Magnesium Ion-dependent Activation of the RecA Protein Involves the C Terminus*

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Optimal conditions for RecA protein-mediated DNA strand exchange include 6–8 mM Mg²⁺ in excess of that required to form complexes with ATP. We provide evidence that the free magnesium ion is required to mediate a conformational change in the RecA protein C terminus that activates RecA-mediated DNA strand exchange. In particular, a “closed” (low Mg²⁺) conformation of a RecA nucleoprotein filament restricts DNA pairing by incoming duplex DNA, although single-stranded overhangs at the ends of a duplex allow limited DNA pairing to occur. The addition of excess Mg²⁺ results in an “open” conformation, which can promote efficient DNA pairing and strand exchange regardless of DNA end structure. The removal of 17 amino acid residues at the Escherichia coli RecA C terminus eliminates a measurable requirement for excess Mg²⁺ and permits efficient DNA pairing and exchange similar to that seen with the wild-type protein at high Mg²⁺ levels. Thus, the RecA C terminus imposes the need for the high magnesium ion concentrations requisite in RecA reactions in vitro. We propose that the C terminus acts as a regulatory switch, modulating the access of double-stranded DNA to the presynaptic filament and thereby inhibiting homologous DNA pairing and strand exchange at low magnesium ion concentrations.

The RecA protein of Escherichia coli plays a central role in the processes of homologous DNA recombination and DNA repair. RecA is a DNA-dependent ATPase that catalyzes an in vitro DNA strand exchange reaction between single-stranded (ssDNA) and homologous double-stranded DNA (dsDNA) molecules. The DNA strand exchange reaction takes place in several stages (Fig. 1). The RecA protein forms a nucleoprotein filament that completely encompasses the circular ssDNA. This filament then aligns the bound single strand with a homologous duplex DNA to form a DNA pairing intermediate often referred to as a joint molecule. 1000 base pairs of DNA can be aligned and exchanged in a joint molecule under the empirically defined optimal reaction conditions, which typically include 1–3 mM ATP and about 10 mM magnesium ion. All steps to this point, including the formation of joint molecules, require ATP but not ATP hydrolysis. ATP hydrolysis is needed only to complete the late stages of strand exchange of long DNA substrates, often derived from bacteriophage DNAs. Whereas DNA pairing, leading to joint molecule formation, can occur at either end of a linear duplex, the subsequent and ATP hydrolysis-dependent extension of the nascently paired regions is unidirectional, proceeding 5’ to 3’ relative to ssDNA initially bound in the filament. Thus, exchange proceeds in one direction along the linear duplex, and joints formed at the “wrong” end in the pairing phase are eliminated. In a DNA strand exchange involving quite long DNAs that leads to nicked circular product formation, the ends of the duplex where the exchange begins and ends are referred to as proximal and distal, respectively (Fig. 1) (1, 2).

Examination of the conditions for an optimal RecA protein-catalyzed DNA strand exchange reaction reveals an unexplored enigma. Magnesium forms a relatively strong 1:1 complex with ATP (3) and should be required at concentrations equal to the ATP added to the reaction. However, optimal rates and yields in RecA-mediated DNA strand exchange require an additional 6–8 mM of “free” Mg²⁺ (4–6). Some of this magnesium ion is associated with the DNA, but the DNA concentration in most experiments is on the order of a few (1–20) μM in total nucleotides (and backbone phosphate) and thus could not complex more than a small fraction of the available magnesium ion. Lower magnesium concentrations, more stoichiometric with the added ATP, are sufficient for primary DNA binding. Some homologous DNA pairing occurs in the presence of ATP-S, an analog that is not appreciably hydrolyzed, but the higher magnesium ion concentrations are required for the generation of long hybrid DNA products with ATP (7). The extra Mg²⁺ is contributing something significant to the reaction, but the effects have not been explained.

The question is intriguing, since the free magnesium ion requirements for RecA protein-mediated DNA strand exchange in vitro appear to exceed what is available in vivo. Mg²⁺ is present at about 100 mM in a bacterial cell (8–10), but almost all of this is bound up in ribosomes and DNA. The level of free magnesium ion is thought to be on the order of 1 or 2 mM at most (11). Based on in vitro data, the RecA protein should be almost inactive in the cell, although the crowding effect of the in vivo environment may moderate the effects of low available Mg²⁺ (12). There is a decreased requirement for magnesium ion in the presence of volume-occupying agents such as polyethylene glycol or polyvinyl alcohol, utilized to approximate the crowded environment of the cell, suggesting that an active conformation of RecA protein can be stabilized by either excess magnesium ion or the presence of crowding reagents at low magnesium (12).

