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Quantitative Analysis of the Kinetics of End-dependent Disassembly of RecA Filaments from ssDNA

Tanya A. Arenson[1], Oleg V. Tsodikov[2] and Michael M. Cox[1]*

[1]*Department of Biochemistry and*

[2]*Department of Chemistry University of Wisconsin-Madison, 433 Babcock Drive Madison, WI 53706, USA*

**Corresponding author*

On linear single-stranded DNA, RecA filaments assemble and disassemble in the 5′ to 3′ direction. Monomers (or other units) associate at one end and dissociate from the other. ATP hydrolysis occurs throughout the filament. Dissociation can result when ATP is hydrolyzed by the monomer at the disassembly end. We have developed a comprehensive model for the end-dependent filament disassembly process. The model accounts not only for disassembly, but also for the limited reassembly that occurs as DNA is vacated by disassembling filaments. The overall process can be monitored quantitatively by following the resulting decline in DNA-dependent ATP hydrolysis. The rate of disassembly is highly pH dependent, being negligible at pH 6 and reaching a maximum at pH values above 7.5. The rate of disassembly is not significantly affected by the concentration of free RecA protein within the experimental uncertainty. For filaments on single-stranded DNA, the monomer k_{cat} for ATP hydrolysis is 30 min^{-1}, and disassembly proceeds at a maximum rate of 60-70 monomers per minute per filament end. The latter rate is that predicted if the ATP hydrolytic cycles of adjacent monomers are not coupled in any way.

Keywords: RecA protein; filament; assembly; disassembly; kinetic analysis

Introduction

The bacterial RecA protein is critical to the processes of recombinational DNA repair, homologous genetic recombination, and the induction of the SOS response to DNA damage (Cox, 1998; Kowalczykowski & Eggleston, 1994; Roca & Cox, 1997). The *Escherichia coli* RecA protein is a polypeptide chain with 352 amino acid residues with a molecular mass of 37,842 Da. RecA is an ancient protein present in virtually all bacteria, with structural and functional homologues in all classes of organisms (Brendel *et al*., 1997; Roca & Cox, 1997).

In vitro, RecA protein promotes a set of DNA strand exchange reactions that mimic its presumed role in recombination and recombinational DNA repair. The active species in this reaction is a helical filament of RecA protein bound to DNA, formed as the first step in the process. There is one RecA monomer bound per three nucleotides or base-pairs of DNA, and six monomers per helical turn. Bound double-stranded DNA (dsDNA) is extended by 50 % and underwound to 18 bp per turn.

An understanding of filament assembly and disassembly is a prerequisite for a broader description of how RecA filaments form where they are needed for recombinational activities *in vivo*. Most models for recombination focus on the invasion of 3′ ends (Anderson & Kowalczykowski, 1997a; Dixon & Kowalczykowski, 1991; Meselson & Radding, 1975; Resnick, 1976; Smith, 1991; Szostak *et al*., 1983), in part because of their potential utility in priming DNA synthesis. A 3′ end bias in DNA pairing reactions has been demonstrated with bacterial enzymes *in vitro* (Dixon & Kowalczykowski, 1991; Dutreix *et al*., 1991; Konforti & Davis, 1991), attributed to the 5′ to 3′ polarity of RecA filament assembly (Register & Griffith, 1985; Shan *et al*, 1997) which would be expected to leave 3′ ends more uniformly coated with RecA protein than 5′ ends. In addition to its implications for recombination models, information about the assembly and disassembly is needed to provide a baseline for the

Abbreviations used: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; EDTA, ethylenediamine tetraacetic acid; OAc, acetate ion; SSB, the single-stranded DNA-binding protein of *E. coli*.

E-mail address of the corresponding author: cox@biochem.wisc.edu

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study of the activities of other proteins, such as the RecFOR proteins (Shan *et al.*, 1997; Umezu & Kolodner, 1994; Webb *et al.*, 1997) and RecBCD enzyme (Anderson & Kowalczykowski, 1997b), that affect RecA filament assembly and disassembly.

Filament assembly occurs in at least two major phases. Nucleation is generally rate limiting on all DNAs and under all conditions, and is followed by a rapid and unidirectional extension of the filament until the available RecA protein and/or contiguous DNA is exhausted (Madiraju *et al.*, 1992; Pugh & Cox, 1988; Roca & Cox, 1997; Shan *et al.*, 1997). On single-stranded DNA (ssDNA), nucleation is followed by the 5′ to 3′ extension of the filament (Register & Griffith, 1985; Shan *et al.*, 1997). On dsDNA, the same polarity is observed in the extension process, relative to the strand occupying the DNA binding site in the filament normally occupied by ssDNA (Lindsley & Cox, 1990). Nucleation occurs much faster on ssDNA than on dsDNA at neutral pH conditions. When nucleation occurs in a single-strand gap, the extension continues to envelop contiguous dsDNA in the path of the assembly reaction (Shan *et al.*, 1997; Webb *et al.*, 1997). On circular DNA substrates, filaments are generally quite stable as long as ATP is regenerated.

On linear ssDNA, end-dependent disassembly is observed (Shan *et al.*, 1997). Like assembly, disassembly occurs 5′ to 3′ on ssDNA, with monomers dissociating predominantly from the end opposite to that at which assembly occurs. Assembly and disassembly at opposite ends of filaments is also observed on dsDNA (Lindsley & Cox, 1990). The rate of disassembly from dsDNA is highly pH-dependent, with little dissociation observed at pH 6 and maximum rates observed above pH 7.5 (Lindsley & Cox, 1989). End-dependent disassembly from ssDNA has been documented only recently (Shan *et al.*, 1997), and has not yet been well characterized.

The net addition of RecA monomers to one end and their deletion from the other end in the same test tube has some thermodynamic implications. The monomer-monomer interfaces are presumably the same at either end (and everywhere else) in the filament. As pointed out by Wegner (1982), the K_D for monomer addition to either filament end cannot be different unless an independent source of chemical energy is provided to affect the binding to one end or the other. The RecA protein is a DNA-dependent ATPase. ATP is hydrolyzed by RecA monomers uniformly throughout RecA filaments (Brenner *et al.*, 1987), and there is no evidence for enhanced rates at either filament end. RecA filament assembly, but not disassembly, proceeds readily in the presence of ATP analogues, like ATPγS, that are bound but not hydrolyzed by RecA. Presumably, ATP hydrolysis is somehow coupled to disassembly. The hydrolysis of ATP by interior monomers does not generally result in dissociation, and under some conditions ATP hydrolysis can proceed with no evident dissociation of RecA monomers (Neuendorf & Cox, 1986; Shan *et al.*, 1997; Shan & Cox, 1996). A simple model arises. ATP hydrolysis occurs everywhere, resulting in dissociation only for monomers at the disassembling end, and occurring with a probability that is some function of reaction conditions.

