Effects of Intercalators on Complexation of RecA with Duplex DNA†

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ABSTRACT: To elucidate the binding mode of recombination protein A (RecA) to double-stranded (ds) DNA, the effects on the RecA−DNA interaction of several mono- and bisintercalators of the acridine, phenanthridine, and cyanine classes have been investigated by linear dichroism spectroscopy. Simple monointercalators lacking side chains efficiently promoted the binding of RecA to dsDNA in the absence of nucleotide cofactor, which is otherwise required. Bisintercalators varied in their ability to induce RecA binding, while monointercalators with aminoalkyl side chains proved inefficient. Modification of DNA structure by the intercalator appears to be necessary for induction of RecA binding, but if the intercalator has a bulky minor-groove-binding side chain, it does not induce RecA binding. In detailed studies with acridines, neither the binding geometry of intercalators nor the structure of DNA was significantly modified upon binding of RecA without cofactor. Judged by circular dichroism, similar RecA conformational changes accompanied bis-9-aminoacridine- and ATPγS-induced RecA association with DNA. In the presence of ATPγS, the intercalators inhibited the rate of RecA binding to dsDNA and were extruded from DNA upon binding of RecA. This competitive aspect may suggest that intercalation of some amino acid residue(s) plays a role in nucleotide-induced RecA binding. The stoichiometry of the RecA−DNA−intercalator filament was determined; in the fully formed filament the base pair:intercalator ratio is 2, and the base pair:RecA ratio also 2. This contrasts with a base pair:RecA ratio of 3 in the ATPγS-induced filament, although in both cases the DNA experiences 50% extension.

RecA[1] protein is a cardinal element of homologous recombination in *Escherichia coli* (Clark and Margulis, 1965; Smith, 1989), and proteins of similar sequence and with similar functions have been found in various other organisms (Roca & Cox, 1990; Shinohara et al., 1993; Kowalczykowski & Eggleston, 1994). RecA catalyzes the strand exchange reaction (Shibata et al., 1979; McEntee et al., 1979) and, besides this activity, stimulates synthesis and activity of proteins involved in DNA repair and mutagenesis (SOS induction) (Walker, 1984; Little & Mount, 1982; Sommer et al., 1993). Purified RecA mimics these functions *in vitro* in the presence of ATP (or its analog, ATPγS); it promotes strand exchange between two homologous DNA molecules (Shibata et al., 1979; McEntee et al., 1979) and stimulates autocleavage of LexA and phage repressors and UmuD protein (Little & Mount, 1982; Horii et al., 1981; Nohmi et al., 1988). To effect these reactions, RecA binds cooperatively to DNA, forming a right-handed helical filamentous complex (DiCapua et al., 1982; Williams & Spengler, 1986), with nucleotide cofactor (ATP or its analog ATPγS) required for binding to dsDNA (McEntee et al., 1981).

When RecA protein binds to dsDNA in the presence of its cofactor ATPγS, key features of the resulting filament are a 50% lengthening of the DNA (DiCapua et al., 1982; Williams & Spengler, 1986) and an unwinding of about 15°/base pair (Stasiak & DiCapua, 1982; Chrysogelos et al., 1983; Pugh et al., 1989) with the nucleobases remaining almost perpendicular to the DNA helix axis (Norden et al., 1992a). This is remarkably suggestive of some intercalative interaction of the protein or its cofactor with DNA since similar features are observed when DNA is saturated with ethidium bromide, for instance (Neidle, 1994). However, RecA binds to DNA with a stoichiometry of 3 base pairs/subunit (DiCapua et al., 1982; Takahashi et al., 1989b). RecA thus elongates DNA by 1.5 base pairs/subunit bound, while classical monointercalators, such as ethidium bromide, increase the length of DNA by about 1 base pair unit/intercalation (Neidle, 1994). To reconcile this with a possible intercalation, it is necessary that either an overextension (relative to ethidium) occurs if 1 residue/protein is intercalated or an underextension if two residues intercalate. The first of these possibilities is not unreasonable since it has been reported that 9-aminoacridine binds by monointercalation with a binding site of 2 base pairs and an exceptionally large elongation of 1.5 base pairs/ligand (Wirth et al., 1988) while its dimer binds bisintercalatively with a binding site of *ca.* 4 base pairs but with an elongation of only 2.1 base pairs/ligand (Hansen et al., 1984). The overextension by 9AA may be caused by a tilting of the ligand in the intercalation pocket.

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[1] Abbreviations: 9AA, 9-aminoacridine; $9AA_2$, 9-aminoacridine dimer; 9AA-PRO, 9-aminoacridine with side-arm linker chain; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); au, absorption units; bp, base pair; CD, circular dichroism; dsDNA, double-stranded DNA; EB, ethidium bromide; EB_2, ethidium dimer; LD, linear dichroism; PrI, propidium iodide; *r*, binding ratio (ligand to nucleotide concentrations); RecA, recombination protein A; TO, thiazole orange; TO-PRO, thiazole orange with side-arm linker chain; TOTO, thiazole orange homodimer.

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The possibility that the nucleotide cofactor itself intercalates has recently been excluded (Takahashi & Norden, 1994a), and studies are continuing to determine whether any of the aromatic amino acids of RecA intercalate, with tryptophan and tyrosine already excluded (Hagmar et al., 1992; Eriksson et al., 1993a,b). In the meantime, however, the notion of such an interaction has been encouraged by recent findings that some intercalators including ethidium can, in fact, promote the binding of RecA to dsDNA in the absence of cofactor (Thresher & Griffith, 1990; Kim et al., 1993a). The possibility of RecA intercalation is further strengthened by the observation that several intercalators (covalently or noncovalently bound to DNA) inhibit nucleotide-induced binding and are extruded from their DNA binding sites in the RecA−dsDNA filament in the presence of cofactor (Dombroski et al., 1983; Kim et al., 1993a,b), suggesting some competition for the intercalation site.

One might consider therefore that this behavior could provide some insight into the proposed role of intercalation in RecA binding. However, the role of intercalators in inducing RecA binding is most likely different from that of ATPγS. In the absence of cofactor, an intercalating liquid is required to modify the DNA structure for the binding of RecA. In the presence of cofactor, RecA itself might promote the modification of DNA structure *via* intercalation of some amino acid residue(s). The role of nucleotide cofactor would thus be to alter the conformation of RecA to permit such intercalation. The helical filaments induced by ATPγS (10-nm diameter, 9.5-nm pitch, and 50% extension) and EB (13-nm diameter, 8-nm pitch, and 45% extension) are not identical, as judged by electron microscopy (Thresher & Griffith, 1990). Notably, the most similar feature is the *ca.* 50% lengthening in both cases. It was suggested (Thresher & Griffith, 1990) that while the ATPγS-induced filaments arose from extensive polymerization, those induced by intercalators might have arisen from extensive nucleation. However, different formation mechanisms do not necessarily give rise to different structures but it is likely that there are differences in the local structures of the two filaments.

As an extension of our previous work, which confirmed by linear dichroism spectroscopy that EB induces RecA binding to dsDNA in a binding ratio-dependent manner, we have investigated here how other intercalators can act in this role, to determine whether this is truly a general phenomenon. Moreover, we have taken further steps toward characterization of the intercalator-induced RecA:dsDNA filament by determining the ligand:base pair and RecA:base pair stoichiometries as well as the base pair and intercalator orientations within the filament.