The RecA protein has a small C-terminal domain extending from residue 270 to the protein terminus at residue 352, the function of which has not been fully explored (1, 13). The last 24
DNA species that are similarly labeled in the data figures. A duplex, and intermediates formed at either end can be observed when exchange is initiated. The reaction can occur on either end of the duplex is then aligned with the bound single strand, and a strand ment first forms on the single-stranded DNA (not shown). A linear vitro 16382 published RecA protein crystal structures (14–15 amino acid residues of the RecA protein are disordered in the sequent extension of the hybrid DNA proceeds unidirectionally when ATP joint molecules. The product used most often to analyze reaction pro- those formed on the other (distal) end are eliminated when ATP is mediates formed on one end (proximal) proceed to products, whereas branches reaction intermediates are referred to as those formed on the other (distal) end are eliminated when ATP is hydrolyzed. The branched reaction intermediates are referred to as joint molecules. The product used most often to analyze reaction pro- gress is the nicked circular duplex. The S₁, S₂, I, and P labels identify DNA species that are similarly labeled in the data figures.

amino acid residues of the RecA protein are disordered in the published RecA protein crystal structures (14–16). We refer to this region as the RecA C terminus (as opposed to the entire C-terminal domain). Within this region, 7 of 25 amino acid residues are negatively charged. In addition to a general lack of structural information about the RecA C terminus, there has been little indication that this part of the protein has functional significance. Several C-terminal deletion mutants of the E. coli RecA protein have been characterized. Deletion from the C terminus of either 25 amino acid residues (17), or a fragment making up about 15% of the RecA polypeptide (18), resulted in faster nucleation, leading to filament formation on dsDNA. A proposal was advanced that the effect could be attributed to the elimination of electrostatic repulsion between the negatively charged residues in the C terminus and the phosphates in the DNA (17, 18). Both C-terminal deletion mutants promoted DNA strand exchange under at least some conditions, and the larger deletion exhibited an enhanced DNA strand exchange in the absence of single-stranded DNA-binding protein (SSB) (17, 18). The larger deletion exhibited ATPase and ssDNA binding activities similar to wild-type (18). The 25-residue deletion bound to ssDNA more tightly in some assays (17). A 25-residue C-terminal deletion of the RecA protein of Proteus mirabilis also exhibited improved binding to dsDNA (19).

Additional C-terminal deletions of RecA protein have also been constructed and studied, but without detailed biochemical characterization. A construct that removes most of the C-terminal domain (75 residues), when expressed with wild-type RecA, interferes with recombinational DNA repair and increases UV sensitivity slightly (20). A 17-residue C-terminal deletion mutant was shown not to affect UV resistance, induc- tion of the SOS response, recombination, or Weigle reactivation when expressed on its own (21). A small effect on conjugal recombination was observed when the same mutant and wild-type proteins were both present in vivo (21). Removal of 18 residues from the C terminus resulted in a substantial conformational change in RecA filaments bound to dsDNA, suggesting an allosteric relationship between the C terminus and the RecA core domain (22).

In the previous paper (23), we characterized a set of RecA C-terminal deletion mutant proteins designed to systemati- cally test the role of the acidic amino acids located in the C terminus of the RecA protein. Removal of the last 13 (RecAAC13), 17 (RecAAC17), or 25 amino acid residues (RecAAC25) increased the rate of binding to dsDNA. The C-terminal deletions also produced a profound effect on the pH dependence of RecA protein-promoted DNA strand exchange reactions relative to the wild-type protein. Ionizable groups in the C-terminal region and others elsewhere in the protein appear to contribute to the pH reaction profile.

In the present study, we show that the negatively charged C terminus of the RecA protein has a modulating function on the DNA strand exchange activity of RecA protein. In the absence of free magnesium ion, the C terminus locks the protein in a conformation in which the initiation of DNA strand exchange is inhibited. When the free magnesium ion concentration is increased to 6–8 mM, the DNA strand exchange function of RecA is activated. The results suggest that magnesium ion interacts directly with RecA protein, altering the conformation of the C terminus.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Biochemicals—**The wild-type E. coli RecA, RecAAC6, RecAAC13, and RecAAC17 proteins were purified as described (23). A plasmid containing the recA E343K mutant (pEAW166) was con- structed using PCR site-directed mutagenesis. The RecA E343K point mutant protein was expressed and purified as described for the wild- type RecA protein (23). The concentrations of the purified RecA proteins were determined from the absorbance at 280 nm using the extinction coefficient of 2.23 × 10^4 M^−1 cm^−1 (24). E. coli SSB was purchased from Sigma. The concentration of the purified SSB protein was determined from the absorbance at 280 nm using the extinction coefficient of 2.83 × 10^4 M^−1 cm^−1 (25). Unless otherwise noted, all reagents were purchased from Fisher. ATP-γ-S was purchased from Roche Molecular Biochi- micals. Dithiothreitol was obtained from Research Organics. Phosphoenolpyruvate, pyruvate kinase, ATP, b-phenethyl alcohol, and NADH were purchased from Sigma. Restriction endonucleases EcoRI, PstI, and SmaI were obtained from New England Biolabs.