The binding of RecA protein to ssDNA is a cooperative process (Kowalczykowski & Eggleston, 1994; Menetski & Kowalczykowski, 1985). In addition, the binding of ATP or ATPγS to individual filament subunits can stimulate NTP hydrolysis in neighboring subunits (Lee & Cox, 1990; Menge & Bryant, 1988). These interactions tend to maintain the entire filament in an extended active state. However, the ATP hydrolytic cycles of adjacent monomers are not coordinated, in the sense that a given step in the hydrolytic cycle of one monomer triggers a particular step in its neighbor. Wild-type RecA protein will form mixed filaments on DNA with the RecA K72R mutant protein that binds but does not hydrolyze ATP or dATP (Shan & Cox, 1996). In most experiments with the mutant, dATP replaces ATP since the mutant protein functions in some RecA assays only in the presence of dATP (Rehrauer & Kowalczykowski, 1993). In these filaments, the observed decrease in dATP hydrolysis by bound RecA protein is directly proportional to the fraction of mutant protein present in the filament (Shan & Cox, 1996). The presence of a mutant monomer does not have any discernible inhibitory effect on its wild-type neighbors with respect to dATP hydrolysis; it simply takes up room in the filament and displaces a wild-type monomer that would otherwise be hydrolyzing ATP (Shan & Cox, 1996). When a second complementary strand of DNA is added (as with bound dsDNA or as a result of DNA strand exchange), the filament state changes. Now, the presence of the mutant exerts an effect on the rate of dATP hydrolysis that is greatly disproportional to its representation in the filament, indicating that the dATP hydrolytic cycles of adjacent monomers are now coupled or coordinated in some way (Shan & Cox, 1996).

The apparent absence of coupling between the ATP hydrolytic cycles of adjacent monomers on ssDNA leads to some quantitative predictions for the disassembly process. The normal hydrolytic cycle will take some time t, and if coupled to dissociation at the disassembly end, a monomer should dissociate from a filament end at some point in the hydrolytic cycle we will label x. For a RecA monomer in a filament on ssDNA, the k_{cat} is about 30 min^{-1}, so that t = two seconds. When one monomer dissociates, the next monomer in line (hydrolyzing ATP independently) could be at any stage of its hydrolytic cycle, but on average it should be halfway through the cycle or one second before the dissociation point x. End-dependent disassembly should therefore occur at a maximal rate of approximately 60 monomers per minute per filament end.

To test this prediction and to fully characterize the end-dependent disassembly of RecA protein from ssDNA, we have developed a comprehensive model for this process. The model leads to a new and quantitative approach to the study of RecA filament dynamics.

Results

Model for RecA protein disassembly from ssDNA

RecA protein disassembles from the filament end nearest the 5′ end of the ssDNA. Although rebinding to ssDNA is usually fast, nucleation of RecA filaments on unbound ssDNA can be slowed considerably by including a single-stranded DNA-binding protein (SSB) in the reaction mixture. The SSB replaces the RecA protein as disassembly proceeds. The overall process is illustrated in Figure 1. RecA filaments are assembled at pH 6.46, where no significant disassembly takes place. RecA is added prior to SSB and ATP to effect maximum DNA binding (SSB inhibits nucleation but not extension of RecA filaments; it also facilitates filament extension by removing secondary structure in the DNA). The pH is then shifted to a higher value by twofold dilution, and end-dependent disassembly begins. For each monomer, the dissociation is described by the first-order rate constant k_{OFF}. The reverse process (rebinding directly to the disassembly end to extend the filament 3′ to 5′), to the extent that it occurs, is described by the second-order rate constant k_{ON}. As end-dependent disassembly proceeds, additional RecA protein can also bind by independent nucleation at any point in the vacated ssDNA, the process that is inhibited by SSB. The nucleation that does occur is described by the second-order rate constant k_{NUC}. Each nucleation event rapidly results in the formation of a new filament from that point extending 5′ to 3′ up to the disassembling end of the preexisting filament, and disassembly then proceeds from the newly created end (this assumption is addressed below). Note that although disassembly is a first-order process, it will not follow classic first-order kinetics. This is because the concentration of reactant, the filament ends, does not change as the reaction progresses, at least until disassembly is essentially complete.

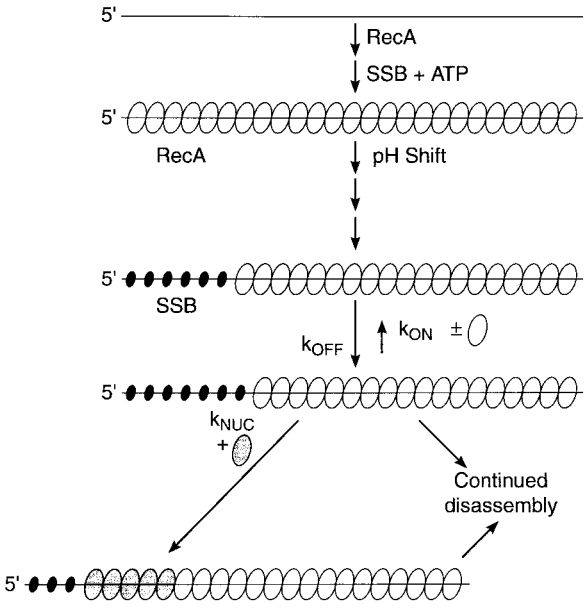

Figure 1. A model for end-dependent disassembly of RecA filaments from ssDNA. RecA protein filaments disassemble only from the end nearest the 5′ end of the DNA (Shan *et al.*, 1997), in a process described by k_{OFF}. As disassembly proceeds, RecA protein is replaced by SSB. Re-binding of RecA to the vacated DNA occurs at a rate described by k_{NUC}, and results in a new filament (shaded) that extends from the point of nucleation to the end of the available DNA to the 3′ side. The direct reversal of disassembly is described by k_{ON}.