MATERIALS AND METHODS

Materials. RecA protein was purified as described previously, using high-performance ion-exchange liquid chromatography (DEAE 5PW, Tosoh) as the final step (Takahashi & Hagmar, 1991). Calf thymus DNA, 9-aminoacridine, ethidium bromide, and propidium iodide were purchased from Sigma, ethidium homodimer-1, TO-PRO-1, and TOTO-1 from Molecular Probes (Eugene, Oregon), and ATPγS from Boehringer Mannheim. These chemicals were used without further purification. 9-Aminoacridine dimer was synthesized as described elsewhere (Wirth et al., 1988). The synthesis of the TO chromophore will be described in a synthetic communication (Per Lincoln, unpublished work). The concentrations were determined spectrophotometrically using the following extinction coefficients: $\epsilon_{280nm} = 2.17 \times 10^4$ M^{-1} cm^{-1} for RecA, $\epsilon_{260nm} = 6.6 \times 10^3$ M^{-1} cm^{-1} (in bases) for DNA, $\epsilon_{260nm} = 1.54 \times 10^4$ M^{-1} cm^{-1} for ATPγS, $\epsilon_{405nm} = 9.3 \times 10^3$ M^{-1} cm^{-1} for 9AA and 9AA-PRO, $\epsilon_{410nm} = 1.8 \times 10^4$ M^{-1} cm^{-1} for $9AA_2$, $\epsilon_{480nm} = 5850$ M^{-1} cm^{-1} for EB, $\epsilon_{493nm} = 5900$ M^{-1} cm^{-1} for PrI, $\epsilon_{492nm} = 8900$ M^{-1} cm^{-1} for EB_2, $\epsilon_{500nm} = 6.85 \times 10^4$ M^{-1} cm^{-1} for TO, $\epsilon_{515nm} = 6.2 \times 10^4$ M^{-1} cm^{-1} for TO-PRO, and $\epsilon_{514nm} = 11.2 \times 10^4$ M^{-1} cm^{-1} for TOTO. The DNA concentration is always referred to in terms of bases. All experiments were performed in a buffer containing 5 mM sodium cacodylate and 20 mM NaCl at pH 6.5 and at ambient temperature (22 ± 2 °C). In some samples 1 mM $MgCl_2$ was also included.

Spectroscopy. Linear dichroism (LD) is defined as the differential absorption of light measured with the polarization vector of the incident light beam oriented parallel and perpendicular relative to the orientation direction (Nordén & Seth, 1985, Nordén et al., 1992b). The sign and amplitude of the LD signal is related to both the orientation of the aligned chromophore with respect to the helix axis and an orientation function which reflects the degree of the orientation of the sample in the flow. The latter depends on the contour length and flexibility of the macromolecule, the flow rate, and the temperature and viscosity of the sample solution. Linear dichroism measurements were performed on a Jasco J-500A spectropolarimeter by the method previously described (Nordén & Seth, 1985), and an Oxley prism was used to convert the circularly polarized light to linearly polarized light. An outer rotating Couette cell (Nordén et al., 1992b) was used to align the samples. The shear gradients used are indicated where appropriate in the text and figure legends.

CD spectra were measured on a Jasco J-720 instrument in a 1-cm cell in the near-UV (250−350-nm) region and a 0.1-cm cell in the 200−260-nm region. All spectra shown are normalized to a 1-cm path length.

RecA−DNA complex formation is accompanied by an increase in the LD signal around 260 nm due to the stiffening of the DNA as a result of the firm support from the surrounding helical RecA filament (Takahashi et al., 1989a; Kim et al., 1993a,b). The kinetics of complex formation could thus be followed by monitoring the LD intensity at 260 nm after the addition of RecA to DNA/intercalator or DNA/ATPγS mixtures. For kinetic studies, LD was monitored using a mild shear gradient of 450 s^{-1} where the signal from uncomplexed DNA is very small. The mixing time of RecA and DNA solutions was always less than 30 s.

Because of the slow association and dissociation of the RecA−DNA complex (Menetski & Kowalczykowski, 1985; Takahashi et al., 1989a), equilibrium conditions could not be easily obtained during titrations. Hence, determinations of binding stoichiometries were carried out by preparing new samples for every RecA:DNA and DNA:intercalator ratio investigated. Samples were equilibrated for 2.5 h at ambient temperature (22 ± 2 °C), after which the LD signal was measured. After incubation, no significant change of the LD signal with time was observed.

The concentration of bound acridine in the stoichiometric experiments was estimated using binding data from the literature, adjusted for ionic strength according to the standard

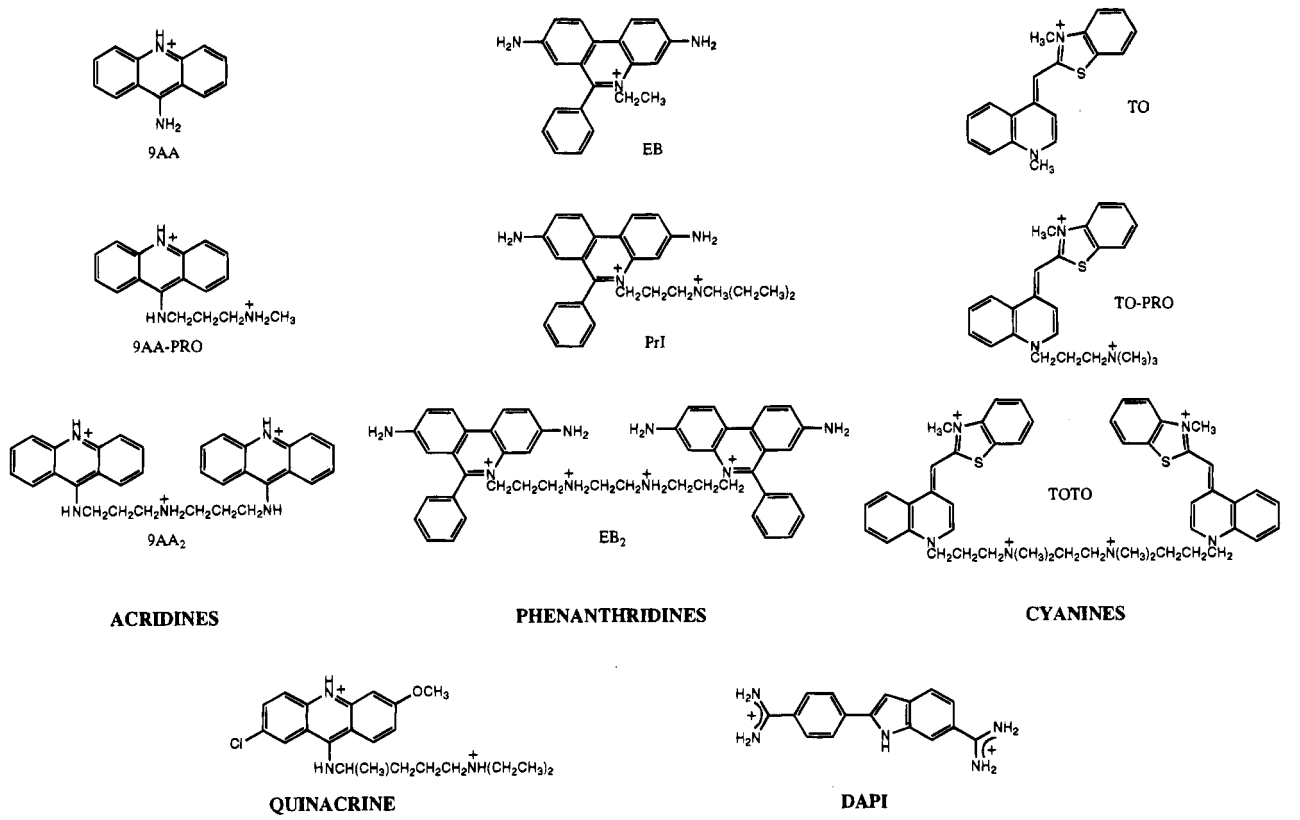

FIGURE 1: Chemical structures and abbreviated nomenclature for the ligands used in this study.

