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Base Orientation of Second DNA in RecA·DNA Filaments

ANALYSIS BY COMBINATION OF LINEAR DICHROISM AND SMALL ANGLE NEUTRON SCATTERING IN FLOW-ORIENTED SOLUTION*

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To gain insight into the mechanism of pairing two complementary DNA strands by the RecA protein, we have determined the nucleobase orientation of the first and the second bound DNA strands in the RecA·DNA filament by combined measurements of linear dichroism and small angle neutron scattering on flow-oriented samples. An etheno-modified DNA, poly(dϵA) was adapted as the first DNA and an oligo(dT) as the second DNA, making it possible to distinguish between the linear dichroism signals of the two DNA strands. The results indicate that binding of the second DNA does not alter the nucleobase orientation of the first bound strand and that the bases of the second DNA are almost coplanar to the bases of the first strand although somewhat more tilted (60 degrees relative to the fiber axis compared with 70 degrees for the first DNA strand). Similar results were obtained for the RecA·DNA complex formed with unmodified poly(dA) and oligo(dT). An almost coplanar orientation of nucleobases of two DNA strands in a RecA-DNA filament would facilitate scanning for, and recognition of, complementary base sequences. The slight deviation from co-planarity could increase the free energy of the duplex to facilitate dissociation in case of mismatching base sequences.

The RecA protein plays a crucial role in homologous recombination in *Escherichia coli:* it regulates the synthesis of proteins involved in recombination reaction, including RecA itself, and catalyzes strand-exchange reaction (1). RecA can, in the presence of cofactor ATP, promote pairing of two complementary DNA strands (2) and also perform strand exchange between two homologous DNA molecules *in vitro* (3, 4). In these reactions, RecA first binds with high cooperativity to a single-stranded DNA (5) forming a nucleoprotein filament (6). In the complex, the protein monomers are arranged in a helical manner around the DNA, and the DNA is elongated about 50% in length. This RecA single-stranded DNA nucleofilament can then bind a second DNA (either single- or double-stranded DNA) and pair complementary parts.

From fluorescence measurements of various probes attached to the DNA bases, we have concluded that direct base-base interaction of the Watson-Crick type contributes to the sequence recognition by RecA (7, 8). Base-base interaction can occur between all three DNA strands in the RecA·DNA complex, supporting formation of a triplex DNA structure, as has been suggested by other studies (9, 10). Several models of different base-triads, involved in triplex-structure of recombination intermediates have been proposed (11, 12)

In order to investigate the base-base interactions and understand more about the mechanism of recognition between complementary DNA strands in RecA, we earlier assessed the orientation (roll and tilt angles) of DNA bases in RecA·DNA complexes by linear dichroism (LD)[1] spectroscopy combined with small-angle neutron scattering (SANS) and chromophore-replacement studies (13–15). LD measures, on an aligned sample, the absorption difference of light polarized parallel and perpendicular to the sample orientation direction (16). The signal is related to both the local orientation of the chromophore in the macromolecule and to the degree of alignment of the whole molecule. LD can therefore be used to estimate the local orientation of the DNA bases in the RecA·DNA nucleofilament once the degree of alignment (the orientation factor) of the complex filaments under LD measurement condition is known. The orientation factor can be independently determined by SANS measurements on the same sample aligned under the identical conditions (13).

Chromophore-replacement analysis has been used to estimate the LD signal of a given chromophore in a complex which has more than two chromophores exhibiting overlapping signals. The analysis is based on the replacement of one of the components in the complex by an analog whose spectroscopic character differs from that of the original chromophore. Comparison of original and replaced spectra allows determination of the signal of a particular constituent (*e.g.* DNA) despite overlapping signals from other chromophores of the complex (*e.g.* ATP and RecA).

Here we have determined selectively the orientation of the second DNA strand bound in a RecA·DNA filament using this approach. Poly(dϵA), absorbing at higher wavelengths than normal DNA (around 320 nm), was used as the first DNA strand in the RecA·DNA filament to be able to distinguish its LD signal from that of the second DNA (oligo(dT)) in the com-

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[1] The abbreviations used are: LD, linear dichroism; ATPγS, adenosine 5′-*O*-3-thiotriphosphate; poly(dϵA), poly(1,N^6-etheno-deoxyadenosine); SANS, small angle neutron scattering.