**Bacteriophage M13 DNA Substrates—**Circular single-stranded and supercoiled circular duplex DNAs from bacteriophage M13mp8 (7229 bp) were prepared using previously described methods (26–28). Except where specifically noted otherwise, full-length linear duplex DNA was generated by the digestion of M13mp8 supercoiled bacteriophage DNA with the EcoRI restriction endonuclease, using conditions suggested by the enzyme supplier. The digested DNA was extracted with phenol/ chloroform/isooamyl alcohol (25:24:1), followed by ethanol precipitation. The concentrations of ssDNA and dsDNA solutions were determined by absorbance at 280 nm, using 66 and 50 μg ml^−1 A₂₈₀, respectively, as conversion factors. All DNA concentrations are given in μM nucleotides.

**DNA Three-strand Exchange Reactions Promoted by the Wild-type and Deletion Mutant Proteins—**Three-strand exchange reactions were carried out in 25 mM Tris-OAc buffer (80% cation) or 25 mM MES buffer (33% anion) (reaction pH 7.3 and 6.0, respectively, after the addition of all reaction components). 1 mM dithiothreitol (5% (v/v) glyc erol), 3 mM potassium glutamate, and the indicated concentration of Mg(OAc)₂. Reactions also contained an ATP regeneration system of 10 units/ml pyruvate kinase and 3.1 mM phosphoenolpyruvate. All incubations are carried out at 37 °C. The wild-type RecA, RecAAC6, RecAAC13, or RecAAC17 proteins (6.7 μM) were preincubated with 20 μM M13mp8 circular ssDNA for 10 min. SSB protein (2 μM) and the indicated amount of ATP were then added, followed by another 10-min incubation. The reactions were initiated by the addition of M13mp8 linear dsDNA to 20 μM. A 10-μM aliquot was removed to use as a zero time point, the reaction was incubated, and at the indicated time points 10-μl
 aliquots were removed and the reaction was stopped by the addition of 5 μl of a solution containing 60 mM EDTA, 6% SDS, 25% (w/v) glycerol, and 0.2% bromphenol blue. Samples were subjected to electrophoresis at 10–20 mA in 0.8% agarose gels with 1× TAE buffer (40 mM Tris-OAc, 80% cation, and 1 mM EDTA), stained with ethidium bromide, and exposed to ultraviolet light. Gel images were captured with a digital CCD camera utilizing GelExpert software (Nucleotech). DNA bands were quantitated with the software package TotalLab version 1.10 from Phoretix.

**RESULTS**

**Experimental Design**—The purpose of this study was to examine the magnesium ion dependence of DNA strand exchange reactions promoted by RecA C-terminal deletion mutants. We focused on the RecΔC6, RecΔC13, and RecΔC17 proteins described in the previous paper (23). Under the standard reaction conditions for DNA strand exchange promoted by wild-type RecA protein, the C-terminal deletion mutants promote a significantly slower reaction (23). We set out to find reaction conditions that improve the efficiency of the mutant protein-catalyzed DNA strand exchange in an attempt to understand the role of the negatively charged C terminus in the RecA-promoted DNA strand exchange reaction.

The RecA C-terminal Deletion Mutants Require Less Magnesium Ion for Optimal Strand Exchange than the Wild-type RecA Protein—In an attempt to find the optimal strand exchange conditions for the C-terminal deletion mutants, magnesium titrations were carried out. DNA strand exchange reactions were carried out in Tris-OAc buffer (reaction pH 7.3) at 3 mM ATP and magnesium ion concentrations from 0 to 40 mM (Fig. 2). For each protein, the final extent of strand exchange increases to an optimum and then decreases as the concentration of magnesium ion is increased. For the wild-type RecA protein, this optimum occurs at about 10 mM magnesium ion, in line with many results published over a period of two decades (4–6). Significantly, the deletion lacking 17 amino acids (RecΔC17) exhibits optimal activity at magnesium ion concentrations that are now roughly equivalent to the ATP concentration. Higher magnesium ion concentrations are inhibitory. The optimal magnesium ion concentrations observed for the RecΔC6 and RecΔC13 mutants fall between the other two, so that the magnesium ion requirements for the reaction decline as more of the C terminus is removed. When reactions are compared at their respective optima, strand exchange reactions progress to similar extents for all of the wild-type and mutant RecA proteins.

