

The L2 loop peptide of RecA stiffens and restricts base motions of single-stranded DNA similar to the intact protein

Tassadite Selmane^a, Pernilla Wittung-Stafshede^{1,b}, Fabrice Maraboef^a, Oleg N. Voloshin^c, Bengt Nordén^b, Daniel R. Camerini-Otero^c, Masayuki Takahashi^{a,*}

^aUnité Mixte de Recherche 216, Institut Curie and CNRS, F-91405 Orsay, France

^bDepartment of Physical Chemistry, Chalmers University of Technology, S-41296 Gothenburg, Sweden

^cGenetics and Biochemistry Branch, NIDDK, National Institutes of Health, Bethesda, MD 29892 USA

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Abstract The L2 loop in the RecA protein is the catalytic center for DNA strand exchange. Here we investigate the DNA binding properties of the L2 loop peptide using optical spectroscopy with polarized light. Both fluorescence intensity and anisotropy of an etheno-modified poly(dA) increase upon peptide binding, indicate that the base motions of single-stranded DNA are restricted in the complex. In agreement with this conclusion, the peptide-poly(dT) complex exhibits a significant linear dichroism signal. The peptide is also found to modify the structure of double-stranded DNA, but does not denature it. It is inferred that strand separation may not be required for the formation of a joint molecule.

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Key words: RecA protein; Homologous recombination; DNA binding; Peptide; DNA base motion

1. Introduction

Homologous recombination, the exchange of DNA between two DNA molecules of identical or similar base sequences, plays a crucial role in DNA repair and DNA segregation in many organisms [1]. The reaction is catalyzed by RecA in *Escherichia coli* and by RecA homologues, including Rad51 and Dmc1, in eukaryotes [2,3]. For this reaction, RecA first binds to single-stranded DNA with a strong cooperativity and forms a filamentous complex with the protein subunits arranged in a helical manner around the DNA, which is elongated about 50% [4,5]. This nucleofilament then binds a second DNA (usually double-stranded DNA), forming a so-called joint molecule [6–8]. RecA thus positions two DNA molecules close to each other and promotes strand exchange between homologous DNAs. Rad51 forms a similar filamentous complex with DNA [9–11], indicating a similar reaction mechanism.

Several pieces of evidence indicate that the L2 loop of the protein is the reaction center. Proteolytic digestion of RecA-DNA complexes [12], mutation analysis [13], photocrosslink-

ing [14–16] and fluorescence measurements of a tryptophan residue inserted into this loop [17] showed that the L2 loop indeed interacts with DNA. Furthermore, it was demonstrated that the peptide alone promotes homologous DNA pairing, though with less efficiency than intact RecA [18]. The structure of the L2 loop has not yet been determined by X-ray crystallographic analysis of RecA, as it adopts multiple conformations in the crystal [19]. In order to characterize the interactions of this peptide with DNA in this study we pursue linear dichroism (LD) and fluorescence measurements of a tryptophan residue inserted into the peptide in the place of a phenylalanine. Also, the peptide-DNA interactions are studied using a fluorescent analog of poly(dA), poly(dεA). These techniques have previously been successful to demonstrate that the DNA bases are immobilized and oriented in one direction in the RecA-DNA complex [8,17,20]. Analysis of L2 loop peptide-DNA interactions shows that the peptide interacts with single-stranded DNA in a manner similar to intact RecA protein.

2. Materials and methods

2.1. Materials

L2 loop peptide in which phenylalanine 203 is replaced by tryptophan (NQIRMKIGVMWGNPETTTGG) was synthesized on the ABI Model 430A synthesizer with the use of standard f-moc chemistry and purified on a C18 reverse phase column (218TP1022, Vydac). A control peptide, which has the same amino acid composition as the above peptide but a scrambled sequence (IPEQTKGGRNTMNVW-GMGIT) [18] was synthesized by Eurogentec. The identity and purity of the peptides were confirmed by mass spectroscopy and analytical HPLC. Poly(dA), poly(dT) and poly(dA):poly(dT) were from Pharmacia and calf thymus DNA was from Sigma. Poly(dεA) was prepared by the chemical modification of poly(dA) with chloroacetaldehyde as described by Chabbert et al. [20]. Fluorescein-labelled F-ACT CCG CCT GGT GCA TCT and rhodamine-labelled complementary R-AGA TGC ACC AGG CGG AGT oligonucleotides were obtained from Genset. Duplex DNA was formed by mixing equimolecules of the two oligonucleotides in 20 mM sodium phosphate buffer with 100 mM NaCl and slow cooling after 5 min heating at 60°C. The duplex DNA was stable up to 40°C in the experimental conditions.