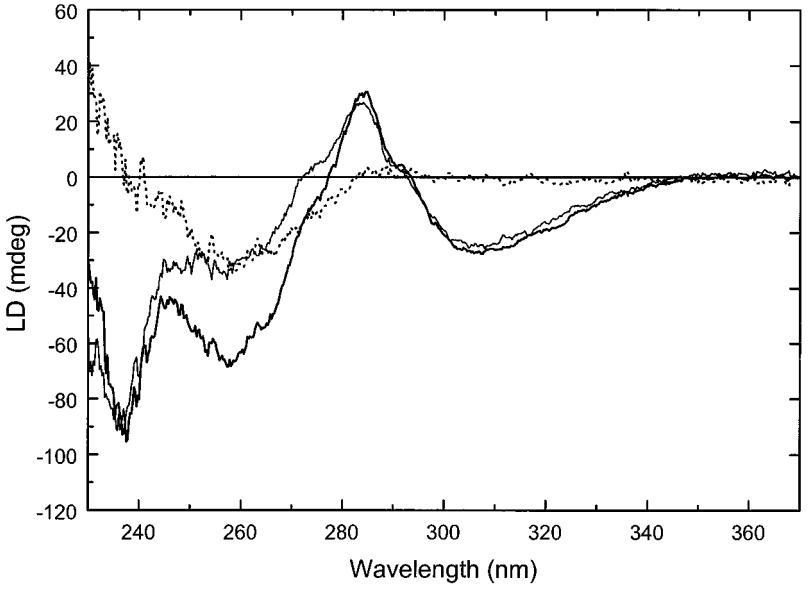

FIG. 1. **Linear dichroism spectra of RecA·poly(dϵA) and RecA·poly(dϵA)·oligo(dT) complexes.** Shown are the LD spectra of RecA·poly(dϵA) (*thin line*), RecA·poly(dϵA)·oligo(dT) (*thick line*) measured as described in the text, and the difference between these LD spectra (differential LD, *dotted line*) computed to assess the base orientation of oligo(dT) in the RecA·poly(dϵA)·oligo(dT) complex. The samples were diluted 1:5 for the LD measurements (10 μM RecA).

plex. The nucleobases of the second DNA are found to be rather immobile and oriented less perpendicular to the axis of the protein filament but still rather similar to the bases of the first DNA strand. An almost coplanar base arrangement could favor base-base contacts between two DNA strands in the RecA filament and may facilitate the search for complementary sequences within the DNA molecules.

EXPERIMENTAL PROCEDURES

RecA and poly(dϵA) were prepared as described previously (17, 18). Poly(dA) and oligo(dT) were from Amersham Pharmacia Biotech. The size of polynucleotides was about 350 bases and that of the oligonucleotides about 20 bases. ATPγS was from Boehringer Mannheim and freshly dissolved solution was used without further purification. The concentrations were determined from UV absorption with $\epsilon_{280\ nm}$ = 21700 M^{-1} cm^{-1} for RecA, $\epsilon_{264\ nm}$ = 8520 M^{-1} cm^{-1} in base for oligo(dT), $\epsilon_{260\ nm}$ = 8600 m^{-1} cm^{-1} in base for poly(dA), and $\epsilon_{257\ nm}$ = 3800 M^{-1} cm^{-1} in base for poly(dϵA).

RecA·DNA complexes were formed in 20 mM sodium phosphate, pH 6.8, with 50 mM NaCl, 1 mM $MgCl_2$, and 400 μM ATPγS, all in D_2O. Initially, RecA was dialyzed against the D_2O buffer mentioned above. A first RecA·DNA complex was formed by mixing 50 μM RecA and 150 μM poly(dϵA) in the above buffer and by incubation at room temperature. To form a complex with two DNA strands, oligo(dT) was added to the preformed RecA·poly(dϵA) complex and further incubated. In another experiment, RecA·poly(dA) complex was first formed, and subsequently oligo(dT) was added in stoichiometric amounts in order to form a RecA complex with two DNA strands.

Absorbance, LD, and SANS were measured on each sample. Absorbance was measured in a Cary 2000 spectrophotometer (Varian) with a bandwidth of 2 nm. Linear dichroism was measured in a modified Jasco J-500 spectropolarimeter (19) with a bandwidth of 2 nm. No smoothing procedure was applied to any of the spectra. SANS data were collected in the facility at Riso National Laboratory, Denmark. The sample to detector distance was 3.86 m, and neutron wavelength was 0.723 nm, as described in Nordén *et al*. (13). LD and anisotropic SANS measurements were performed on the sample aligned by an inner rotating Couette cell of the same dimension and with identical shear gradients, 620 s^{-1}.