Just as the magnesium ion concentration needed for an optimal reaction declines as the C terminus of RecA is truncated, the magnesium ion concentrations needed to see inhibition of the strand exchange reaction also decline. At magnesium ion concentrations above 15 mM, strand exchange products by the wild-type RecA protein include large DNA complexes that do not readily enter the gel. These large complexes are protein-dependent (data not shown), are not resolved in 120 min, and appear at successively lower magnesium ion concentrations for the C-terminal deletion mutants.

The yield of DNA strand exchange products produced by each protein after 60 min, at magnesium ion concentrations between 0 and 10 mM was quantitated (Fig. 3). The products in this experiment include the bands corresponding to the complex species in the well, joint molecules, and nicked circular products of DNA strand exchange. These were totaled and divided by the amount of all dsDNA (the above bands plus the linear dsDNA substrate) in the lane. The bands at the well were included in the quantitation because they are protein-dependent and because they are needed to account for all of the DNA in the lane. The magnesium ion concentration required for maximum conversion of substrate DNA into these products is ~10 mM for wild-type RecA, as has been reported by several laboratories (4–6). As amino acid residues are removed from the carboxyl terminus, the mutant proteins promote an optimal DNA strand exchange reaction at progressively lower concentrations of magnesium ion (Fig. 3).

**Fig. 2. Magnesium ion effects on DNA strand exchange reactions promoted by the wild-type RecA, RecΔC6, RecΔC13 and RecΔC17 proteins.** Shown at the top is a linear sequence representation that highlights the C-terminal region of RecA protein. The core domain, which includes the P-loop (ATP binding motif), is shown in white. The shaded and black regions of the sequence correspond to the N- and C-terminal domains, respectively. The primary structures of the C-terminal 17 amino acids of the RecA protein is diagrammed below the linear sequence. The hexagons highlight the high concentration of negatively charged amino acids in this region. The arrows indicate points of truncation in the deletion mutants: RecΔC6, RecΔC13, and RecΔC17. Reactions were carried out as described under “Experimental Procedures” and contained RecA protein or RecA mutant protein (each at 6.7 μM) and 3 mM ATP. The substrates of the reaction are circular ssDNA (S1, 20 μM) and linear duplex DNA (S2, 20 μM). The intermediates of the reaction are joint molecules (I), and the final products are nicked circular duplex molecules (P). The reactions were carried out at pH 7.3 and with the concentration of Mg(OAc)2 indicated.

The RecΔC17 Deletion Mutant Promotes an Optimal DNA Strand Exchange Reaction When the Magnesium Ion Concentration Is Approximately Equal to the ATP Concentration—In order to determine whether the RecΔC17 deletion mutant’s optimal strand exchange conditions were dependent on the ATP concentration, strand exchange reactions were carried out at several ATP concentrations (1, 3, and 6 mM), with the reaction extent examined as a function of magnesium acetate con-
Low Magnesium Ion Concentrations

...type RecA Protein-promoted DNA Strand Exchange Reaction at the concentration of ATP. Served at a magnesium ion concentration that closely parallels the protein, the optimal DNA strand exchange reaction is observed at 3 mM. This result appeared to contradict not only the terminal deletion mutants are plotted as a function of Mg(OAc)$_2$ concentration. The quantitation was carried out as described under "Experimental Procedures."