We have monitored filament disassembly by electron microscopy, sucrose gradient sedimentation, and spectrophotometry (Shan *et al.*, 1997). All three methods provide reasonably consistent results, but only the last is sufficiently accurate to determine rate constants. The most convenient method is to monitor declines in the DNA-dependent ATP hydrolysis of RecA protein using the coupled spectrophotometric assay. Although indirect, the method correlates very well with DNA binding measured in other ways (Lindsley & Cox, 1989; Pugh & Cox, 1988; Shan *et al.*, 1997). It is one of the few methods available for RecA, which provides a real-time measure of DNA binding, yet requires no artificial alterations in RecA or DNA structure. The ATP concentration is maintained at high levels so that each DNA-bound RecA monomer is functioning at its k_{cat}. The k_{cat} employed is that observed in the presence of longer random sequence ssDNA molecules. Under conditions used in this series of experiments, the k_{cat} for ATP hydrolysis is 28(±2) min^{-1}.

Once initiated, disassembly proceeds not to completion but to a steady state in which disassembly and rebinding are balanced. To focus our analysis on the disassembly process, it is necessary to minimize the background ATPase rates due to filament reassembly on the vacated DNA. This can be done by including SSB in the reaction and by limiting the length of the DNA. The rate of nucleation is a function of the concentrations of free RecA protein and potential DNA binding sites. Every nucleation event results in a filament from that point to the end of the available DNA. If DNA molecules are shortened but the total concentration of DNA binding sites is kept constant, the number of nucleations will stay the same. However, the length of the filaments generated by each nucleation will be reduced, and thus the total amount of re-bound RecA and its accompanying ATP hydrolysis will be reduced. Shortening the DNA too much has the disadvantage of abbreviating the time during which disassembly can be measured, and very short DNAs (less than 60 nt) reduce the initial

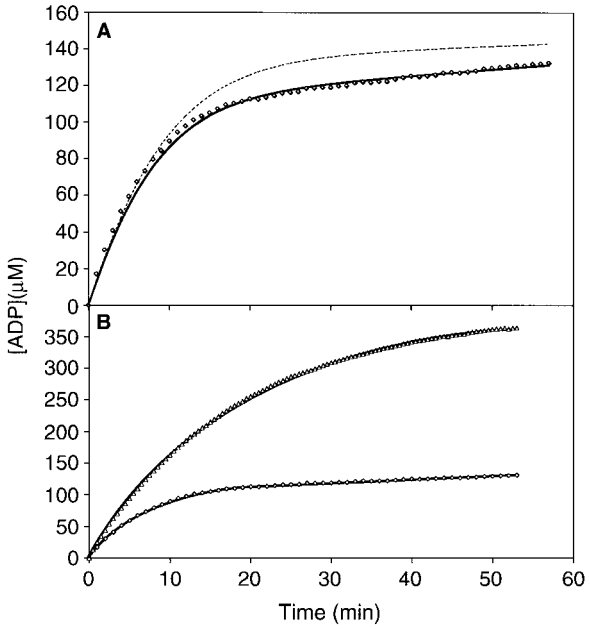

Figure 2. End-dependent RecA filament disassembly monitored by changes in ATP hydrolysis. ATPase reactions were started at pH 6.46 (Mes/NaOH; 56 % anion). After 20 minutes (time = 0 in the Figure), 200 μl of each was added to 200 μl of buffer (Tris-acetate ion (OAc) 30 % cation) to give a final pH of 7.98. The final DNA concentration (in total nucleotides) after the pH shift was 1.5 μM. The final RecA protein and SSB concentrations were 1.5 μM and 0.15 μM, respectively. (a) Disassembly reaction under standard reaction conditions. Data for disassembly from a 2693 base ssDNA fragment of ϕX174 DNA. The best-fit line to equation (8) is shown by the continuous line. The line obtained from equation (8) if k_{OFF} is decreased by 10 %, and then the curve is fitted for k_{NUC}, is shown by the broken line. (b) Effects of DNA length on filament disassembly. Both reactions contained the same DNA, SSB, and RecA concentrations as described above. The ϕX174 substrate (lower curve) is the 2693 base substrate described in (a). The M13mp8 substrate (upper curve) is full-length M13mp8 (7229 bases) linearized with EcoRI. The best-fit parameters for k_{OFF} and k_{NUC}, respectively, are 71.2(±0.8) min^{-1} and 1.20(±0.03) × 10^{-4} μM^{-1} min^{-1} (lower curve), and 74.2(±1) min^{-1} or 1.5(±0.1) × 10^{-5} μM^{-1} min^{-1} (upper curve).

binding so much that the analysis becomes very difficult. The DNA substrate chosen is thus a compromise between a length sufficient for convenient and reliable measurement but short enough to minimize rebinding. Most of these experiments were carried out with ϕX174 ssDNA, cleaved with *Bss*SI as described in Materials and Methods to produce two nearly equal fragments 2641 and 2745 nt in length. For the analysis below, the length is assumed to be the average of these, or 2693 nt.

Determination of kinetic parameters

To monitor disassembly, we are following the production of ADP. To develop an expression for the production of ADP in terms of the two primary unknowns, k_{OFF} and k_{NUC}, we need to define several additional parameters. These are: [D-ends] = the concentration of disassembling DNA ends = the concentration of DNA molecules; [RecA] = the total concentration of RecA monomers; [$RecA_B$] = the concentration of RecA that is bound to the DNA; [DNA] = the concentration of available (unbound) DNA binding sites; n_{TOT} = the total number of RecA binding sites per DNA molecule = 2693/3 or 898; n_{BOUND} = the average number of binding sites occupied by RecA per DNA molecule; n_{GAP} = the average number of unoccupied binding sites per molecule; and k_{cat} in all cases refers to the k_{cat} for ATP hydrolysis by the RecA protein.

