recA Protein Filaments Bind Two Molecules of Single-stranded DNA with Off Rates Regulated by Nucleotide Cofactor

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To probe the role of nucleotide cofactor in the binding of single-stranded DNA to recA protein, we have developed a sedimentation assay using 5'-labeled [32P]-poly(dT). recA-poly(dt) complexes sediment quantitatively when centrifuged at 100,000 × g for 45 min, whereas free poly(dT) remains in the supernatant. In the absence of ATP, between 6 and 7 bases cosediment per recA monomer; but when ATP is present or in the absence of added nucleotide cofactor, only 3–3.5 bases/recA monomer cosediment. In competition experiments in which recA-[32P]-poly(dt) complexes are incubated with unlabeled poly(dt), we again find 3–3.5 bases of labeled poly(dt) cosedimenting per recA monomer when no nucleotide cofactor is present. However, when the same experiment is performed with ATP, only half of the expected 6–7 bases of labeled poly(dt) remain bound to the DNA, demonstrating that half of the poly(dt) in the complex exchanges rapidly with free poly(dt), whereas the other half elutes slowly, like poly(dt) in the absence of nucleotide. The rate of exchange of the second more tightly bound poly(dt) is accelerated when ADP is present. Our observations are rationalized by a model in which each recA protein helical filament binds two strands of poly(dt) with a stoichiometry of 3–3.5 bases/recA monomer/strand.

The role of recA protein from Escherichia coli in homologous recombination has been studied extensively (1, 2). In strand exchange reactions in vitro, recA protein initially binds cooperatively to ssDNA, and this nucleoprotein filament then aligns with a homologous region of duplex DNA in a process called synapsis. recA protein then catalyzes displacement of the homologous strand of the duplex in an ATP-dependent reaction with concomitant replacement by the recA protein-bound ssDNA to create a new DNA hybrid. The ternary complex of recA protein, ssDNA, and double-stranded DNA has been observed by electron microscopy (3–6).

The mechanistic details of the homology search and strand exchange reactions remain a focus of current research efforts. Essential information for any mechanistic model includes the stoichiometry, affinity, and cooperativity of recA-recA and recA-DNA interactions. Although numerous attempts to extract this information have been made (see, for example, Refs. 1 and 2), there are still unresolved discrepancies in the observed site size and state of oligomerization of recA protein prior to binding DNA (6–8) that make quantitative analysis equivocal (see, for example, Refs. 9 and 10).

The DNA binding stoichiometry has been determined by electron microscopy (11), filter binding (12), titration monitoring of the DNA-dependent ATPase of recA protein (13, 14), titration monitored via fluorescently modified ssDNA (9, 10, 14, 15), and linear flow dichroism (15). Observed stoichiometries fall in two ranges: 3–4 and 6–8 DNA bases/recA monomer. Bryant et al. (12) first suggested that these different stoichiometrics imply that recA protein might bind two strands of ssDNA under some conditions.

We have developed a sedimentation assay to assess directly DNA binding by recA protein. The results indicate that recA protein filaments indeed bind two strands of ssDNA under usual in vitro conditions. Moreover, we find that nucleotide cofactor has a pronounced effect on the rate at which both strands of bound DNA exchange with exogenous DNA.

MATERIALS AND METHODS

Protein—RecA protein was purified from E. coli strain G6645 (a gift of George Weinstock, University of Texas Medical School, Department of Biochemistry and Molecular Biology, Houston, TX) by a modification of the method of Griffith and Shores (16) as described (6). recA protein concentration was determined using an ε200 of 23,200 M−1 cm−1.

DNA—Single-stranded DNA from M13 phage was prepared by standard procedures (3). Poly(dT) (P-L Biochemicals) was labeled at the 5'-end by dephosphorylation with calf intestinal alkaline phosphatase (Boehringer Mannheim) and rephosphorylation with [γ-32P] ATP (Du Pont-New England Nuclear) using T4 polynucleotide kinase (U. S. Biochemical Corp.). After removal of proteins and free [γ-32P]ATP by chromatography on a Nensorb column (Du Pont-New England Nuclear), the end-labeled poly(dT) was diluted with unlabeled poly(dt) and size-fractionated on a Sepharose 6B column (Du Pont-New England Nuclear), the end-labeled poly(dT) was diluted with unlabeled poly(dt) and size-fractionated on a Sepharose 6B column (Du Pont-New England Nuclear). The size range of the labeled poly(dt) was 300–600 bases, and its initial specific activity varied from 7 to 20 × 106 cpm/mmol.

All ssDNA concentrations were determined using an ε200 of 8520 M−1 cm−1 (17) for poly(dt) and of 9350 M−1 cm−1 (18) for M13 ssDNA.