A 3' Overhang on the dsDNA Substrate Enhances the Wild-type RecA Protein-promoted DNA Strand Exchange Reaction at Low Magnesium Ion Concentrations—One of the results in Fig. 2 conflicts with a recently published result from our laboratory (29), in which the wild-type RecA protein promoted the formation of significant levels of nicked circular product in DNA strand exchange reactions throughout a range of 1–11 mM Mg(OAc)$_2$ (although the optimal reaction was still seen with 11 mM Mg$^{2+}$, the reaction was significant but much reduced at 1 mM). As in many of the current trials, the ATP concentration was 3 mM. This result appeared to contradict not only the results above, but also other published results indicating that wild-type RecA protein does not promote DNA strand exchange reactions at low magnesium ion concentrations (7). We investigated this apparent inconsistency in results. The DNA strand exchange reaction results in Fig. 6 address this issue. The DNA substrates utilized in the Rice et al. study were derived from dX174 bacteriophage, and the linear dsDNA substrate was generated by digestion of circular dsDNA with the PstI endonuclease (29). This treatment generates 3' overhangs at the DNA ends, which are distinct from the 5' overhangs in the duplex DNA substrates used in Figs. 2–5. To determine whether the overhangs play a role in the reaction, we carried out DNA strand exchange reactions with M13mp8 linear dsDNA substrates generated by circular dsDNA digestion with the restriction enzyme PstI, EcoRI, or SmaI to generate 3' overhangs, 5' overhangs, or blunt ends, respectively (Fig. 6). PstI and EcoRI both leave 4-nucleotide overhangs. At 10 mM magnesium ion, the results of wild-type RecA reactions using the different linear dsDNA ends are virtually indistinguishable, whereas at 3 mM magnesium ion, appreciable amounts of the nicked circular final product of strand exchange can be seen only when 3' overhangs are utilized. We note that the wild-type mediated reaction with the EcoRI-cleaved DNA (5' overhangs) did result in more reaction intermediates than the blunt-ended DNA, although they were not converted to quantitatable final products. The 5' overhangs would be complementary to the circular ssDNA, and thus enhance DNA pairing, at the distal end of the linear duplex. The RecAΔC17 protein appears to not require any particular end, regardless of the magnesium ion concentration.
The RecAΔC17 Deletion Mutant Requires Low Magnesium Ion for Joint Molecule Formation with ATPγS—DNA strand exchange (with long DNA substrates) generally does not proceed past the formation of joint molecules when ATP is not hydrolyzed. However, the initiation of DNA pairing in the presence of ATPγS should occur in a manner similar to the more extended DNA strand exchange. As is the case for the DNA strand exchange reaction with ATP, the optimal conditions for RecA protein-promoted formation of joint molecule intermediates in the presence of ATPγS include magnesium ion concentrations in significant excess relative to the ATPγS that is present (30). We examined the effect of DNA overhangs on both wild-type RecA and RecAΔC17-promoted DNA strand exchange reactions. The linear dsDNA substrates described above for Fig. 6 were used, with 3 mM ATPγS and either 3 or 10 mM Mg(OAc)₂ (Fig. 7). At a magnesium ion concentration of 10 mM, joint molecules formed by wild-type RecA, using the different linear dsDNA ends, are virtually indistinguishable. However, at 3 mM Mg(OAc)₂, appreciable amounts of joint molecule products can be seen only when 3′ overhangs, 5′ overhangs, or blunt ends, respectively. PstI and EcoRI both leave 4-nucleotide overhangs. The labels on the gel are described in the legend to Fig. 2.

In contrast, RecAΔC17 appears to not require any particular end at 3 mM magnesium, but it is unable to form joint molecules at 10 mM Mg(OAc)₂. The absence of observable joint molecules in this reaction at the higher concentration of magnesium ion, although a more complete DNA strand exchange reaction (albeit somewhat suboptimal) is seen under these conditions, is taken up under “Discussion.”

RecAΔC17 Protein Is Unable to Promote Complete DNA Strand Exchange Reactions with ATP or Promote Joint Molecule Formation with ATPγS at pH 6, Even at Low Magnesium Ion Concentrations—In an accompanying paper (23) characterizing the various C-terminal deletion mutants of the RecA protein, we showed that the mutants were deficient in their ability to generate nicked circular products in DNA strand exchange reactions carried out at pH 6. In light of the results above showing that the optimal magnesium ion concentration for the RecAΔC17 protein-promoted reaction is ~3 mM, we tested the lower magnesium ion concentrations in DNA strand exchange reactions with ATP or ATPγS at pH 6 (Fig. 8). The RecAΔC17 protein is unable to promote a complete strand exchange reaction with ATP or promote joint molecule formation with ATPγS at pH 6, even at 3 mM magnesium ion. Thus, the effects of magnesium ion are distinct from the pH effects noted elsewhere (23), at least by this criterion. However, the wild-type RecA reaction at low magnesium is somewhat stimulated by the decreased pH.

The RecA E343K Point Mutant Requires Less Magnesium Ion for Optimal Strand Exchange than the Wild-type RecA Protein—To begin to determine whether the negatively charged residues of the RecA C terminus are contributing to the requirement for excess magnesium in wild-type RecA-mediated DNA strand exchange reactions, we constructed a mutant that replaces the acidic glutamate residue at position 343 with a basic lysine residue (E343K). Position 343 lies in the protein segment that is deleted in the RecAΔC13 mutant but is present in the RecAΔC6 truncation mutant (see Fig. 2). DNA strand exchange reactions were carried out with wild-type RecA and the RecA E343K mutant at 3 mM ATP and magnesium ion concentrations from 1 to 15 mM (Fig. 9). The E343K mutant promotes the strand exchange reaction at lower magnesium ion concentrations than the wild-type protein. Using the criteria described for the deletion mutants above, it appears that the optimal reaction occurs for the E343K mutant at about 5–8 mM Mg²⁺, similar to that of RecAΔC6 protein. This experiment was carried out twice with consistent results. Converting one of the Glu residues in the C terminus to a positively charged Lys residue thus has an effect similar to the deletion of 6 residues from the C terminus.