All concentrations are in μM and all times are in minutes. The rate constant k_{OFF}, is reported in units of min^{-1}, and is thus the number of monomers to dissociate per minute per filament end. Likewise, k_{NUC} is reported in units of μM^{-1} min^{-1}. The concentration of RecA protein is kept in sufficient excess relative to binding sites on the DNA (threefold or more) so that to a good approximation, the concentration of free RecA is equal to its total concentration. This is especially true under conditions in which nucleation of new filaments would have its greatest effect on the kinetics, i.e. when disassembly is nearly complete. The disassembly mediated change in bound RecA protein is then described by equation (1):

$$\frac{\partial[RecA_B]}{\partial t} = k_{NUC}[DNA][RecA] - k_{OFF}[D\text{-ends}] \quad (1)$$

The concentration of available DNA binding sites will be equal to the number of dissociating ends, times the average number of binding sites in the vacated DNA (n_{GAP}):

$$[DNA] = [D\text{-ends}](n_{GAP}) \quad (2)$$

Because each nucleation event results in propagation of a filament from the nucleation point to the end of the gap and, on average, the nucleation point is midway through the gap, the change in concentration of RecA due to k_{NUC} must be further modified by the term $n_{GAP}/2$. After including this term, and substituting equation (2) into equation (1), we obtain:

$$\frac{\partial[RecA_B]}{\partial t} = k_{NUC}[D\text{-ends}](n_{GAP})\left(\frac{n_{GAP}}{2}\right)[RecA] - k_{OFF}[D\text{-ends}] \quad (3)$$

The change in [$RecA_B$] with time is a function of both disassembly and reassembly onto the vacated DNA. The term n_{GAP} is related to the concentration of bound RecA protein ([$RecA_B$]) by the following expression:

$$n_{TOT} = n_{GAP} + n_{BOUND} = n_{GAP} + \left(\frac{[RecA_b]}{[D\text{-ends}]}\right) \quad (4)$$

Substituting n_{GAP} from equation (4) into equation (3) yields:

$$\frac{\partial[\mathrm{RecA_B}]}{\partial t} = k_{NUC}[\text{D-ends}]\left((n_{TOT} - \frac{[\mathrm{RecA_B}]}{[\text{D-ends}]}\right)^2\frac{[\mathrm{RecA}]}{2} - k_{OFF}[\text{D-ends}] \quad (5)$$

By integrating equation (5), and taking into account that at $t = 0$, all binding sites on the DNA are occupied by RecA protein, i.e. $[\mathrm{RecA_B}] = n_{TOT}[\text{D-ends}]$, we obtain:

$$[\mathrm{RecA_B}] = [\text{D-ends}]\left(n_{TOT} - \sqrt{\frac{2k_{OFF}}{k_{NUC}[\mathrm{RecA}]}} \times \tanh\left(\sqrt{\frac{k_{NUC}[\mathrm{RecA}]k_{OFF}}{2}}t\right)\right) \quad (6)$$

Equation (6) is substituted into the expression:

$$\frac{\partial[\mathrm{ADP}]}{\partial t} = k_{cat}[\mathrm{RecA_B}] \quad (7)$$

The resulting expression is then integrated to yield:

$$[\mathrm{ADP}] = k_{cat}[\text{D-ends}]\left(n_{TOT}t - \left(\frac{2}{k_{NUC}[\mathrm{RecA}]}\right) \times \ln\left(\cosh\left(\sqrt{\frac{k_{NUC}[\mathrm{RecA}]k_{OFF}}{2}}t\right)\right)\right) \quad (8)$$

Data obtained from the ATPase assays were converted to [ADP], plotted as a function of time, and fit to equation (8) using the non-linear least-squares program NONLIN (Johnson & Frasier, 1985). As described in Materials and Methods, the k_{cat} for ATP hydrolysis was always measured independently from the rate of ATP hydrolysis before shift in pH, and therefore it was held constant during fitting. The terms [D-ends], [RecA], and n_{TOT} were known in any given experiment and also held constant during fitting, leaving k_{OFF} and k_{NUC} as the fitting parameters.

A representative time-course is shown in Figure 2(a) for a reaction carried out under standard reaction conditions at pH 7.98. The plot of [ADP] *versus*. time is non-linear, reflecting the decline in the rate of ATP hydrolysis due to disassembly. The curve is fit very well by equation (8) (continuous line in Figure 2(a)), yielding a k_{OFF} in this instance of 71.2(±0.8) $\mathrm{min^{-1}}$ and a k_{NUC} of $1.20(\pm0.03) \times 10^{-4}$ $\mu\mathrm{M^{-1}}$ $\mathrm{min^{-1}}$. The data fitting was as good or better than that shown in Figure 2(a) for all data reported here. The broken line in Figure 2 illustrates the fit obtained if the best-fit k_{OFF} is decreased by 10 %, fixed, and then the data is fit for k_{NUC}. In general, a satisfactory fit of the data for both parameters is not obtained if the values of either k_{OFF} and k_{NUC} are varied by more than 5 %. Figure 2(b) illustrates the effect of DNA length. With a ssDNA more than twice as long (i.e. full-length M13mp8 linear ssDNA), the dissociation proceeds over a longer time-course, consistent with an end-dependent process. The final concentration of bound RecA protein in the steady state is greater than that observed with the shorter DNA for reasons described above. However, the measured k_{OFF} (74.2(±1) $\mathrm{min^{-1}}$) generated from this experiment is quite comparable to those obtained with the shorter DNA, as expected. The parameter k_{NUC} ($1.5(\pm0.1) \times 10^{-5}$ $\mu\mathrm{M^{-1}}$ $\mathrm{min^{-1}}$) makes less of a contribution with the M13mp8 DNA, although this parameter was generally reproducible in experiments carried out with a particular DNA substrate as shown below.

SSB was added to these experiments at a final concentration of 0.15 μM, providing one SSB monomer per ten nucleotides of ssDNA. SSB inhibits the nucleation of RecA filaments on ssDNA such that net disassembly is generally not observed if it is not included (Shan *et al*., 1997). Doubling the SSB concentration had no significant effect on the measured rates of RecA filament disassembly (data not shown).