Assessment of nucleobase orientation of DNA strands in RecA filaments was made as follows. LD is defined as the differential absorption, $LD = A_{parallel} - A_{perpendicular}$, between orthogonal forms of plane polarized light. The reduced dichroism, $LD^r = LD/A_{iso}$ for DNA (with A_{iso} the normal, isotropic absorbance) at a given wavelength, is related to the angle between the light-absorbing transition moments in the DNA and orientation axis of the RecA·DNA complex (*i.e.* the fiber axis) (16),

$$LD^r = 3/2\ S\ (3\cos^2\alpha - 1) \qquad \text{(Eq. 1)}$$

where S, the orientation factor describing the degree of alignment of the fibrous complex, is determined from SANS anisotropy data (13). For the RecA·DNA complex with poly(dϵA), LD^r could be straightforwardly determined for the 320 nm absorption band (polarized in the plane of the etheno-adenine chromophore (20)) since, at this wavelength, neither protein nor cofactor displays any interfering absorption. Combining LD^r data with SANS-determined orientation factor S allows us to estimate an effective value of α, the angle the nucleobase planes make with the protein-filament axis (see Fig. 3).

The SANS and LD samples referred to identical flow cell geometry and flow rates. But in order to not exceed a maximum absorbance of 2, some LD sample was diluted 1:5. Control experiments, in the absorption region where the absorbance did not exceed about 2 absorbance units, showed that the orientation of the RecA·DNA fibers was not significantly dependent on the concentration (14).

RESULTS

We first investigated whether the orientation of the nucleobases of a first bound DNA strand in a RecA·DNA filament is altered upon binding of a second DNA strand. We measured LD of RecA·DNA complexes with poly(dϵA) before and after addition of a second DNA, oligo(dT). Poly(dϵA) exhibits an extra absorption band above 320 nm in which wavelength region the signals from normal DNA, ATPγS, and RecA are negligible (21). The LD signal above 320 nm is, thus, only related to the orientation of etheno-adenine, with no interference from other components. As a second DNA, we used short oligonucleotides (oligo(dT) around 20 bases long) instead of polynucleotides, to avoid formation of network structures which may prevent orientation of the complexes in the shear flow (22).

The LD signal of the ATPγS·RecA·poly(dϵA) complex above 320 nm was negative (Fig. 1), as observed previously (23), in quantitative agreement with a rather perpendicular base orientation relative to the protein-filament axis. Upon addition of oligo(dT) to saturate the second DNA-binding site in RecA, the intensity of the negative signal around 260 nm increased, however, without modifying the LD signal in the region above 320 nm (Fig. 1). Binding of a second DNA to the RecA·DNA complex apparently does not affect the average orientation of the nucleobases of poly(dϵA) bound in the first DNA-binding site of RecA.

Thus, assuming an essentially unchanged base orientation of the first bound DNA strand, the base orientation of the second DNA strand in the complex could be assessed from the difference (LD spectrum of ATPγS·RecA·poly(dϵA)·oligo(dT) complex) – (LD spectrum of ATPγS·RecA·poly(dϵA) complex). This difference spectrum had a maximum centered around 260 nm and had a shape similar to that of the oligo(dT) absorption spec-

trum which is centered at 264 nm (Fig. 1). This observation of similarity between spectral shapes supports the idea that the increase in negative LD around 260 nm upon oligo(dT) binding to the RecA·poly(dϵA) complex is indeed due to the base orientation of the second DNA. Since the LD is negative, the nucleobases of the second DNA too are oriented rather perpendicular to the filament axis.

As mentioned under "Experimental Procedures," one can quantitatively determine the effective angle of the nucleobases relative to the orientation axis of the RecA·DNA filament from the LD signal, provided information about the degree of alignment of the overall fiber is available (see Fig. 3). From SANS anisotropy measured under identical flow conditions, the second-moment orientation function can be estimated from a comparison of the experimental SANS data with the anisotropy calculated for flow model orientation distributions (13). The SANS pattern of RecA·poly(dϵA) as well as of RecA·poly(dϵA)·oligo(dT) complexes exhibited a certain anisotropy in the presence of flow (Fig. 2). As a control that nonhomogeneous detector sensibility is not the cause of virtual anisotropy, the difference in SANS pattern (differential SANS) between flow-oriented and randomly oriented sample was recorded, showing a systematic difference (Fig. 2*B*). The measurements, in fact, show that the degrees of flow alignment of the RecA·poly(dϵA) and RecA·poly(dϵA)·oligo(dT) complexes were similar, corresponding to an orientation factor S in Equation 1 equal to about 0.12 ($\pm$ 0.02).