Record model (Record et al., 1978). Adjusting three different literature values (Fornasiero & Kurucsev, 1985; Drummond et al., 1965; Kubota & Motoda, 1980) for the binding constant of 9AA with dsDNA gave an average value of $K = 3.5 (\pm 0.5) \times 10^5$ M^{-1} in 25 mM Na^+ (5 mM cacodylate/20 mM NaCl). The binding constant of $9AA_2$ is considerably higher than that of the monomer, and the reported literature value (Wirth et al., 1988) may be adjusted to give $K = 4.5 \times 10^7$ in 25 mM Na^+. On the basis of these binding constants and site sizes of $n = 0.25$ for 9AA and 0.125 for $9AA_2$, we estimate that *ca.* 50% of the 9AA is bound and 99% of the $9AA_2$ for the range of ligand and DNA concentrations used in these experiments.

Normally, samples were prepared directly before the experiments were carried out. They were stored in polyethylene tubes and were protected from laboratory light as much as possible. Samples containing bisintercalators were equilibrated in the dark at 50 °C for 3 h to ensure homogeneous distribution of the dye between the DNA molecules (Carlsson et al., 1995). The effects of exposure of the complexes to UV/vis radiation were studied by comparing samples (a) kept exclusively in the dark during incubation, (b) samples exposed to laboratory light (fluorescent tubes) during incubation, and (c) samples exposed to 260-nm irradiation from the linear dichroism spectrometer during incubation.

RESULTS

Not Every Intercalator Promotes RecA Binding to Duplex DNA in the Absence of Cofactor. Thresher and Griffith (1990) screened a number of DNA intercalators and found that some but not all induced RecA binding to duplex DNA in the absence of cofactor. In an attempt to systematically determine what features are required of an intercalator to promote RecA binding, we have examined the effects of related ligands in three classes of intercalators: acridines, phenanthridines, and cyanines (Figure 1).

Binding of RecA to dsDNA results in an enlargement of the negative LD signal at 260 nm with time (Takahashi et al., 1989b), as previously observed when RecA was added to an EB/DNA mixture (Kim et al., 1993a). This increase is certainly due to the formation of a RecA−DNA complex which is accompanied by stiffening of the DNA, thus generating an LD signal of large intensity; the increase in LD magnitude is particularly dramatic at mild shear gradients where uncomplexed DNA exhibits almost no LD signal.

At a binding ratio (r) of 0.25 the duplex should be essentially saturated with intercalator (nearest-neighbor exclusion), and the effect of various ligands on the interaction of RecA and dsDNA at this binding ratio are shown in Figure 2. The three monointercalators with no bulky side chains induce RecA binding quite efficiently, while of the monointercalators with aminoalkyl side chains, only 9AA-PRO causes RecA binding and that is relatively inefficient. The cyanine dimer TOTO does not induce any RecA binding at all, while $9AA_2$ is approximately as efficient as its monomer, and EB_2 induces binding only inefficiently. At a lower binding ratio of 0.05 the same trend was observed but with different kinetics. In addition to these classes of ligands, the effects of two other ligands were tested at $r = 0.20$—DAPI, a minor-groove binder, and quinacrine, an acridine intercalator with a bulky side chain (Figure 1). Neither ligand promoted any binding of RecA to DNA in the absence of cofactor.

Since the acridine family of dyes appeared to be the most efficient at inducing RecA binding, these ligands were

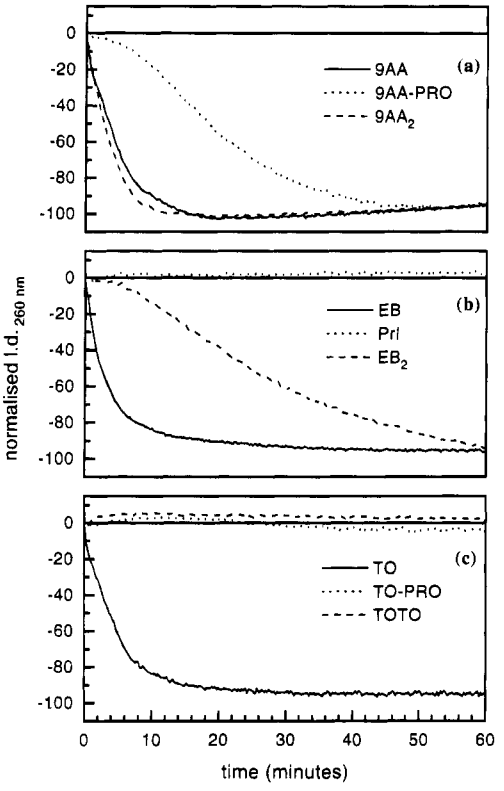

FIGURE 2: Time dependence of LD signal at 260 nm after addition of RecA to DNA:intercalator complexes in the absence of ATPγS. (a) Acridine dyes, P/D = 4. (b) Phenanthridine dyes, P/D = 4. (c) Cyanine dyes, P/D = 4. [DNA] = 20 μM, [RecA] = 7 μM, buffer is 5 mM cacodylate/20 mM NaCl (pH 6.5), shear gradient = 400 s^{-1}. To facilitate comparison of various data, amplitudes of the signals for samples where the LD increased were normalized at 60 min. Signals which showed no increase were scaled by the same factor as the signal for the analogous monointercalator.

subjected to further studies to characterize the ternary complex RecA−dsDNA−intercalator. Of particular interest was the comparative behavior of the mono- and bisintercalators which might provide insight into the basic interaction of the RecA subunit with DNA.

Effects of UV/Vis Irradiation on Intercalator-Induced Binding of RecA to dsDNA. Thresher and Griffith (1990) showed that the binding of RecA to dsDNA induced by intercalators is strongly affected by irradiation of the samples, even with laboratory light. They attributed the deleterious effects of light to photoinduced inactivation of RecA by the dyes, probably *via* a free radical mechanism. Since in the LD method the sample is exposed to spectrometer 260-nm radiation during the course of the incubation, it is possible that the observed effects (either the ability of some dyes to induce binding or the inability of others) could result from photochemical reactions in the system. For instance, these dyes could photosensitize DNA damage (Kochevar & Dunn, 1990) which RecA might recognize.