Further discussion of model assumptions

It is useful to comment on some of the assumptions made in developing the model of Figure 1. First, in simplifying the rebinding that might occur during disassembly to a single term k_{NUC}, we are making the assumption that nucleation is rate limiting and filament extension relatively fast, such that each nucleation event rapidly results in a new filament extending from the nucleation point to the disassembling end. This would effectively create a longer filament with a new disassembling end. The most important part of this assumption is that filament extension is fast enough so that any nucleation on DNA vacated by disassembly is likely to ''catch-up'' with the disassembling end. Many different observations support this view, including: (a) complete RecA filaments form on M13 ssDNA in a few minutes (Griffith & Harris, 1988; Roca & Cox, 1997), suggesting rates of filament extension of 1000 monomers per minute or more, rates that are at least an order of magnitude faster than disassembly. (b) If filament extension were not much faster than disassembly, then late nucleation events would create filaments that would contain long breaks. These breaks should be visible in the electron microscope, but are rarely observed when monitoring disassembly by electron microscopy (Shan *et al*., 1997). (c) At early times after RecA addition, long filaments are formed on linear bacteriophage ssDNA encompassing nearly its entire length at pH 7.6 even without the pH shift protocol (Shan *et al*., 1997). These long contiguous filaments, formed in the presence of the same SSB concentrations used in the present experiments, could not form unless filament extension was much faster than disassembly. (d) Filament assembly has been characterized as a nucleation-limited process (Chabbert *et al*., 1987). In this study, binding occurred more rapidly when longer DNAs were

used. If filament extension limited the rate at which DNA was coated with RecA, the opposite result might be expected. We have observed the same effect on SSB-coated ssDNA. Under the conditions used in this study, the lag in ssDNA binding to SSB-coated DNA (as indicated by rates of ATP hydrolysis) is several minutes shorter on circular M13mp8 ssDNA (7249 nt in length) than it is when circular ϕX174 ssDNA (5386 nt in length) is bound, when the same concentration of DNA (in total nucleotides) is included in each experiment (T.A.A., unpublished data). (e) Finally, we note that substoichiometric levels of RecOR proteins greatly stimulate the binding of RecA protein to SSB-coated ssDNA (Umezu & Kolodner, 1994; Shan *et al*., 1997). A number of results in these studies strongly suggest that the slow step affected by RecOR is nucleation.

Second, the model assumes that measurable disassembly is occurring at only one point on each DNA molecule. Each RecA monomer binds to three nucleotides of DNA, such that filaments can bind to DNA in one of three binding frames, much like a gene can be translated in any of three reading frames. When a new filament is nucleated on the DNA vacated by a disassembling filament, it is possible that it would not be in frame with the disassembling filament ahead of it. This would create a small gap at the junction of the two filaments, where the first filament would continue to disassemble. Such gaps should have no effect on the disassembly kinetics we are measuring in the current study, because any RecA that dissociated at such a gap would be immediately replaced by extension of the second filament with no net change in bound RecA protein. Our assay only measures net changes in the hydrolysis of ATP by bound RecA protein, and net changes will occur at only one point per DNA molecule.

Third, the experimental protocol we are using assumes that the k_{cat} for ATP hydrolysis does not vary over the pH range of the pH shift experiments. Early measurements indicated that rates of ATP hydrolysis generally do not vary between pH 5.5 and pH 9 (Weinstock *et al*., 1981). More detailed examination of the pH dependence of k_{cat} has not been reported. We therefore measured the k_{cat} for ATP hydrolysis by RecA protein as a function of pH. In five total measurements distributed between the pHs 6.4 and 7, the measured k_{cat} for ATP hydrolysis varied only from 27.2 to 29.4 min^{-1}. All of the experiments were carried out in the presence of SSB under conditions used in the disassembly studies, using circular ϕX174 ssDNA as a DNA substrate. Under these conditions, it is difficult to achieve complete binding of ssDNA at pH 7.5 and above, leading to rates of ATP hydrolysis that appear to be about 20% lower than expected. To circumvent this problem, a series of experiments was carried out in which filaments were formed on circular ssDNA at pH 6.4 where complete binding was readily achieved, and then the pH was shifted to either 7.4 (one trial) or 8 (four trials). The ATP concentration in these trials was 3 mM, more than an order of magnitude higher than any reported K_M for RecA protein bound to ssDNA. The rate of ATP hydrolysis did not change in these pH shift experiments (i.e. it decreased by exactly the twofold factor of the dilution used to effect the pH shift). If the observed rates are taken as reflecting a state of near V_{max}, the k_{cat} for ATP hydrolysis in these experiments ranged from 28.6 to 29.9 min^{-1}. These experiments on circular ssDNA directly indicate that the pH shift protocol used in the present experiments does not result in any significant change in the rate at which ATP is hydrolyzed by bound RecA protein.

Direct binding of RecA to the disassembly end is minimal

Even though coupled to ATP hydrolysis, the end-dependent disassembly of filaments cannot be completely irreversible. The rate of end-dependent disassembly might be underestimated if direct rebinding to the disassembly end (the reverse of the k_{OFF} process, described by k_{ON} in Figure 1) is significant but neglected. As for k_{NUC}, k_{ON} should be a second-order process dependent on RecA concentration. To evaluate the effect of direct reversal of the disassembly process, we examined the effect of RecA protein on the measured rate of disassembly.

The effect of RecA concentration on the rate of disassembly is shown in Figure 3(a). The effect of high RecA concentrations is quite small. A best-fit line is drawn through the data for the full data set. If affected at all, the observed k_{OFF} would be altered by direct reversal according to the expression:

$$(k_{OFF})_{obs} = k_{OFF} - k_{ON}[\text{RecA}] \quad (9)$$

The intrinsic k_{OFF} can be estimated by extrapolating the data in Figure 4 back to zero RecA concentration. The parameter k_{ON} is then described by the equation:

$$k_{ON} = \frac{k_{OFF} - (k_{OFF})_{obs}}{[\text{RecA}]} \quad (10)$$

If we take the observed k_{OFF} at 6 μM RecA protein (where the concentration of free RecA is least affected by DNA binding), then we obtain a k_{ON} value of 1.875(±1.46) μM^{-1} min^{-1}. Since $K_D = k_{OFF}/k_{ON}$, this result gives an apparent K_D of 29.9 μM. The limit for K_D within the error of the experiment is 22 μM. Therefore, to the extent it occurs at all, rebinding to the disassembly end is quite weak, and affects the disassembly data minimally under our normal reaction conditions. The error in this experiment is such that the minimum effect of direct reversal is very close to zero (broken line in Figure 3(a)). To a good approximation, the reassembly at the disassembly end proceeds at a rate no greater than that expected for the rare renucleation at the specific site adjacent to the disas-