Buffers—Experiments were performed in 10 mM HEPES, 30 mM NaCl, 12 mM MgCl2 (HNM buffer) with or without added nucleotides (5 mM ADP, 5–10 mM ATP (Sigma), or 0.2 mM ATPγS (Boehringer Mannheim)) to allow comparison to typical conditions for in vitro strand exchange and DNA binding reactions.

Sedimentation Assay—For sedimentation of 5'-labeled recA protein, a 600 µl sample of 92 recA protein at the desired concentration in HNM buffer was prepared. A 50-µl aliquot was taken and assayed for radioactivity. Buffer or 100 µM M13 ssDNA (total volume of 6.75 µl) was added to 200 µl of the recA protein solution, and 175-µl portions were placed in 200-µl Airfuge tubes (Beckman Instruments). The tubes were incubated for 5 min at room temperature and then spun at 100,000 × g for 30 min. Nonsedimented recA protein was removed by airfuge.
determined by assaying the radioactivity in a 100-µl aliquot of the supernatant.

For experiments using 32P-poly(dT), all samples were made to a final volume of 250 µl. A stock solution of recA protein in HN buffer, with or without nucleotide, was placed in a microcentrifuge tube and mixed with water and poly(dT) to bring it to volume. The 32P-poly(dT) was added in steps of 3-6 µM, with stirring, to the desired concentration. We found it necessary to add the DNA in small aliquots to allow nucleation and growth of a minimal number of recA protein clusters. If the DNA was added in a single aliquot, it sedimented anomalously presumably due to the formation of smaller protein clusters widely dispersed on the DNA. A 175-µl portion was centrifuged at room temperature in a Beckman Airfuge at 100,000 x g. Samples (50 µl) of the supernatant before and after centrifugation were assayed for radioactivity. The maximum total handling time for a recA/ATP/DNA mixture, including centrifugation, was 2 h.

In some experiments, a low-speed sedimentation assay (19) was used in which samples, prepared as described above, were centrifuged in a microcentrifuge (Fisher) at ~12,000 x g for 15 min at room temperature.

RESULTS

To determine the recA-poly(dT) binding stoichiometry, we attempted to separate recA-ssDNA complexes from unbound species using centrifugation and either monitoring labeled protein (Fig. 1) or labeled ssDNA (Fig. 2, upper). In the absence of DNA, recA protein exhibited concentration-dependent assembly into sedimentable aggregates (Fig. 1 and see Ref. 6). The addition of poly(dT) did, as expected for a complex, cause an increase in the amount of recA protein sedimentable at low protein concentrations; but the difference between the sedimentation curves in the presence and absence of poly(dT) was not sufficiently sensitive to measure the stoichiometry of the complex.

More satisfactory results were obtained using poly(dT) labeled at the 5'-end with 32PO4 (Fig. 2, upper). The poly(dT) was size-fractionated after end labeling (see "Materials and Methods"), and only material in the range of 300-600 bases was used for the sedimentation experiments. In the absence of recA protein, all of the poly(dT) remained in the supernatant after centrifugation at 100,000 x g for 45 min. In the presence of recA protein and ATP,2 the sedimentation curve as a function of poly(dT) concentration was biphasic. Essentially all of the DNA sedimented below a critical value of the poly(dT) concentration. Above that critical value, the additional DNA was all found in the supernatant. The amount of DNA bound at saturation by a given amount of recA protein was determined as described in the text from data such as that shown in the upper portion (see also Table I).

2 The ATP concentration in these experiments was chosen to ensure that, assuming ATP hydrolysis at the maximum reported rate for recA protein (30 molecules of ATP hydrolyzed per recA monomer/min at 37 °C (17)), more than 60% of the initial ATP remained at the end of an experiment. There was no difference in the site size in sedimentation experiments with 1 µM recA protein and 5 or 10 mM ATP.
is thus simply determined by measuring the difference between points on the binding curve and the standard curve above the critical protein concentration, or equivalently by extrapolating the experimental binding curve back to zero DNA in the supernatant. At a recA protein concentration of 1 μM, we found that 5.8 ± 0.2 μM poly(dT) bound at saturation, giving a site size of 5.8 bases/recA monomer (see Table I). Similar site sizes of 6.3 ± 0.2 and 6.5 ± 0.1 bases were determined for recA protein concentrations of 2.0 and 3.0 μM, respectively (Fig. 2, upper, and Table I). The small but systematic increase in site size with protein concentration was investigated in more detail (Fig. 2, lower). The result shows that the correct site size can only be obtained by carrying out the experiment at protein concentrations above the apparent Kd for the interaction.

