damaged throughout the series of the reactions. Moreover, preliminary observations suggest that this sequestered recA protein can dissociate and act again under some conditions.\(^6\)

These observations suggest that the dissociation of D-loops and the inactivation of form I DNA result from binding of free recA protein to form I DNA bearing D-loops; free recA protein may bind cooperatively from the site of a D-loop.

Based on results of the present study and our previous ones (11, 12, 16–18), all identified steps of reactions involving form I DNA, homologous single-stranded fragments, and recA protein in the presence of ATP can be expressed as follows;

\[
\begin{align*}
S & \rightarrow SA_n R \\
R & \rightarrow D \\
SA_n & \rightarrow RA_m
\end{align*}
\]

where \(R, S, A,\) and \(D\) denote form I (RF I) DNA, single-stranded fragments, recA protein, and form I DNA bearing a D-loop, respectively, and \(n\) and \(m\) are the number of molecules of recA protein. \(SA_n R\) denotes a ternary complex of single-stranded fragments, recA protein, and double-stranded DNA in which the double strand is partially unwound (15). The formation of a ternary complex does not require homology between single-stranded fragments and double-stranded DNA (15, 17, 18).

The model proposed here includes the idea that recA protein is a component of the product of the reaction. This idea is supported by our previous observations; 1) D-loops made from form II DNA and homologous single-stranded fragments by recA protein are stable in the standard reaction mixture at 37 °C. 2) However, if the proteins were denatured by treatment with detergent, D-loops in form II DNA were very unstable at 37 °C (12, 36)\(^7\). The observations indicate that recA protein which remains bound to D-loops prevents thermal branch migration. Thus, recA protein behaves not like a classical catalyst or enzyme but as one of the substrates in the formation of D-loops.

There are two ways to explain the subsequent dissociation of D-loops by recA protein. Model I, recA protein binds to the displaced strand of the D-loop, unwinds the heteroduplex region in the D-loop, and then pairs the displaced strand with its original complementary strand. This is similar to the mechanism for the formation of D-loops (17). However, this model does not easily explain why the amount of recA protein required for the dissociation of D-loops is an amount that is sufficient to cover all of the form I DNA, and why D-loops in

\(^6\) Unpublished observations.

\(^7\) Unpublished observation.
form II DNA are not dissociated by excess recA protein (Fig. 4).

We prefer a second model. Model II, unidirectionally cooperative binding of free recA protein to form I DNA from the site of D-loops stimulates unidirectional unwinding of form I DNA. Since the extent of unwinding of form I DNA is topologically limited, unidirectional unwinding of form I DNA causes rewinding at the trailing end of the unwound region. As a result, the displaced strand and its complementary strand are rewound at one end of the D-loop and the single-stranded fragment is gradually dissociated from the form I DNA.

Unidirectional unwinding of the double helix by cooperative binding of proteins was proposed as a mechanism of ATP-dependent unwinding by DNA helicase II of E. coli (37). The unwinding by helicase II is initiated at a single-stranded tail of duplex DNA, whereas the unwinding by recA protein is initiated at the site of a D-loop. Polarity in the reaction promoted by recA protein has been demonstrated by Kahn et al. (38).

The unidirectional unwinding of double-stranded DNA by recA protein might have roles also in the formation of long heteroduplex joint between linear double-stranded DNA and homologous single-stranded circular DNA in vitro (11, 38, 39), and play roles in general recombination in vivo, such as promoting branch migration and enlarging initial heteroduplex regions (see Ref. 29).

Acknowledgments—We thank Drs. R. Kahn, R. P. Cunningham, C. DasGupta, and C. M. Radding for sending us their manuscript before publication, and Dr. Charles M. Radding for valuable advice and critical reading of this manuscript.

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J. Biol. Chem. 1982, 257:370-376.

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