Using this value, we could compute the orientation angles of the first and second DNA from the LD data (Fig. 3). At a given degree of alignment of molecules (S), a reduced LD (LD/normal UV absorption) corresponds to an orientation degree of the chromophore to the alignment axis (angle α in Equation 1). The nucleobase orientation of first DNA (poly(dϵA)) was estimated from reduced LD at 320 nm and found to be approximately 70 degrees as observed previously (13). Because of uncertainty on the S value (0.12 $\pm$ 0.02), the orientation could be between 68 and 78 degrees (Fig. 3). Since we found that the base orientation of the first DNA strand (poly(dϵA)) was not altered upon the binding of the second DNA, it is reasonable to assume that the spectral difference (LD of RecA·poly(dϵA)·oligo(dT) complex – LD of RecA·poly(dϵA) complex) reflects directly the signal from oligo(dT) at the same S value. We thus estimated the reduced LD signal (at 260 nm) of oligo(dT) bound to the second site. The value of the reduced LD for oligo(dT) was significantly smaller than that for poly(dϵA). This is not an effect of partial binding of oligo(dT) to the RecA·poly(dϵA) filament because the titration controls showed that the second site was saturated at the addition of 3 bases/RecA of oligo(dT) (not shown), as expected for almost complete binding of oligo(dT). We therefore conclude that the bases of the second strand are on average more tilted than those of the first bound DNA strands. An angle of about 60 degrees was estimated relative to the long axis of the RecA filament (Fig. 3). Estimation is more precise at this α value (60 $\pm$ 2 degrees).

We also examined the complex formed between RecA and two complementary, unmodified DNA strands, poly(dA) and oligo(dT). LD spectra of ATPγS· RecA·poly(dA) complex before and after addition of oligo(dT) as well as the differential LD spectrum for the oligo(dT) are shown in Fig. 4. For a quantitative analysis, the orientation factors of each complex were also determined by SANS measurements on the same samples under the same conditions as for the LD measurements. In this case, however, we could not experimentally distinguish the signal of oligo(dT) from that of poly(dA). We must, therefore, assume that the binding of second DNA does not alter the base orientation of first DNA, as was the case of the Rec A·poly(dϵA)·oligo(dT) complex, to be able to estimate the base orientation of the second DNA.