Samples of DNA:9AA were incubated with RecA (conditions as in Figure 2) for 3 h under three sets of lighting conditions: (a) in darkness, (b) in the LD spectrometer with the kinetics monitored at 260 nm, and (c) exposed to broad-spectrum laboratory light (fluorescent tubes). LD spectra recorded after incubation (data not shown) confirmed the observation of Thresher and Griffith (1990) that exposure to UV/vis radiation results in lower yields of the RecA−DNA−intercalator filament. Careful examination of Figure 2a reveals, in fact, that after reaching a maximum the LD signal decreases slowly and such behavior was observed to continue slowly with extended monitoring. After 3 h of incubation with 260-nm monitoring, the LD spectrum resembled that of the sample incubated in the dark but with an amplitude about 30% lower. By contrast, for the sample incubated with laboratory light exposure, the LD spectrum remaining after 3 h was like that recorded before RecA addition, indicative of no net filament formation, consistent with the observations of Thresher and Griffith (1990). However, the time scale of filament degradation due to the spectrometer 260-nm irradiation is much slower than that due to laboratory irradiation and does not significantly affect interpretation of the data presented here, which is largely qualitative. According to the data of Thresher and Griffith (1990), even with laboratory irradiation which rapidly degrades RecA, approximately 100% coverage of the DNA is achieved in most cases before degradation becomes apparent. We believe that with the less intense spectrometer irradiation, this is also likely to be the case.

A similar experiment was carried out for the DNA/PrI system, where no induction of RecA binding was observed. In this case, the exposure of the sample to light during incubation had no effect on the LD spectrum recorded after 3 h. Hence, the inability of certain dyes to induce RecA binding is not likely to be related to either DNA or RecA photosensitized damage.

Samples of 9AA−DNA were then incubated for 3 h without RecA under the same three irradiation conditions. RecA was then added and the samples were incubated for a further 3 h in darkness. LD spectra recorded at the end of this incubation period were almost identical, demonstrating that RecA recognizes DNA modified by dye intercalation rather than photosensitized damage.

Dependence of RecA Binding to Duplex DNA on the Binding Ratio of Acridines. Both the kinetics and the extent of RecA binding induced by acridines were found to depend on the ligand binding ratio; this was similar to previous observations with EB (Kim et al., 1993a). The rate of increase of the LD signal varied with DNA:intercalator ratio and became greater at higher binding ratios for both the mono- and bisintercalators (Figure 3). The extent to which the LD was increased also became larger at higher DNA:intercalator ratios, which is evident from the variation of the final LD amplitude with binding ratio (Figure 4).

DNA binding promoted by these intercalators occurred faster than cofactor-induced DNA binding at pH 6.5 and, notably, exhibited little lag time, in contrast to the nucleotide-induced binding (compare Figures 3 and 6), particularly at higher ligand concentrations. $9AA_2$ induces RecA binding significantly faster than 9AA (Figure 3, panels b and a, respectively), although the variation of final amplitude with binding ratio (Figure 4) is very similar for the two compounds when corrections are made for the estimated concentration of bound ligand (see Materials and Methods).

The observation that acridines induce RecA binding in the absence of nucleotide cofactor confirms our previous conclusion (Kim et al., 1993a) that some structural change of dsDNA, induced by intercalation of a ligand, promotes the binding of RecA to dsDNA. The shortening of the initial lag time when acridines induce binding of RecA suggests that the same structural change may be the limiting step of cofactor-induced dsDNA binding of RecA.

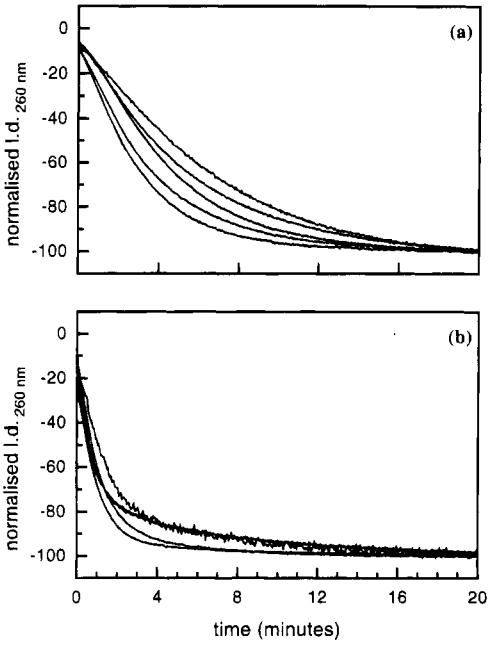

FIGURE 3: Variation of LD signal at 260 nm with time after RecA was mixed with DNA:acridine complexes in the absence of ATPγS. (a) 9-Aminoacridine: [9AA] = 5.0, 6.0, 8.0, 9.0, and 10.0 μM, respectively, from slow to fast kinetics. (b) 9-aminoacridine dimer: [$9AA_2$] = 0.5, 1.0, 2.0, 3.0, and 3.5 μM, respectively, from slow to fast kinetics. [DNA] = 20 μM, [RecA] = 7 μM, [$MgCl_2$] = 1 mM, buffer is 5 mM cacodylate/20 mM NaCl (pH 6.5), shear gradient = 450 s^{-1}. The LD amplitudes were normalized at 25 and 30 min, respectively, in (a) and (b).

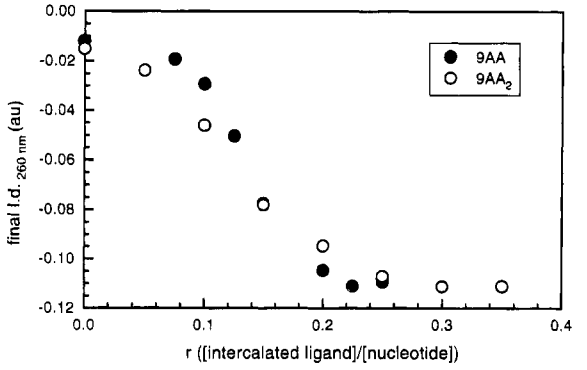

FIGURE 4: Final LD amplitudes at 260 nm of the RecA:DNA:9AA (●) and the RecA:DNA:$9AA_2$ (○) mixtures at various estimated acridine:DNA binding ratios. [DNA] = 20 μM, [RecA] = 7 μM, [$MgCl_2$] = 1 mM, buffer is 5 mM cacodylate/20 mM NaCl (pH 6.5), shear gradient = 1800 s^{-1}. [9AA] = 0, 1.5, 2.0, 2.5, 3.0, 4.0, 4.5, and 5.0 μM (assuming *ca.* 50% is bound; see Materials and Methods) and [$9AA_2$] = 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 μM.

RecA−dsDNA Filaments Retain the Intercalator with both Ligand and Base Pairs Oriented Perpendicular to the Helix Axis. Linear dichroism spectroscopy is an excellent technique for obtaining information about the conformation of a DNA−ligand complex (Nordén et al., 1992b). The DNA base pairs are stacked with their planes approximately perpendicular to the DNA helical axis (*i.e.*, to the flow direction); hence, the LD signal due to $\pi \rightarrow \pi^*$ transitions of the DNA bases is negative (Nordén et al., 1992b). The in-plane transition moments of an intercalated ligand also produce negative LD signals because the plane of the intercalator is parallel to that of DNA bases and is thus perpendicular to the flow direction (Nordén et al., 1992b).