Similar experiments were carried out with 32P-poly(dT) in the absence of added nucleotide or in the presence of 5 mM ADP (Fig. 3, upper), and site sizes were determined as described above (Table I). The site size in both cases was approximately half of that seen with ATP: 3.3 ± 0.3 bases/recA monomer in the absence of added nucleotide and 2.9 ± 0.5 bases/recA monomer in the presence of ADP. As shown in Fig. 3 (lower), the smaller site size was not the result of carrying out the experiment at too low a recA protein concentration. A long incubation of recA protein and poly(dT) with ATP prior to centrifugation, in which a large fraction of the ATP (>40%) had been hydrolyzed to ADP, yielded results identical to experiments performed with ADP (data not shown). In Fig. 4, it is shown that the lower stoichiometry does not increase with longer centrifugation. Likewise, in the presence of ATP, the stoichiometry of ~3-3.5 bases/recA monomer was independent of centrifugation time beyond the minimum of 30 min at 100,000 × g necessary for complete pelleting of the complexes.

No recA-poly(dT) complexes, with either ATP or ADP or in the absence of nucleotide, were sedimentable in the low-speed microcentrifuge assay used to study recA-DNA network formation (19) (data not shown). We should note that much larger DNA substrates were used in those experiments. With poly(dT), large nucleoprotein networks, sedimentable at low speed, were formed in the presence of 0.2 mM ATP and 0.1 mM ADP. These complexes contained a nonstoichiometric amount of DNA (>7-10 bases/recA monomer), with an apparent site size that increased considerably with higher centrifugal force.

To test whether the DNA in recA-poly(dT) complexes can exchange with free DNA, recA 32P-poly(dT) complexes were preformed, and then unlabeled poly(dT) was added, and the samples were incubated for 5 min and centrifuged. As shown in Fig. 5, the amount of labeled poly(dT) sedimented in these experiments depended on the nucleotide present during the incubation. In the presence of ATP, the amount of 32P-poly(dT) sedimented decreased from ~6 to ~3 bases/recA monomer as the amount of unlabeled DNA was increased (Fig. 5, upper). On the other hand, when no nucleotide was present, ~3-3.5 bases of 32P-poly(dT) were sedimented independent of the amount of added unlabeled competitor poly(dT) (Fig. 5, lower). With ADP or no nucleotide cofactor (W), all other conditions were the same as those described for Fig. 2 (upper). Lower, the site size as a function of recA protein concentration in the absence of added nucleotide was determined from experiments such as shown in the upper portion. (The data for 5 mM ADP are not shown, but are described in Table I.)

![Fig. 3. Sedimentation of 32P-poly(dT) complexes with recA protein in presence of ADP or no nucleotide. Upper, the amount of 32P-poly(dT) in solution after sedimentation was observed with either 5 mM ADP instead of ATP (○) or no nucleotide cofactor (■); all other conditions were the same as those described for Fig. 2 (upper). Lower, the site size as a function of recA protein concentration in the absence of added nucleotide was determined from experiments such as shown in the upper portion. (The data for 5 mM ADP are not shown, but are described in Table I.)](http://www.jbc.org/)

| Table I |
|---|
| The recA-poly(dT) stoichiometry determined by centrifugation |
| [recA] Nucelotide stoichiometry<sup>a</sup> <br> μM bases/recA |
| ATP | 3.3 ± 0.3 (6) |
| ADP | 2.9 ± 0.5 (7) |
| None | 5.8 ± 0.2 (22) |
| ATP | 6.3 ± 0.2 (4) |
| ATP | 6.5 ± 0.1 (3) |

<sup>a</sup> When present, nucleotide concentrations were 5 mM.

<sup>b</sup> Stoichiometry was calculated from the amount of DNA sedimented by a fixed amount of recA protein when excess DNA was present. The parenthetical values are the number of experiments used to calculate the average.
bound 3–3.5 bases/recA monomer largely exchange on the time scale of this experiment (the solid curve represents the expected result if the recA protein-bound DNA were in equilibrium with exogenous DNA).

**DISCUSSION**

The difference between the apparent site size in the presence of ATP versus ADP (or absence of nucleotide) can be explained by a simple model in which each recA nucleoprotein filament can contain either one or two strands of poly(dT), with each strand having a stoichiometry of 3–3.5 bases/recA monomer. Two strands would be tightly bound in the presence of ATP, but with ADP (or no nucleotide) present, the second strand would be bound more weakly and lost from the complex during the sedimentation assay.