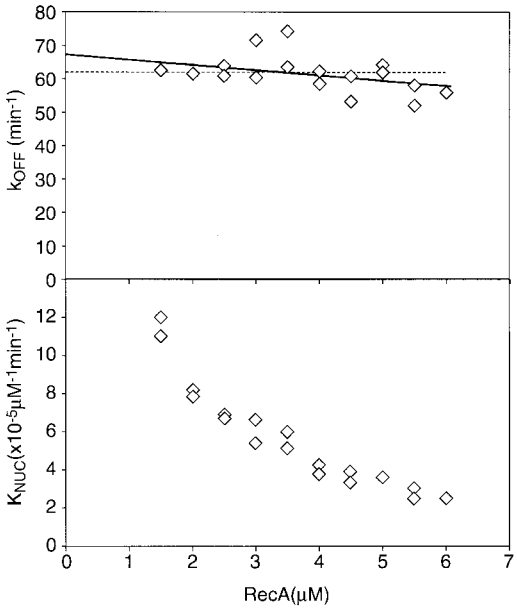

Figure 3. Effects of RecA protein concentration on kinetic parameters. The pH shift reactions were carried out with a final DNA concentration of 1.5 µM at varying RecA concentrations as described in Materials and Methods and the legend to Figure 2. The final concentration of SSB was 0.15 µM in all cases. The reactions were started at pH 6.46 and shifted to a pH of 7.98 after 20 minutes. (a) Effects of RecA protein concentration on observed rates of end-dependent filament disassembly. Values for k_{OFF} were calculated as described in the text. The continuous line represents a linear least-squares fit of the entire data set. The broken line is horizontal, representing the lower limit (0) for the effects of RecA protein concentration. The slope and y-intercept of this line were used to calculate k_{ON} and K_D as described in the text. (b) Effects of RecA protein concentration on rates of renucleation of RecA filament formation on vacated DNA. Values for k_{NUC} were calculated as described in the text.

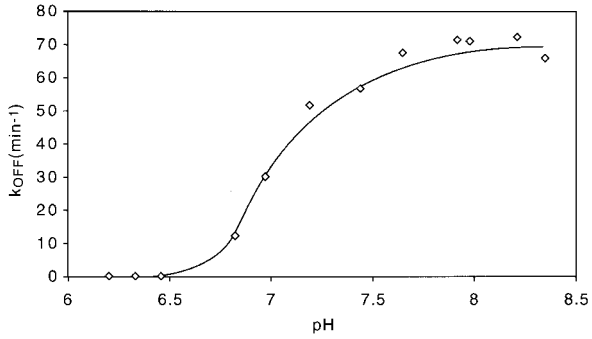

Figure 4. Effects of pH on rates of end-dependent RecA filament disassembly. pH shift reactions were performed as described in Materials and Methods. The final reactions contained 1.5 µM ϕX174 DNA, 1.5 µM RecA, and 0.15 µM SSB. The pH shift buffers contained Tris-Oac ranging from pH 7.25 (85% cation) to pH 9.1 (10% cation). At time $t = 0$ (20 minutes after the reaction was started), 200 µl of each reaction started at pH 6.46 was added to 200 µl of each of the pH shift buffers. Values for k_{OFF} were calculated as described in the text.

sembly end, or $k_{ON} \approx k_{NUC}$. We have therefore not corrected for the effect of k_{ON} in the values for k_{OFF} reported below.

The best-fit values for k_{NUC} decrease with increasing RecA concentration (Figure 3(b)). We attribute this phenomenon to the formation of higher-order filamented RecA species in solution at high RecA concentrations, an effect that tends to compete with DNA binding (Morrical & Cox, 1985).

End-dependent RecA filament disassembly is pH and temperature-dependent

Derived values of k_{OFF} exhibit a strong pH dependence (Figure 4), with essentially no net disassembly observed at pH conditions below 6.4, and maximum rates of disassembly observed above pH 7.5. The change in the rate of disassembly seen between pH 6.5 and 7.2 in Figure 4 indicates that the dissociation of one RecA monomer is accompanied by the release of one or two protons. Similar observations have been made in the assembly and disassembly of RecA filaments on dsDNA (Lindsley & Cox, 1989; Pugh & Cox, 1988).

The temperature dependence of the disassembly process is shown in Figure 5(a) in the form of an Arrhenius plot. The plot is linear over the temperature range 35-45 °C, with no breaks that might signal a change in rate-limiting step. The slope of the plot yields an Arrhenius activation energy of 24.1(±2.4) kcal mol^{-1}. We did not obtain results for temperatures below 34 °C, because of anomalies introduced by the PEP/pyruvate kinase ATP regenerating system at lower temperatures (Bedale & Cox, 1996).

The Arrhenius plot for the process described by k_{NUC} is given in Figure 5(b). The plot is again linear and yields an Arrhenius activation energy of 22.1(±5.0) kcal mol^{-1}.

End-dependent filament disassembly is coupled to ATP hydrolysis

It has long been observed that RecA filaments bind stably to ssDNA and dsDNA in the presence of the ATP analogue ATPγS, which is only very slowly hydrolyzed by RecA protein. To further illustrate the link between ATP hydrolysis and dissociation of RecA protein at the disassembly end of a filament, we investigated the effects of very low concentrations of ATPγS. The reported concentrations of ATPγS are those present before the pH shift; they were diluted twofold by the shift. At 0.5 and 1.0 µM ATPγS, disassembly was inhibited partially even though these concentrations of the analog had little effect on overall ATP hydrolysis (data not shown). With 2.0 µM ATPγS, disassembly was suppressed almost entirely, even though the overall rates of ATP hydrolysis before the pH shift

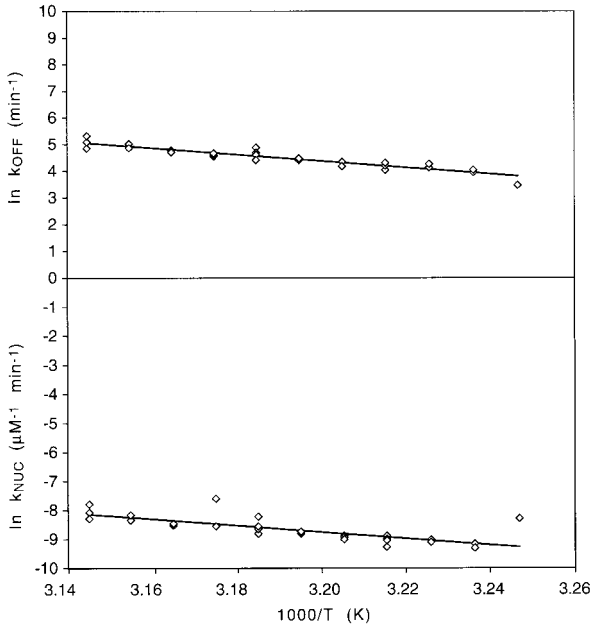

Figure 5. Effects of temperature on rates of end-dependent RecA filament disassembly and renucleation. The pH shift reactions were carried out with a final DNA concentration of 1.5 μM. RecA and SSB concentrations were 1.5 μM and 0.15 μM, respectively. Reactions carried out at the indicated temperatures were started at pH 6.46 and shifted to a pH of 7.98 after 20 minutes. Lines represent a least-squares fit of the data, and were used in the calculation of the Arrhenius activation energies for end-dependent disassembly (a) and re-binding (b).