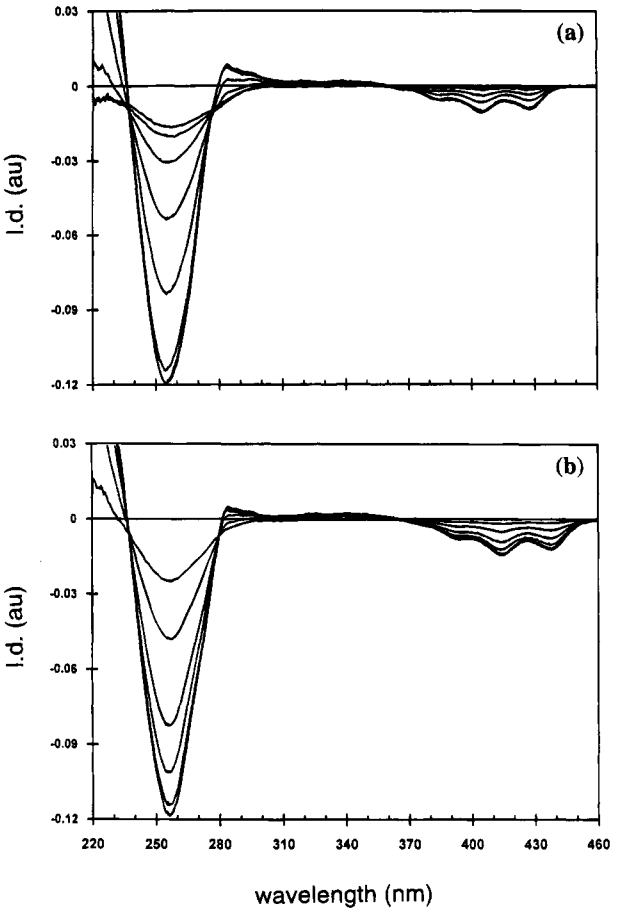

FIGURE 5: Linear dichroism spectra of RecA:DNA:acridine mixtures in the absence of ATPγS after 30 min of incubation. (a) 9-Aminoacridine: [9AA] = 0, 3.0, 4.0, 5.0, 6.0, 8.0, 9.0, and 10.0 μM (uncorrected for fraction of dye bound). (b) 9-Aminoacridine dimer: [$9AA_2$] = 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 μM, respectively, from bottom to top for each ligand. In both panels, the spectra at the two highest ligand concentrations are indistinguishable. [DNA] = 20 μM, [RecA] = 7 μM, [$MgCl_2$] = 1 mM, buffer is 5 mM cacodylate/20 mM NaCl (pH 6.5), shear gradient = 1800 s^{-1}.

The LD spectra around 260 nm of the RecA/DNA/9AA mixtures (Figure 5a) are negative; in this absorption region the signal from the DNA bases is dominant. Therefore, the negative signal indicates a rather perpendicular orientation of the DNA bases. Furthermore, the spectra are negative in the absorption region between 370 and 460 nm, where only the ligand among the constituents of mixture exhibits absorption [arising from short-axis polarized transitions (Matsuoka & Nordén, 1982)]. Strong absorptions of the long-axis polarized transitions (240−280 nm) are hidden under the DNA absorption, making it difficult to determine their orientation. However, the final LD amplitude at 260 nm for the fully formed RecA−DNA−$9AA_2$ complex is more negative than that for the RecA−DNA−ATPγS complex (Figure 7), suggesting that the acridine chromophores also make a strong negative contribution in this short-wavelength region of the spectrum. Hence, the ligand is also oriented in the plane rather perpendicular to the helix axis, an observation which is compatible with an intercalative geometry of the ligand. This indicates, therefore, that the binding of RecA modifies neither the binding geometry of the ligand nor the structure of DNA significantly. The same conclusion is reached for the complex with

the monointercalator, where the spectra are also entirely negative (Figure 5b). Both complexes exhibit positive LD signals around 295 nm where the absorption from the protein (due to tryptophan residues) is dominant. This positive sign is consistent with an orientation of RecA tryptophan residues rather parallel to the helix axis, as previously observed for the cofactor-induced RecA−DNA complex (Hagmar et al., 1992).

The final LD intensities (*i.e*, when no further changes occurred with time) in both the nucleobase absorption (around 260 nm) and the ligand absorption (around 400 nm) regions increase as the ligand concentration is raised. Since the LD intensity increases at 405 nm as well as at 260 nm when RecA is added, this indicates that the ligand is intimately involved in the RecA−dsDNA filament and does not simply remain bound to uncomplexed regions of the DNA. Hence, as observed previously in the case of ethidium bromide (Kim et al., 1993a), when 9AA and $9AA_2$ induce RecA association in the absence of ATPγS, the bound ligand remains in its intercalation pocket within the helical RecA−DNA filament.

The LD spectrum in the ligand absorption region exhibits negative peaks at 385, 405, and 428 nm for the RecA−DNA−9AA complex while these peaks are red-shifted by 8 nm for the RecA−DNA−$9AA_2$ complex. The binding geometries of the chromophores in the mono- and bisintercalators may be slightly different since the reduced linear dichroism spectra for the two compounds when bound to DNA are not identical (Hansen et al., 1984); a somewhat different binding geometry for the dimer might occur to facilitate better the accommodation of the rather short linker to allow for bisintercalation.

Acridines Inhibit RecA−ATPγS Binding to dsDNA and Are Extruded from Their DNA Binding Sites in the Presence of ATPγS. Ethidium bromide only slightly alters the rate of cofactor-induced DNA binding of RecA at pH 6.5 but is extruded from the intercalation pocket upon the binding of RecA under these conditions (Kim et al., 1993a). By contrast, when DNA is complexed with either 9AA or $9AA_2$, the association of RecA with DNA in the presence of cofactor, ATPγS, becomes significantly slower than in the absence of these ligands (Figure 6). Both ligands also increase the initial lag time of the RecA−DNA association.

In the presence of 9AA and $9AA_2$, the incubation times required to achieve half the maximum LD amplitude were about 1.5 and 3 times longer, respectively, than in the absence of ligand. In the presence of 9AA and $9AA_2$ the initial lag time also increased (about 400 and 700 s, respectively, compared to *ca.* 100 s with no dye). Thus, both 9AA and $9AA_2$ act as inhibitors of ATPγS-promoted RecA association.

This inhibiting effect was common to all the ligands studied, and those which did not induce RecA binding in the absence of cofactor had particularly strong inhibiting effects on the association of RecA in the presence of ATPγS. Furthermore, all ligands were extruded from their binding sites on association of RecA−ATPγS, even those that were not intercalated (Tuite and Sehlstedt, unpublished results).

The inset of Figure 6 shows the final LD spectrum for the complex formed when RecA with ATPγS is added to a DNA−$9AA_2$ complex (an identical spectrum is seen with 9AA). The negative LD signal in the region between 370 and 460 nm was insignificant compared with the RecA−DNA−$9AA_2$ complex (compare with Figure 5). Furthermore, addition of ATPγS to the preformed RecA−DNA−$9AA_2$ complex results in an extrusion of the ligand and a final LD spectrum like that of RecA−DNA−ATPγS. These results demonstrate that the intercalated ligands are extruded from DNA upon binding of RecA with ATPγS. There appears to be, therefore, some form of competition between RecA and ligands for DNA binding in the presence of ATPγS. This suggests that intercalation of one or more amino acid residues of RecA may occur in nucleotide-induced binding to dsDNA.