**DISCUSSION**

The primary conclusion of this study is that the C terminus of the RecA protein modulates the protein’s DNA strand exchange activity. The last 17 amino acid residues are responsible for the observed requirement for excess magnesium ion, above that necessary to form complexes with ATP, in a wild-type RecA protein-promoted DNA strand exchange reaction in vitro (31–34). Removal of these residues eliminates the measurable requirement for the excess Mg²⁺. These results strongly suggest that the RecA C terminus has a regulatory role in RecA protein activity. At low magnesium ion concentrations, the C
A change in state is needed to facilitate DNA pairing and strand exchange at low Mg\(^{2+}\) concentrations.

We propose that the state of RecA protein, bound to ssDNA at low Mg\(^{2+}\) (or Mg\(^{2+}\) levels commensurate with the ATP present) be designated Ac. The status of RecA on single-stranded DNA in the presence of ATP has historically been referred to as activated (40-42), hence the “A.” The state present at low Mg\(^{2+}\) is relatively closed to interaction with incoming duplex DNA, hence the “c.”

A change in state is needed to facilitate DNA pairing and strand exchange. In most studies carried out with the E. coli RecA protein, the change is brought about by adding Mg\(^{2+}\) in excess to the ATP concentration. We propose that the resulting
RecA protein with 1 mM magnesium ion and 0.5 or 1 mM ATP require excess magnesium ion to bind to ssDNA. The rates of data have suggested that the wild-type RecA protein does not nucleoprotein filaments that seem to be most affected. Previous stages, but it is the stages after the formation of RecA-ssDNA to overcome the barrier and allow some DNA pairing to occur. Magnesium ion could affect DNA strand exchange at many stages, but it is the stages after the formation of RecA-ssDNA nucleoprotein filaments that seem to be most affected. Previous data have suggested that the wild-type RecA protein does not require excess magnesium ion to bind to ssDNA. The rates of ssDNA-dependent ATP hydrolysis catalyzed by the wild-type RecA protein with 1 mM magnesium ion and 0.5 or 1 mM ATP and no SSB protein are close to the rates measured at 10 mM magnesium in the presence of SSB protein (43, 44). Given the similarities in ssDNA binding and ATP hydrolysis, the structural differences between the proposed Ac and Ao states may be subtle. The key functional distinction is that the low Mg\(^{2+}\) conditions do not support initiation of a robust DNA strand exchange reaction.

There is additional evidence in the literature that Mg\(^{2+}\) concentrations affect RecA conformation. RecA-ssDNA nucleoprotein filaments have been observed in the electron microscope in the presence of 1 mM magnesium. Notably, the contour lengths of these filaments were measured to be 116–120% relative to duplex DNA in the presence of 1 mM ATP\(\gamma\)S (7) and 137% relative to duplex DNA in the presence of 1.3 mM ATP (45), which translates to less filament extension than the >150% extension observed when the filaments are formed with 10 mM magnesium ion (23, 46, 47). It is possible that this reduced filament extension seen at low Mg\(^{2+}\) concentrations is a result of an inhibitory conformation of the C terminus. Elevated Mg\(^{2+}\) levels also result in the formation of RecA filament bundles observable by electron microscopy (46, 48, 49). Finally, with elevated Mg\(^{2+}\) levels, RecA protein better resists displacement by SSB (50).

The RecA24C17 protein exhibits a substantially altered pH reaction profile in DNA strand exchange reactions (23). We have determined that this pH dependence is not affected by magnesium, since, at pH 6, the RecA24C17 protein is deficient in homologous pairing with ATP\(\gamma\)S and in the formation of nicked circular product with ATP at low (Fig. 8) or high magnesium levels (23).

Combining the data from this study and the previous paper (23) allows us to refine the protein flap model for the function of the C-terminal domain of RecA protein. We propose that the C-terminal amino acid residues of wild-type RecA protein are inhibitory not only to the primary binding of RecA protein to establish a filament on dsDNA (as previously proposed) (17, 18) but also to DNA pairing of duplex DNA with a single strand bound within a RecA filament. These may be quite distinct processes, since the barrier to direct binding of RecA protein monomers to a duplex DNA should be different from the binding of a duplex DNA to a RecA filament already formed on a single strand. The barrier to direct binding of RecA protein to dsDNA is overcome at pH 6 with wild-type protein, presumably reflecting the protonation of one or more residues in the C terminus, but is not overcome by excess magnesium ion at neutral pH values. This again indicates that the pH- and magnesium ion-mediated changes in protein state are to some degree distinct, albeit both involve the C terminus in some way.