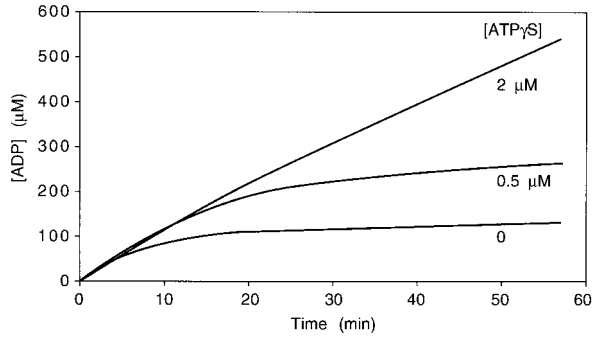

Figure 6. Effects of low concentrations of ATPγS on end-dependent disassembly of RecA filaments. The pH shift reactions were carried out with a final DNA concentration of 1.5 μM. RecA and SSB concentrations were 1.5 μM and 0.15 μM, respectively. ATPγS is added to indicated concentrations before the shift in pH ($t = -10$ minutes). No additional ATPγS is included in the pH shift buffer, so that the concentration of ATPγS after shift in pH is one-half of that indicated next to each curve.

were decreased only by 4.7 % (Figure 6). This result indicates that the binding of a non-hydrolyzed analogue to even a very small fraction of the RecA monomers in a filament effectively blocks disassembly.

Discussion

When combined with the results of earlier studies, the experiments described here support and begin to give substance to the model in Figure 1. Previous studies have shown that RecA filaments disassemble unidirectionally from ssDNA (Shan *et al.*, 1997), that ATP is hydrolyzed uniformly throughout such filaments (Brenner *et al.*, 1987), and that adjacent RecA monomers hydrolyze ATP independently (Shan & Cox, 1996). Here, we provide the first quantitative description of the disassembly process. We also demonstrate that the rate of end-dependent disassembly (60-70 monomers per filament end per minute at pH conditions above 7.5) is almost exactly that expected if there is little or no coupling between the ATP hydrolytic cycles of adjacent monomers in the filament.

The major assumption we must make in reaching this quantitative conclusion is that the rates of disassembly reflect a situation (above pH 7.5) in which the end-most monomer always dissociates when it hydrolyzes an ATP. That is, dissociation at the disassembly end of the filament is coupled to ATP hydrolysis by the corresponding RecA monomer. The pH dependence of the reaction can be explained in terms of an increase in the probability of monomer dissociation as the pH increases from 6.4 to 7.5, reflecting the ionization state of one or two groups on the protein. It is tempting to speculate that the residue(s) involved might include one or both of the two His residues of RecA protein, both of which have been subjected to mutational analysis (Muench & Bryant, 1991; Nguyen *et al.*, 1993). The leveling-off observed above pH 7.5 is most easily explained if the probability of dissociation reaches 100 % at the higher pH conditions, as the titration of the ionizable group(s) in question is completed. If the probability of dissociation of the end monomer is less than 100 % under these conditions, then the k_{cat} for ATP hydrolysis in the end monomers would have to be enhanced relative to the rest of the filament. There is no evidence for such an end-dependent enhancement, and given the lack of coupling between the ATP hydrolytic cycles of adjacent monomers (Shan & Cox, 1996), it is difficult to envision a mechanism by which it might occur. If the hydrolysis of ATP were instead suppressed at filament ends in a manner heretofore undetected, we would have to propose that some monomers dissociate without hydrolyzing ATP. The stability of RecA filaments formed in the presence of weakly hydrolyzed ATP analogues (like ATPγS) argues against this idea. The effect of even very low concentrations of ATPγS (enough to inhibit ATP hydrolysis as a whole by only 4.7 %) in suppressing disassembly (Figure 6) also reinforces the link between ATP hydrolysis and dissociation at the disassembly end of the filament.

Two parameters are obtained directly from the data generated in a disassembly experiment. One is the first-order rate constant for monomer dissociation at the disassembly end, k_{OFF}. The other is the second-order rate for nucleation of a new filament on vacated DNA, described by k_{NUC}. We show that rebinding at the disassembly end (the direct reversal of disassembly) occurs at approximately the same rate as nucleation of a new filament at the site adjacent to the disassembly end, and is thus well-described by the nucleation rate constant k_{NUC}. Disassembly rates should be independent of the sequence of the DNA, and the rates obtained have been quite consistent from one experiment to another. The other parameter is k_{NUC}, which describes the re-nucleation of RecA filaments at random locations on the vacated DNA as disassembly proceeds. The nucleation process is affected by many things, including SSB concentration and DNA sequence (Kowalczykowski *et al.*, 1987; Tracy & Kowalczykowski, 1996). The apparent k_{NUC} decreases by about eightfold when M13mp8 ssDNA is substituted for our cleaved ϕX174 DNA substrates. We do not know the reason for the change, but different DNA sequences and a potentially different arrangement of SSB protein on the DNA would seem to offer the most likely possibilities.

In solution there is a wide range of RecA aggregation states. The populations of the various oligomers and polymers change with RecA concentration and solution conditions (Brenner *et al.*, 1988, 1990; Morrical & Cox, 1985). It has therefore been difficult to define the RecA species that is added or subtracted from a RecA filament during assembly and disassembly. If no unusual assumptions are made about enhancement or suppression of ATP hydrolysis at filament ends, a straightforward involvement of monomers provides the simplest explanation of the data. If a higher-order unit (dimer, hexamer, etc.) is the active species, then all subunits within the unit must hydrolyze ATP to dissociate. At a minimum, the suppression of DNA binding seen at high RecA concentrations (Morrical & Cox, 1985; Figure 3(b)) indicates that very large aggregates of RecA protein do not bind directly to DNA. Some recent evidence indicates that RecA monomers can function in filament assembly on ssDNA (Masui *et al*, 1998).