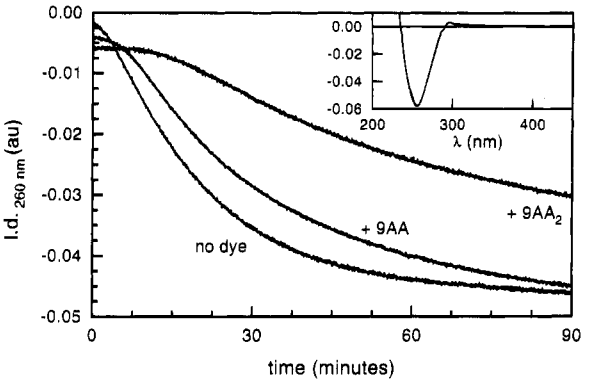

FIGURE 6: Variation of LD at 260 nm, reflecting kinetics of RecA:DNA complex formation in the presence of ATPγS with and without the monomeric and dimeric acridine dyes. Inset: LD spectrum of the RecA:DNA:$9AA_2$ mixture after completion of the complexation reaction in the presence of ATPγS. Absence of significant negative signal around 400 nm (by contrast with Figure 5) demonstrates that the intercalator has been extruded from the DNA. The chromophore to nucleobase ratio is 0.4 for both ligands. [DNA] = 20 μM, [RecA] = 7 μM, [ATPγS] = 60 μM, buffer is 5 mM cacodylate/20 mM NaCl (pH 6.5), shear gradient = 450 s^{-1}.

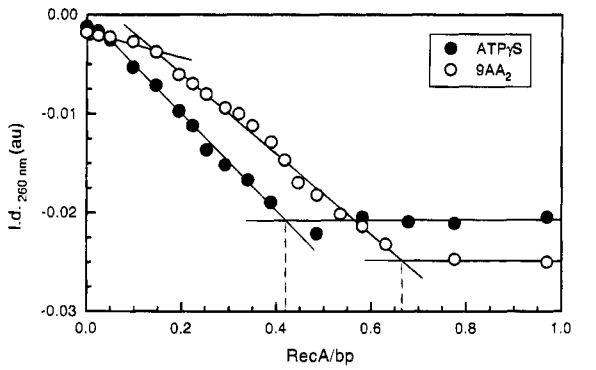

FIGURE 7: Variation of the LD signal at 260 nm with RecA to DNA ratio for complexation induced by either 9-aminoacridine dimer or ATPγS. The DNA concentration was kept constant at 40 μM and RecA was added as required to a mixture of DNA, Mg^{2+} (2 mM), and either $9AA_2$ (5 μM) or ATPγS (60 μM). The LD amplitude of each sample was measured after 2.5 h of equilibration. Shear gradient = 150 s^{-1}.

Stoichiometry of the RecA−dsDNA Complex Induced by an Intercalator. In order to further characterize the ternary RecA−DNA−intercalator complex, the RecA:dsDNA binding stoichiometry was determined for the complexes induced both by ATPγS and by $9AA_2$ (Figure 7). It is quite clear that the RecA:bp ratio is higher in the $9AA_2$-induced filament than in the ATPγS-induced filament. In fact, the difference is a factor of about 1.5 (9AA and $9AA_2$ were found to behave similarly; data not shown). Analyzing the data gives a bp:RecA ratio of *ca.* 2.4 for the ATPγS-induced filament. This is somewhat lower than the value of 3 which was expected

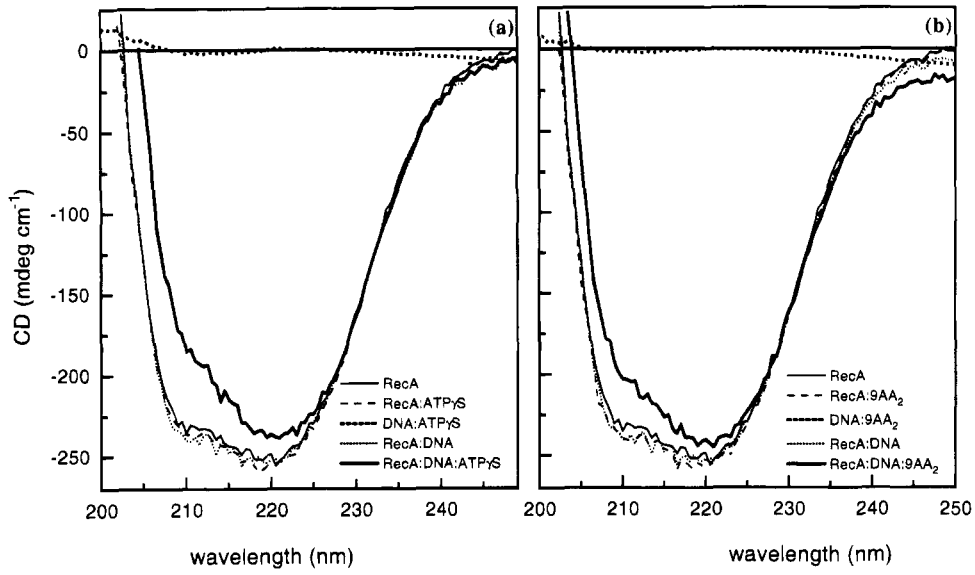

FIGURE 8: CD spectra of RecA:DNA complexes induced by ATPγS or $9AA_2$ under the conditions used in the LD experiments. (a) RecA:dsDNA:ATPγS system. (b) RecA:dsDNA:$9AA_2$ system. [ATPγS] = 60 μM, [$9AA_2$] = 5 μM, and for all spectra, [RecA] = 7 μM, [DNA] = 40 μM, and [Mg^{2+}] = 2 mM.

and which has been observed using different techniques (DiCapua et al., 1982; Dombroski et al., 1983; Takahashi et al., 1989b). This may be due to some of the RecA being inactive in the preparation used. From Figure 7, we obtain bp:RecA = 1.5 for the $9AA_2$-induced filament; when we correct for the inactive RecA (× 3/2.4), we obtain a value of 1.9. This is approximately equivalent to 1 RecA bound/acridine chromophore, suggesting a monointercalation requirement for binding of each RecA subunit.

Conformational Effects Associated with $9AA_2$- and ATPγS-Induced RecA−DNA Filament Formation. It was recently shown by CD (Wittung et al., 1995) that significant protein conformational changes do not occur when RecA binds ATPγS but rather when the RecA−ATPγS complex binds dsDNA. It was thus of interest to investigate whether any such changes accompany acridine-induced RecA binding to dsDNA, and similar experiments were carried out with $9AA_2$ replacing the nucleotide cofactor.

In the 200−250-nm region of the RecA spectrum, CD arises principally from the chiral arrangement of peptide chromophores and therefore reports on the secondary RecA structure. In this wavelength region, the CD spectra of the dsDNA/ATPγS and dsDNA/$9AA_2$ mixtures are hardly significant compared to that of the protein (Figure 8). Hence, any changes observed at these wavelengths are attributed to modification of RecA structure upon binding of dsDNA (Wittung et al., 1995). Figure 8 shows that RecA spectral changes did indeed accompany formation of the RecA−dsDNA−$9AA_2$ filament. The changes were similar to those occurring with ATPγS but of a slightly lower magnitude. Notably, similar changes had also been observed on binding of single-stranded poly(dT) by RecA in the presence of ATPγS (Wittung et al., 1995).