2.2. Spectroscopic measurements

LD was measured in modified Jasco J-500 and J-720 spectropolarimeters [21]. The samples were oriented in an outer rotating Couette cell [22]. Circular dichroism (CD) was measured in a Jasco J-710 spectropolarimeter equipped with a Peltier effect temperature controller. Absorption was measured in a V-530 spectrophotometer (Jasco).

Fluorescence was measured in a Jasco F-777 spectrofluorometer. For the observation of tryptophanyl fluorescence the sample was excited usually at 295 nm with a bandwidth of 3 nm. The emission spectra were measured from 300 to 450 nm with a bandwidth of 1.5 nm. The effect of the increase of the inner filter effect upon the addi-

*Corresponding author. Fax: (33) (1) 69863011.

E-mail: masa.takahashi@curie.u-psud.fr

¹Present address: Chemistry Department, Tulane University, New Orleans, 70118-5698 LA, USA.

Abbreviations: LD, linear dichroism; CD, circular dichroism; Poly(dεA), poly(1,N⁶-etheno-deoxyadenosine)

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tion of DNA is corrected by the following formula

$$F_{\text{corrected}} = F_{\text{measured}} \times 10^{(Abs \times 0.6)}$$

The fluorescence of poly(dεA) was observed at 410 nm with a bandwidth of 10 nm upon the selective excitation at 320 nm with a bandwidth of 10 nm. For the observation of energy transfer from fluorescein to rhodamine, emission spectra were measured with a bandwidth of 1.5 nm upon excitation of fluorescein at 493 nm (bandwidth 3 nm).

The anisotropy was measured at 20°C in a F-777 spectrofluorometer using an automatic anisotropy measurement device (ADP 301, Jasco). Excitation and emission wavelengths were set, respectively at 295 and 340 nm (bandwidth 10 nm), for the fluorescence of the peptide, and 320 and 420 nm (bandwidth of 10 nm) for poly(dεA). The 10 measurements of 0.5 s each were averaged. The anisotropy r was defined as

$$r = (I_{VV} - G \times I_{VH}) / (I_{VV} + 2 \times G \times I_{VH})$$

where G is a correction factor.

2.3. Experimental conditions

DNA binding experiments were generally performed in a buffer containing 4 mM sodium phosphate pH 6.9, except the fluorescence measurements (20 mM sodium phosphate). For the thermal denaturation of poly(dA):poly(dT), the samples were extensively dialyzed against 100 times the volume of the buffer for an accurate comparison. The denaturation was followed by a change in absorption at 260 nm (bandwidth 2 nm) or a CD signal at 248 nm (bandwidth 2 nm) with temperature increase. The temperature was increased from 15°C at a rate of 1°C/min with the help of a DNA denaturation program kindly provided by Jasco.

3. Results

3.1. Binding of L2 loop peptide to single- and double-stranded DNA

The study was performed with a L2 loop peptide in which phenylalanine is replaced with tryptophan. This peptide is even more active for joint molecule formation than the original L2 loop [18]. Addition of various DNA (poly(dA), poly(dT), poly(dA):poly(dT) or calf thymus DNA) decreased the intensity of the peptide fluorescence with a slight blue shift of the emission spectrum (Fig. 1, Fig. 2). The intensity decrease was accompanied by an increase of the fluorescence anisotropy (not shown), thus clearly indicating binding of the peptide to both single- and double-stranded DNA. The