It has been hypothesized that recA protein or recA protein complexes must bind two strands of DNA based on models for the mechanism of strand exchange reactions (21) and on trying to rectify the two observed site sizes (1). The recent work of Muller et al. (5) has shown that recA protein filaments can bind two strands of nonhomologous DNA (single- or double-stranded) in the presence of the nonhydrolyzable ATP analog ATPγS. We have shown that recA protein filaments can bind two strands of poly(dT) in the presence of the naturally occurring ligands ATP and ADP or in the absence of nucleotide. Our competition results show that ATP hydrolysis provides a mechanism for differentially modulating the affinity of the recA protein filament for the two strands. This result is consistent with the observations of Bryant et al. (20) that nucleotide is not required for the initial strand pairing event, but accelerates the formation of DNA networks, probable intermediates in the search for complementary partners. Other recent studies using flow linear dichroism and fluorescence (15) and fluorescence in conjunction with recA protein-dependent ATPase activity (14) are consistent with a model of two DNA strands binding to recA protein. The presence of two DNA-binding sites/filament does not necessarily require two binding sites/recA monomer as in the model of Howard-Flanders et al. (21); it is equally plausible that the alternate DNA strands are bound to every other monomer in a filament. What is clear from our results is that two strands of poly(dT) are bound and exchange with free DNA at different rates. Therefore, either there are two distinct binding sites on the filament, or the binding of the first strand results in an allosteric change altering the second site.

Menetski and Kowalczykowski (22) have observed that binding assays based on recA protein ATPase and etheno-M13 ssDNA fluorescence give site sizes that differ by a factor of 2. The fluorescence assay gave the higher value of 7 ± 1 bases/recA monomer, in agreement with preliminary reports from our laboratory (14). Menetski and Kowalczykowski (22) rationalized these results by suggesting that each recA monomer has a binding site for ~7 bases of ssDNA, but that the ATPase of a second recA monomer can be activated by binding to the first recA protein filament-ssDNA complex, without direct interaction with the DNA. Our results strongly support an alternative model in which a single recA protein filament can be fully activated for ATP hydrolysis by the binding of a single strand of DNA, but that such an activated filament can bind a second strand of DNA, with both bound strands showing a fluorescence increase. Our data clearly demonstrate two distinct classes of binding sites that have differential rates of exchange with free DNA, strongly suggesting that there are two sites, each of which binds 3–3.5 bases, rather than a single site binding 6–7 bases. In addition, since dimeric recA protein filaments have never been observed...
via electron microscopy, since the helical nature of the protein filament does not suggest a likely dimer interface, and since evidence already exists for single recA protein helices containing both a single and a double strand of DNA (3-5), we favor the model of a single protein helix with two ssDNA-binding sites. The recent work of Muller et al. (5) shows that the ssDNA-binding sites can also bind duplex DNA.

Our data on the exchange of bound for exogenous DNA, interpreted with an emphasis on site size, may more fully explain the elegant kinetic experiments of Menetski and Kowalczykowski (23), who observed the exchange of recA protein-bound fluorescently labeled ssDNA with free unlabeled DNA. The exchange rates were found to be biphasic, with roughly half the DNA exchanging at the faster rate. Nucleotide cofactor was found to affect both fast and slow rates. The relative exchange rates were ranked as: $\text{Fast}_{\text{OF}} > \text{Fast}_{\text{ATP}} \approx \text{Slow}_{\text{ADP}} > \text{Slow}_{\text{ATP}}$, where the subscript refers to cofactor and $\phi$ is no nucleotide. We can now identify the slow kinetic component with the slowly exchanging DNA strand found bound to recA protein with or without ATP or ADP. The fast component corresponds to the fast exchanging DNA strand found only in the presence of ATP in the sedimentation assay. This is also consistent with the site size of 7 bases found using the fluorescence of ssDNA, where excess labeled DNA is in equilibrium with recA protein, independent of the cofactor (14).

It is now clear why previous measurements of recA-DNA binding stoichiometries gave variable results. In assays where the binding equilibrium is perturbed, such as sedimentation or filter binding followed by high salt washes to remove exogenous DNA (12), at least one or, with ADP, both strands of DNA may be removed from the nucleoprotein complex. Also, the analysis of recA-DNA binding curves is complex; not only does the protein oligomerize and bind cooperatively to DNA, it can do so to two different DNA lattices with different apparent affinities in a nucleotide-dependent manner. Even the direct application of such elegant formalisms as that of McGhee and Von Hippel (24) for cooperative binding of a protein to an infinite DNA lattice does not allow a simple disentanglement of the binding constants and cooperativities. A re-examination of the binding isotherms with a model including a second DNA lattice will be required before quantitative binding parameters can be deduced.
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