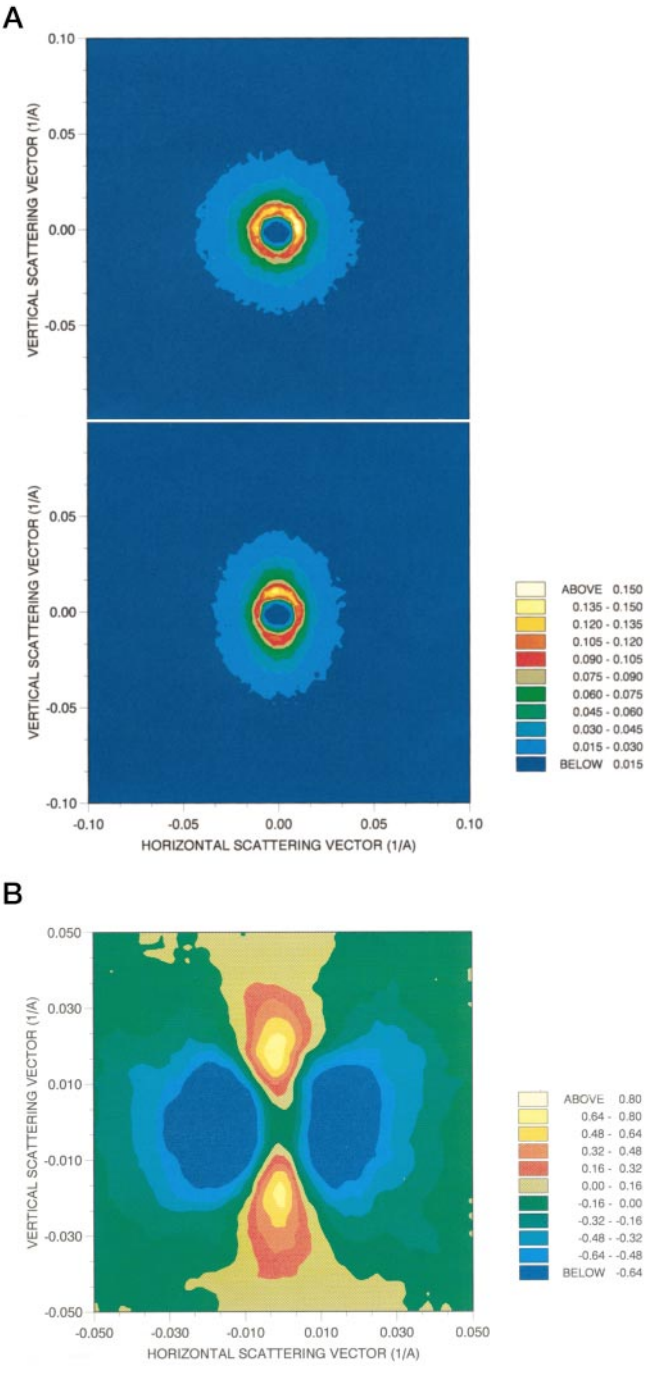

FIG. 2. **Small angle neutron scattering pattern of RecA·poly(dϵA) complex.** *A,* SANS of RecA·poly(dϵA) complex measured in a Couette cell with (*lower panel*) and without (*upper panel*) a shear flow gradient of 620 s^{-1}. *B,* differential SANS for RecA·poly(dϵA) complex (SANS at shear – SANS at rest) was computed.

The results show that the average orientation of the first DNA bases was about 68 degrees and that of the second DNA bases was 60 degrees relative to the filament axis. The results were thus very similar to those for the RecA-poly(dϵA)-oligo(dT) complex.

DISCUSSION

To gain better insight into the pairing mechanism of complementary DNA strands by RecA and the base-base interactions occurring between the two DNA strands in the RecA filament, we have investigated the orientation of DNA bases in the

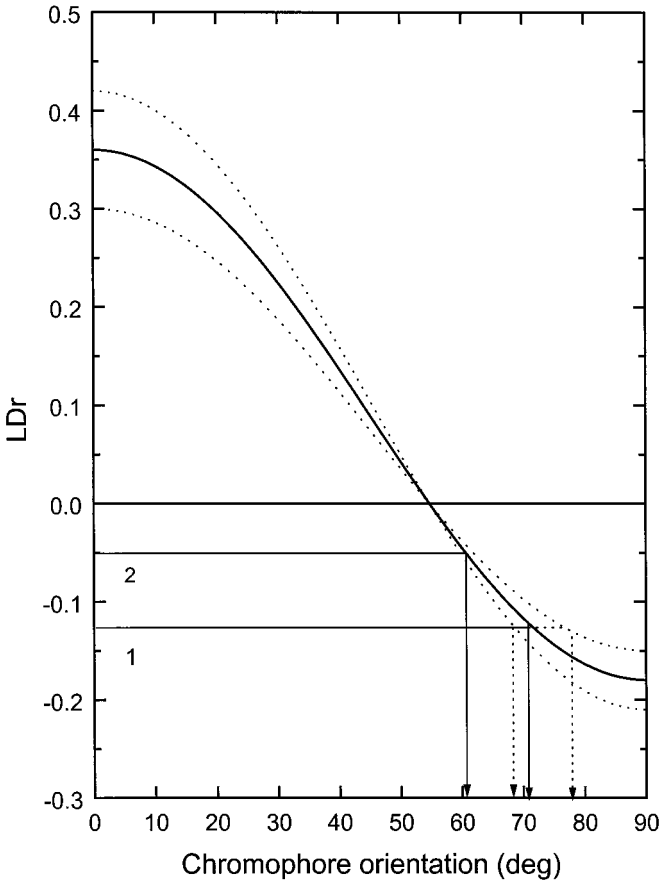

FIG. 3. **Determination of nucleobase orientation from the reduced linear dichroism values.** Theoretical variation of reduced dichroism value as a function of chromophore orientation (α) was computed for $S = 0.12$ (*continuous line*) as well as $S = 0.10$ and 0.14 (*broken lines*). *Arrow 1* corresponds to the reduced LD value of poly(dεA) bound to the first site, and *arrow 2* corresponds to that of oligo(dT) bound to the second site.