The filaments formed on ssDNA do not reflect the situation observed during DNA strand exchange. When homologous duplex DNA is introduced, the rate of ATP hydrolysis in the RecA filaments abruptly decreases by about 30 % (Schutte & Cox, 1987), and the filament becomes more dynamic (Shan & Cox, 1997). There is also now a demonstrable coordination in the ATP hydrolytic cycles of adjacent RecA monomers in the filaments, as is also observed with RecA filaments bind to dsDNA (Shan & Cox, 1996, 1997). The present results provide a baseline with which to explore changes in the filament which accompany the commencement of DNA strand exchange.

Materials and Methods

Enzymes and reagents

E. coli RecA protein was purified to homogeneity as described (Cox *et al.*, 1981). *E. coli* SSB was purified to homogeneity as described (Lohman & Overman, 1985), except that an additional step utilizing DEAE-Sepharose chromatography was included to ensure removal of single-stranded exonucleases. The RecA and SSB concentrations were determined by absorbance at 280 nm, using extinction coefficients of $\varepsilon_{280} = 0.59\ A_{280}\ \text{mg}^{-1}$ ml (Craig & Roberts, 1981) and $\varepsilon_{280} = 1.5\ A_{280}\ \text{mg}^{-1}$ ml (Lohman & Overman, 1985), respectively. RecA and SSB preparations were free of detectable endo- and exonuclease activities on double or single-stranded DNA. Restriction endonucleases were purchased from New England Biolabs. Tris buffer was purchased from Fisher. DEAE-Sepharose was purchased from Pharmacia Biotech Inc. Pyruvate kinase, phosphoenolpyruvate, lactate dehydrogenase, ATP, β-NADH, and Mes buffer were purchased from Sigma. Microcon-100 microconcentrators were purchased from Amicon.

DNA

ϕX174 virion DNA was purchased from New England Biolabs. Circular single-stranded DNA from bacteriophage M13mp8 was prepared using methods described (Messing, 1983; Neuendorf & Cox, 1986). The concentration of DNA stock solutions was determined by absorbance at 260 nm, using 36 μg ml^{-1} A_{260}^{-1}, as a conversion factor. DNA concentrations are expressed in terms of total nucleotides unless specified otherwise.

Linear single-stranded substrates were generated by restriction enzyme digestion after 18 base oligonucleotides complementary to restriction enzyme sites were annealed to the circular ssDNA. After digestion, residual protein was removed by sequential 1:1 extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). Excess oligonucleotides were separated from substrate DNAs by passing the DNA solutions through a microconcentrator (microcon 100; sufficient for the oligos of up to 300 nt but not the large DNA substrates to pass through) until the volume was reduced twofold. The concentrated solution was then diluted twofold and the process was repeated twice. Substrates were then concentrated by precipitation in ethanol.

Reaction conditions

All reactions were carried out in 25 mM buffer, 10 mM magnesium acetate, 5 % (v/v) glycerol, 1 mM DTT, 3 mM ATP, an ATP regenerating system (10 units/ml of pyruvate kinase, 3 mM phosphoenolpyruvate, and 3 mM potassium glutamate), and concentrations of RecA protein, SSB, and ssDNA as described below and in the Figure legends. The coupled spectrophotometric assay also contained 10 units/ml lactate dehydrogenase and 3 mM NADH. Specific buffers used are described below and in the Figure legends. DNA and protein concentrations are indicated for each experiment. Reactions were incubated at the indicated reaction temperature for ten minutes before a mixture of ATP and SSB was added

to start the reaction. All reactions were carried out at 37 °C, except where indicated.

ATPase assay

A coupled spectrophotometric assay was used to measure ATP hydrolysis by the RecA protein (Morrical *et al.*, 1986). The assay was carried out using a Varian Cary 100 dual beam spectrophotometer equipped with a temperature controller and 12 position cell changer. The cell path-length and band pass were 0.5 cm and 2 nm, respectively. The regeneration of ATP from ADP and phosphoenolpyruvate can be coupled to the oxidation of NADH and followed by the decrease in absorbance of NADH at 380 nm. Absorbances were measured at 380 nm, instead of 340 nm (the absorbance maximum for NADH) to remain within the linear range of the spectrophotometer for the duration of the experiment. An extinction coefficient of $\varepsilon_{380} = 1.21\ \mathrm{mM^{-1}\ cm^{-1}}$ was used to calculate the rates of ATP hydrolysis, using the equation:

$$[\mathrm{ADP}] = A_{380}/(0.5\ \mathrm{cm}\ 1.21\ \mathrm{mM^{-1}\ cm^{-1}})$$

Filament disassembly reactions

ATPase reactions were started as described above in a Mes/NaOH (56 % anion, pH 6.46) buffer. The reactions were incubated for approximately 20 minutes to reach a stable steady state of ATP hydrolysis. This rate provided a starting point and reflected virtually complete binding of RecA protein to the ssDNA. We routinely achieve rates reflecting an apparent k_{cat} for bound RecA protein (one monomer per three available DNA nucleotides) of 26-30 $\mathrm{min^{-1}}$. Those few reactions displaying initial rates reflecting a k_{cat} of less that 26 $\mathrm{min^{-1}}$ were discarded. After preincubation, a 200 µl portion of the reaction was diluted with gentle mixing into 200 µl of a solution (also preincubated at 37 °C), containing 25 mM Tris-acetate, 10 mM magnesium acetate, 5 % (v/v) glycerol, 3 mM ATP, and an ATP regenerating system. The Tris-acetate-containing buffers used ranged in pH from 7.25 (85 % cation) to 9.10 (10 % cation). The final pH of the reactions, after the pH shift, ranged in pH from 6.2 to 8.35. Following the pH shift, the production of ADP was followed spectrophotometrically as the RecA filaments disassembled. In all cases, the reported pH values of reaction mixtures are those measured in control mixtures, made up and treated like the experimental mixtures but substituting appropriate storage buffers for DNA and protein components.

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