Kowalczykowski and colleagues (18) previously proposed that the C-terminal domain had a role in modulating DNA assimilation and strand exchange. However, their more substantial deletion led to an enhancement of DNA strand exchange (with no SSB present) under conditions similar to those in which the deletions studied here decrease the efficiency of strand exchange.

Shibata and colleagues (51) have put forth a model for homologous pairing in which the “gateway” for dsDNA binding to the presynaptic filament lies in the filament groove that is made up of, on one side, the C-terminal domain of the RecA protein. Mutations of some of the many basic residues in this cleft have been shown to abolish homologous pairing. The residues involved include Arg\(^{243}\), Lys\(^{245}\) (52, 53), Lys\(^{296}\), and Lys\(^{302}\) (51) (Fig. 10). These residues all lie within 20 Å of the last residue of the RecA protein seen in the apoenzyme crystal structures, Leu\(^{328}\) (14, 15). There are 24 C-terminal residues (including the seven negative charges that we have removed in the current study) that were disordered in those structures. It is possible that the negative charges of the C terminus can form salt bridges with basic residues in this cleft, thereby restricting the access of dsDNA to the presynaptic filament. These interactions may be part of a network of surface salt bridges. A network would help to explain the gradual reduction in the requirement for magnesium seen with the progressive removal.
in the negative charges of the C terminus. Each salt bridge disruption could affect the strength of the next. Such a network would explain the effects of the RecA E343K mutant, which exhibits a behavior similar to RecAΔC6 although that mutation is not in the region removed with RecAΔC6. Magnesium could act by disrupting those salt bridges, enabling homologous pairing. In this way, the C-terminal tail of the RecA protein could form a flap that regulates accessibility to the nucleoprotein filament. The coupling of protein surface salt bridge disruption to DNA binding is a common mechanism used by DNA-binding proteins and has been reviewed recently (54).

As mentioned in the Introduction, the levels of free Mg\(^{2+}\) available in the cell are insufficient to bring about the activation seen in vitro. It is possible that the molecular crowding in the cell substitutes for the effects of high Mg\(^{2+}\) levels (12). Alternatively, another molecule may replace Mg\(^{2+}\) in the cell as an activating agent.