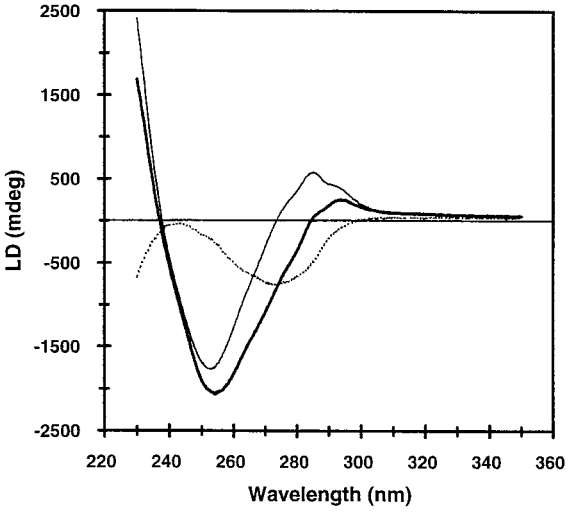

FIG. 4. **Linear dichroism of RecA·poly(dA) and RecA·poly(dA)·oligo(dT) complexes.** LD of RecA·poly(dA) (*thin line*), RecA·poly(dA)·oligo(dT) (*thick line*) measured as described in the text. Differential LD for oligo(dT) (*dotted line*) is also shown. The concentration of RecA was 50 μM.

complex. The base orientations were determined by combining LD and SANS data as described previously (13).

The results indicate for both of the types of DNAs investigated that the nucleobases of the second DNA are somewhat more tilted than those of the first DNA, but the difference is small so that the nucleobases of the two DNA strands are almost coplanar, although we cannot exclude the possibility that the base tilts of the two DNA strands are in opposite direction (Fig. 5). This is also the case when the two DNA molecules are not complementary in sequence. An almost coplanar structure is logical since it may facilitate effective base-base hydrogen bondings between the two DNA strands with the purpose of comparing sequences and of search for complementarity.

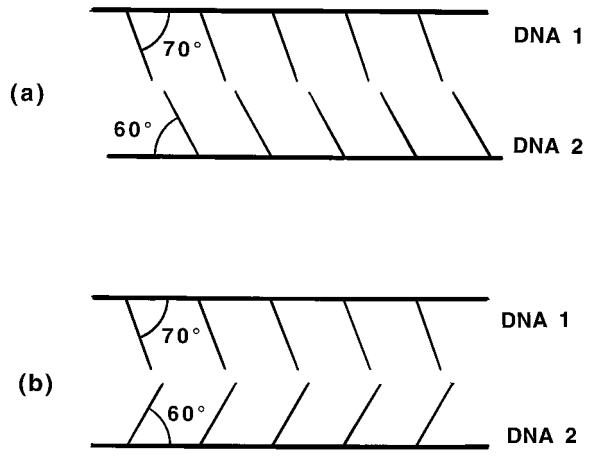

FIG. 5. **Schematic presentation of RecA·DNA structure showing the orientation of the nucleobases.** The bases of the first bound DNA strand are oriented at about 70 degrees and those of the second DNA at about 60 degrees relative to the fiber axis. In model *a,* the bases of the two DNA strands are tilted in the same direction and in *b* are in opposite directions (a less coplanar arrangement). For reasons of clarity, the RecA helix has been omitted, and the DNA backbones are represented by *thick straight lines* and only the orientations of base plans are shown.

The quantitative evaluation indicates that bases are neither perfectly perpendicular to the fiber axis nor coplanar to each other. This must be taken into account upon model building of the base-base interactions in the RecA·DNA complex filament. Most models of base triads of RecA-promoted triplexes are based on the assumption that all the nucleobases of the three DNA strands are perpendicular to the main DNA axis and are thus perfectly coplanar to each other, although the bases of conventional poly(dT):poly(dA):poly(dT) triplexes are generally not perfectly perpendicular to the helix axis (24, 25). Our observation that the bases of the second bound DNA strand are more tilted than those of the first DNA could indicate a mechanism by which the RecA filament acts as a scaffold preventing the formation of classical Watson-Crick-Hoogsteen hydrogen bonding, keeping the free energy of the complex higher than that of an ideal triplex. Such an arrangement would facilitate dissociation, in case of mismatch, and also facilitate longitudinal sliding of the DNA molecules relative to each other, which would be required for an efficient search-for-homology mechanism.

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