DISCUSSION

Structural Requirements of an Intercalator for Induction of RecA Binding to dsDNA in the Absence of Cofactor. Several intercalators are shown here to mimic the behavior of ethidium bromide (Thresher & Griffith, 1990; Kim et al., 1993a) and induce the bindig of RecA to dsDNA in the absence of cofactor. However, not all the intercalating ligands tested were capable of performing this function, which poses the question: what features differentiate these two types of ligand?

The three simple intercalators—9AA, TO, and EB—were all efficient at inducing RecA binding, but when their basic chromophores were modified with aminoalkyl side chains, which we believe all lie in the minor groove, only the acridine induced any RecA binding and relatively inefficiently. Considering the structures of the side chains (Figure 1), the common feature of TO-PRO and PrI is that they have bulky groups, methyl and ethyl, respectively, branching from the main chain. This differentiates them from 9AA-PRO, which has only a single methyl group at the end of the chain. It is quite telling, then, that when a different acridine with a bulky side chain—quinacrine—is used, it does not induce RecA binding in the absence of cofactor. For ligands with side chains in the minor groove, it is reasonable that bulky side chains interfere with induction of RecA binding since the bulky minor-groove-binder DAPI does not induce RecA−dsDNA binding. One anomalous result then, is that ethidium bromide, which has a phenyl group in the minor groove, does induce RecA binding; however, this group may be aligned in the groove in a different manner than the side chains so that it does not interfere with RecA.

The idea that it is the bulk of the side chain which determines the ability of an intercalator to induce nucleotide-free RecA binding is borne out by the behavior of the bisintercalators. TOTO, which has a side-chain structure similar to TO-PRO, does not induce binding, while EB_2, which has a significantly less bulky side chain than PrI, can induce binding although relatively inefficiently. $9AA_2$ has a nonbulky side chain like 9AA-PRO and is even more efficient at inducing RecA binding. In fact, the number of atoms in the $9AA_2$ side chain approaches the lower limit required for bisintercalation and will probably be held quite rigidly within the groove, whereas the side chain of 9AA-PRO may be more loosely bound and have the possibility of fluctuating out into solution. Following this line of

thought, the longer EB_2 side chains may not be held so tightly in the DNA groove and this may explain why it interferes more with the RecA binding.

Another factor influencing RecA binding could be the charge on the side chain, since one $9AA_2$ side chain will have a lower charge than two 9AA-PRO side chains. However, the EB_2 side chain will have the same charge as two PrI side chains and still promotes RecA binding more efficiently, evidencing that side chain charge is not a critical determinant.

Thus, in summary, these results, indicating that the presence of a bulky group in the minor groove prevents the binding of RecA to dsDNA, are compatible with the idea that RecA interacts with the minor groove (DiCapua & Müller, 1987). Interesting points to note are that the presence of relatively nonbulky side chains or positive charges in the groove do not prevent the RecA−DNA interaction. This may indicate that RecA does not make contact with DNA deep within the groove and that the interaction is not primarily ionic in nature.

Is a RecA Amino Acid Residue Intercalated? The data present here for several ligands confirm a generality in the previous observation (Thresher & Griffith, 1990; Kim et al., 1993a) that intercalators can induce binding of RecA to dsDNA in the absence of nucleotide cofactor. In both RecA−dsDNA−ATPγS and RecA−dsDNA−intercalator complexes, the DNA is lengthened by about 50% overall (Thresher & Griffith, 1990). The intercalators remain bound in their DNA sites within the RecA−dsDNA filament, accounting for the DNA extension in that filament. This strongly suggests that the intercalating ligands mimic some intercalative interaction of RecA, enabled by ATPγS binding to the protein; recent studies have shown that the nucleotide cofactor itself does not intercalate (Takahashi & Nordén, 1994a). RecA binds ATPγS with a 1:1 stoichiometry (Cotterill et al., 1982) and this interaction causes no change in the protein secondary structure (Wittung et al., 1995). However, binding of ATPγS certainly induces some change in the RecA conformation since certain amino acid residues become more susceptible to chemical/enzymatic modification (Kobayashi et al., 1987; Takahashi & Nordén, 1993). ATPγS binding may therefore change the RecA conformation in a manner that realigns certain aromatic amino acids, so enabling them to intercalate in DNA.

The fact that intercalators are extruded from their DNA binding sites when RecA binds in the presence of ATPγS suggests some competitive aspect reflecting the displacement of the intercalator by some amino acid residue. However, more recent studies have shown that other DNA ligands bound in both the minor and major grooves are also displaced upon binding of RecA−ATPγS (Tuite and Sehlstedt, unpublished results), which suggests that ligand extrusion may be more generally associated with conformational changes of DNA accompanying RecA binding (*e.g.*, unwinding). Such DNA conformational changes (Wittung et al., 1995) must be quite subtle since LD and neutron scattering studies have shown that the DNA bases remain essentially perpendicular to the helix axis in the nucleotide-induced filament (Nordén et al., 1990; Nordén et al., 1992a).

However, one critical experiment has shown that the RecA−dsDNA−ATPγS filament can tolerate ligands bound in the minor groove but not those intercalated—when RecA−ATPγS binds to dsDNA covalently modified with (−)-*anti*-BPDE, which is intercalated, the ligand is extruded into a minor-groove binding site similar to that of (+)-*anti*-BPDE (Kim et al., 1993b). This is the strongest support for a model in which RecA needs access to the base-pair pocket in order to bind dsDNA in the presence of ATPγS.

Nature of the Intercalator-Induced RecA−dsDNA Filament. From the electron microscopy work of Thresher and Griffith (1990) we know that, like other RecA structures, the RecA−dsDNA complex induced by ethidium bromide is a right-handed helical filament, although the dimensions are somewhat different from those of the ATPγS-induced filament. This is also the case in solution, as recently observed by small-angle neutron scattering (Wittung et al., unpublished results), where a smaller pitch was observed when the filament was induced by EB compared to ATPγS.

Previous work using LD spectroscopy (Kim et al., 1993a) demonstrated that EB was retained within the RecA−dsDNA filament such that both the ligand and the base pairs were oriented essentially perpendicular to the helix axis. The results presented here show that this is also the case with both 9AA and $9AA_2$ and similar results were found for all the intercalating ligands which promoted RecA−dsDNA binding in the absence of ATPγS (data not shown). As with EB, it has been shown here that for complete binding of RecA to dsDNA in the absence of cofactor the DNA must be fully saturated with intercalator and thus extended by 50%. The final LD intensity decreases as the intercalator:DNA binding ratios decreases below 0.25, indicating that the DNA is only partly covered by RecA under such conditions. Hence, RecA appears to bind only to regions of the DNA which have already been extended by the intercalating ligand, suggesting that ligand-induced filament formation is indeed polymerization-limited rather than nucleation-limited, in aggreement with Thresher and Griffith (1990), who saw multiple filament tracts on each DNA molecule with intercalator but single tracts on each DNA molecule with ATPγS. Unfortunately, it is not possible to determine conclusively from LD data (which represent only an average signal) whether there is a cooperative effect which encourages the intercalator to bind adjacent to RecA−dsDNA tracts to facilitate extension of polymerization. A strong increase in LD is observed only at binding ratios above 0.08 (*ca.* 6 bp/ligand) (Figure 4). This corresponds to approximately 30% coverage of the DNA molecules with randomly distributed acridine chromophores (site size = 2 bp). If the advancing RecA polymer could induce intercalators to bind adjacent to it, an LD signal closer to 30% of the highest value might be expected. However, the magnitude of the LD signal is not necessarily linearly related to the length of DNA (Nordén et al., 1992b), and relatively long tracts of DNA could potentially be covered before an LD signal is strongly manifest.