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REFERENCES

1. Lusetti, S. L., and Cox, M. M. (2002) Annu. Rev. Biochem. 71, 71–100
2. Cox, M. M. (1999) Prog. Nucleic Acids Res. Mol. Biol. 63, 310–366
3. Phillips, R. C., George, P., and Rutman, R. J. (1966) J. Am. Chem. Soc. 88, 2621–2640
4. Cox, M. M., and Lehman, I. R. (1982) J. Biol. Chem. 257, 8523–8532
5. Shiba, T., Das Gupta, C., Cunningham, R. P., and Radding, C. M. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1658–1642
6. Roman, L. J., and Kowalczykowski, S. C. (1986) Biochemistry 25, 7375–7385
7. Honigberg, S. M., Gonda, D. K., Flory, J., and Radding, C. M. (1985) J. Biol. Chem. 260, 11845–11851
8. Kuhn, A., and Kellenberger, E. (1985) J. Bacteriol. 163, 906–912
9. Kuhn, A. H., Jutte, H., and Kellenberger, E. (1985) J. Bacteriol. 162, 413–419
10. Lavery, P. E., and Kowalczykowski, S. C. (1992) J. Biol. Chem. 267, 9307–9314
11. Kowalczykowski, S. C., and Eggleston, A. K. (1994) Annu. Rev. Biochem. 63, 991–1043
12. Story, R. M., Weber, T. S., and Steitz, T. A. (1992) Nature 355, 318–325
13. Story, R. M., and Steitz, T. A. (1992) Nature 355, 374–376
14. Datta, S., Prabhu, M. M., Vase, M. B., Ganesh, N., Chandra, N. R., Munipappa, K., and Vijayan, M. (2000) Nucleic Acids Res. 28, 4961–4973
15. Tuteshi, S., Hiroi, T., Ogawa, T., and Ogawa, H. (1992) J. Biol. Chem. 267, 115–129
16. Benedict, R. C., and Kowalczykowski, S. C. (1988) J. Biol. Chem. 263, 15513–15520
17. Ruse, R. J., Konigsberg, W., and Howard-Flanders, P. (1985) J. Biol. Chem. 260, 949–955
18. Yarranton, G. T., and Sedgwick, S. G. (1982) Mol. Gen. Genet. 185, 99–104
19. Larrinu, F., and Defais, M. (1989) Mol. Gen. Genet. 216, 106–112
20. Yu, X., and Egelman, E. H. (1991) J. Struct. Biol. 106, 243–254
21. Lusetti, S. L., Wood, E. A., Fleming, C. D., Modica, M. J., Korth, J., Abbott, L., Dwyer, D. W., Roca, A. I., Inman, R. B., and Cox, M. M. (2003) J. Biol. Chem. 278, 16372–16380
22. Craig, N. L., and Roberts, J. W. (1981) J. Biol. Chem. 256, 8039–8044
23. Lehman, T. M., and Overman, L. B. (1985) J. Biol. Chem. 260, 3594–3603
24. Davis, R. W., Botstein, D., and Roth, J. R. (1988) Advanced Bacterial Genetics, pp. 70–82, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Messing, J. (1983) Methods Enzymol. 101, 20–78
26. Neuenfeld, S. K., and Cox, M. M. (1966) J. Biol. Chem. 261, 8276–8282
27. Rice, K. P., Egelger, A. L., Sung, P., and Cox, M. M. (2001) J. Biol. Chem. 276, 38570–38581
28. Menetski, J. P., Bear, D. G., and Kowalczykowski, S. C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 21–25
29. Cox, M. M., Solits, D. A., Livneh, Z., and Lehman, I. R. (1983) J. Biol. Chem. 258, 2577–2585
30. Flory, S. S., Tsang, J., Munipappa, K., Bianchi, M., Gonda, D., Kahn, R., Ashderian, E., Eger, C., Shaner, S., and Radding, C. M. (1984) Cold Spring Harbor Symp. Quant. Biol. 49, 513–523
31. Tsang, S. S., Chow, S. A., and Radding, C. M. (1985) Biochemistry 24, 3226–3232
32. Morrical, S. W., Lee, J., and Cox, M. M. (1986) Biochemistry 25, 1482–1494
33. Wedekind, J. E., Poyner, R. R., Reed, G. H., and Raymont, I. (1994) Biochemistry 33, 9333–9342
34. Millburn, M. V., Tong, L., deVos, A. M., Brunger, A., Yamaizumi, Z., Nishimura, S., and Kim, S. H. (1990) Science 247, 959–945
35. VanLoock, M. S., Yu, X., Yang, S., Lai, A. L., Low, C., Campbell, M. J., and Egelman, E. H. (2003) Structure 11, 1–20
36. Yu, X., Jacobs, S. A., West, C. O., S., T., Egelman, E. H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8419–8424
37. Flory, S. S., Lusetti, S. L., and Cox, M. M. (2003) J. Biol. Chem. 278, 16389–16396
38. Roberts, J. W., Roberts, C. W., and Craig, N. L. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4714–4718
39. Sassanfar, M., and Roberts, J. W. (1990) J. Mol. Biol. 212, 79–96
40. Shinagawa, H. (1996) EXS 77, 231–235
41. Kowalczykowski, S. C., Clay, J., Somani, R., and Varghese, A. (1987) J. Mol. Biol. 193, 81–95
42. Morrical, S. W., and Cox, M. M. (1990) Biochemistry 29, 837–843
43. Flory, J., Tsang, S. S., and Munipappa, K. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7026–7030
44. Egelman, E. H., and Stasiak, A. (1986) J. Mol. Biol. 191, 677–697
45. Yu, X., and Egelman, E. H. (1992) J. Mol. Biol. 227, 334–346
46. Egelman, E. H., and Stasiak, A. (1988) J. Mol. Biol. 200, 329–349
47. Yu, X., and Egelman, E. H. (1992) J. Mol. Biol. 235, 193–216
48. Kowalczykowski, S. C., and Krupp, R. A. (1987) J. Mol. Biol. 193, 97–113
49. Kurumizaka, H., Aihara, H., Ikawa, S., Kashiya, T., Bassere, L. R., Kawasaki, K., Sarai, A., Radding, C. M., and Shibata, T. (1996) J. Biol. Chem. 271, 33515–33524
50. Kurumizaka, H., Ikawa, S., and Shibata, T. (1999) Arch. Biochem. Biophys. 365, 83–91
51. Kurumizaka, H., Aihara, H., Ikawa, S., and Shibata, T. (2000) FEBS Lett. 477, 129–134
52. Saecker, R. M., and Record, M. T. (2002)Curr. Opin. Struct. Biol. 12, 311–319