We have found here that the fundamental nature of the intercalator-induced RecA−DNA filament is different to the cofactor-induced filament by comparing the protein subunit:base pair ratio in both cases. The filament induced by $9AA_2$ has a RecA:bp ratio which is only *ca.* $^2/_3$ that of the ATPγS-induced filament. Since it has been well established that the nucleotide-induced filament contains 3 base pairs/RecA, this implies a ratio of 2 base pairs/RecA in the intercalator-induced filament. In other words, in a fully formed filament, one RecA subunit will bind for every intercalator bound. This leads to a tentative suggestion by analogy that in the

Table 1: Summary of Characteristics of Various RecA Filaments

filament	pitch (Å)	diameter (Å)	b(p)/RecA[a]	bases oriented?	b(p)/turn	rise/RecA[b] (Å)	rise/bp[c] (Å)
RecA−ssDNA	75	110−120	4−5	no	24−30	12.5	2.8
RecA−ds/ssDNA−ATPγS	95	100	3	yes	18.6	15.3	5.1
RecA−dsDNA−EB	80	130	2[d]	yes	ca 16[e]	*ca.* 10[f]	4.9
RecA−ADP (crystal)	83	120				13.8	

[a] 10.5 bp/turn in B-DNA. [b] *ca.* 6 RecA/turn in all filaments, where reported. [c] 3.4 Å axial rise/bp in B-DNA. [d] As reported herein. [e] Estimated from pitch and rise/bp. [f] Estimated from rise/bp and bp/RecA.

ATPγS-induced filament, each RecA subunit might bind with one nonclassically intercalated moiety (with greater than 50% lengthening) every 3 base pairs.

Comparison of RecA−dsDNA−Intercalator Complexes with Other RecA Filaments. Since we have now characterized the stoichiometry of the intercalator-induced filament, it is instructive to compare it with other types of RecA filament to place these results in a structural context. For this purpose we have compiled a table of the average characteristics of the different filaments from reports in the literature (Table 1) (Takahashi & Nordén, 1994b; Egelman & Stasiak, 1993; Thresher & Griffith, 1990).

RecA can bind ssDNA both in the absence and in the presence of ATP but requires nucleotide cofactor to bind dsDNA. However, the ssDNA filaments with (*i.e.*, active) and without (*i.e.*, inactive) cofactor are somewhat different (Egelman & Stasiak, 1993); although each has *ca.* 6 RecA subunits/turn, the filament is more extended, the diameter is smaller, and the base pair:RecA stoichiometry is lower in the presence of cofactor (95-Å pitch, 100-Å diameter, 3 well-oriented bases/RecA) than in its absence (75-Å pitch, 110−120-Å diameter, 4−5 randomly oriented bases/RecA) (Takahashi et al., 1989b; Chabbert et al., 1991; Yu & Egelman, 1992; Wittung et al., unpublished results). The two types of filament are not simply interconvertible since depolymerization and reconstruction is observed to occur on addition of ATPγS to the RecA−ssDNA filament (Lee & Cox, 1990; Yu & Egelman, 1992).

The RecA−dsDNA−ATPγS and the RecA−ssDNA−ATPγS filaments (both active) are very similar, while in several respects the RecA−dsDNA−EB filaments appear quite similar to those of the inactive RecA−ssDNA filament. As with the inactive filament, the pitch of the intercalator-induced filament is smaller and the diameter is larger than the active filament (Thresher & Griffith, 1990, Wittung et al., unpublished results). Furthermore, Thresher & Griffith (1990) observed that depolymerization occurs when ATPγS is added to the RecA−dsDNA−EB filament, and we observed a repolymerization to form the RecA−dsDNA−ATPγS filament with the ligand extruded. However, there are differences such as stoichiometry, base separation, and base orientation which show that the RecA−ssDNA and RecA−dsDNA:intercalator filaments are not the same from the viewpoint of DNA structure, although the RecA framework could well be similar.

The same type of CD changes were observed for formation of RecA−dsDNA−$9AA_2$ and RecA−dsDNA−ATPγS filaments, and similar changes were previously observed for binding of RecA to poly(dT) in the presence of ATPγS (Wittung et al., 1995); these changes may reflect RecA−DNA interactions or RecA−RecA interactions arising from protein polymerization. If they arise from RecA−DNA interactions, this suggests that the protein−DNA contact is similar in the filaments induced by both ATPγS and intercalators.

Different Roles for ATPγS and Intercalators in Binding of RecA to dsDNA. The role of ATPγS is to bind to RecA rather than DNA and somehow modify its structure so that it can accommodate dsDNA. This modification does not affect the α-helical content of RecA since the CD spectrum of RecA in the 200−250-nm region is not modified when the cofactor binds but rather when DNA binds (Wittung et al., 1995). The role of intercalator, on the other hand, is to bind to dsDNA and extend it by 50% overall so that RecA, which has not been modified with ATPγS, can bind. Moreover, RecA modified with ATPγS cannot bind the DNA−intercalator complex intact but rather displaces the intercalator from its DNA binding site. Although there appears to be some competitive aspect to formation of the RecA−DNA−ATPγS and RecA−DNA−intercalator filaments, the somewhat different structures indicate that the roles of nucleotide cofactor and intercalator are not identical. Notably, intercalators were found to inhibit the binding of RecA to ssDNA in the absence of ATPγS (Thresher & Griffith, 1990), which further suggests that ATPγS and intercalators do not play identical roles in filament formation.

Acridine-induced binding of RecA to dsDNA is faster than ATPγS-induced binding and careful examination shows a significantly shorter initial lag time in the presence of 9AA and $9AA_2$ compared to ATPγS. The stimulating effect increases with acridine concentration and $9AA_2$ induces notably faster binding than 9AA. These observations suggest that destacking and/or unwinding is an important prerequisite for the RecA−dsDNA interaction and is probably related to the initial slow RecA−dsDNA association step. Conformational differences between the 9AA−DNA and $9AA_2$−DNA complexes may be part of the reason for the different stimulating effects. LD and CD studies showed quite similar nonclassical intercalation binding geometries for the two compounds with the long axis tilted relative to the DNA bases (Wirth *et al.*, 1988), but the larger LD red shift for the dimer (Figure 5) may indicate subtle differences in chromophore orientation that could facilitate RecA binding. Further, the two acridines in the dimer will bind close to each other, causing a larger local unwinding and elongation. However, we have found that the trisintercalating 9AA analogue only weakly induces RecA binding in the absence of nucleotide cofactor, suggesting that only a certain amount of DNA distortion associated with multiintercalation is compatible with RecA binding.

Studies are now in progress to determine whether intercalating ligands can also promote third-strand binding, to determine how closely the intercalator-induced filament really resembles that of the ATPγS-induced filament. Stud-

ies are also continuing to determine whether any RecA amino acid is intercalated in the RecA−dsDNA−ATPγS filamentous complex, with the aim of fully characterizing the structure of the active filament at the molecular level.

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