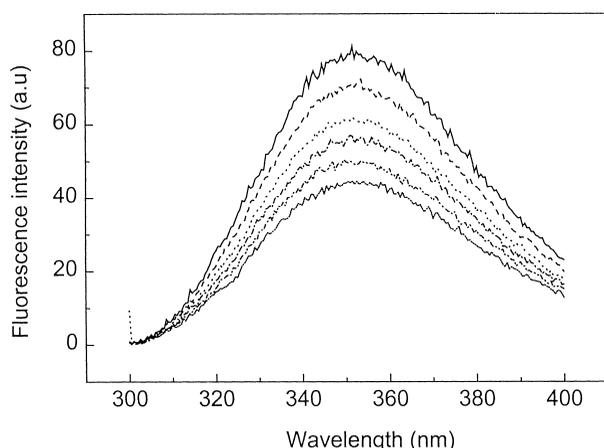


Fig. 1. Fluorescence change of the L2 loop peptide upon DNA binding. Change in the emission spectrum of the peptide (2.5 μM) upon addition of 0 (—), 60 (---), 120 (· · ·), 180 (— · —), 240 (— — —) and 300 μM (— · — · —) poly(dT).

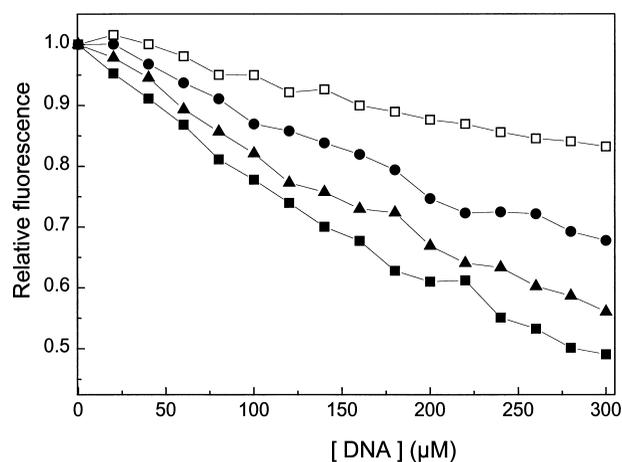


Fig. 2. Decrease in the intensity of peptide fluorescence upon the addition of polynucleotides. Change in the intensity at 340 nm of 2.5 μM L2 loop peptide upon the addition of poly(dT) (▲), poly(dA) (●), poly(dA):poly(dT) (■). Fluorescence change of the scrambled control peptide upon the addition of poly(dA):poly(dT) (□) is also shown.

decrease of fluorescence intensity was not completed even upon adding a large excess of DNA (Fig. 2), revealing a low binding constant. The fluorescence was, however, quenched as much as 80% with an extremely large excess of DNA (1 mM) (not shown). The decrease in fluorescence occurred at lower concentration of poly(dT) than poly(dA) (Fig. 2), suggesting a higher peptide affinity for poly(dT). Thus, the peptide, like RecA protein [23,24], binds preferentially to single-stranded DNA lacking a strong base stacking. However, the affinity of the peptide to duplex DNA (poly(dA):poly(dT) and calf thymus DNA) was similar to that for poly(dT) (Fig. 2). A rough estimate of the binding affinity, based on the concentration of DNA required to half saturate the fluorescence decrease, suggests a binding constant of 2×10^3 – 4×10^3 M. The fluorescence of control (scrambled) peptide also decreased upon the addition of DNA but the change was much smaller (Fig. 2), indicating further a weaker binding affinity or some different binding mode.

3.2. Restriction of single-stranded DNA base motion by L2 loop peptide

We investigated the structure of the DNA-peptide complex by studying the fluorescence change of poly(dεA) upon RecA binding [20]. Free in solution, the nucleobases of poly(dεA) are in motion, as in normal single-stranded DNA, and their fluorescence is quenched by collisions between the bases. Binding of RecA to poly(dεA), especially in the presence of ATP as a cofactor, restricts the base motion: the fluorescence intensity is increased 8-fold and the anisotropy goes up from 0.04 to 0.14 [20]. Addition of the L2 loop peptide to poly(dεA) increased the intensity of the fluorescence more than four times and the anisotropy was increased to 0.13 (Fig. 3). These large fluorescence changes, similar to those obtained by RecA [20], show that the peptide, just like RecA, restricts the motion of the bases so that collisions, and thus quenching, is prevented. These modifications of the fluorescence properties did not reach complete saturation even at a very large excess of the peptide, again indicating a weak binding constant. The

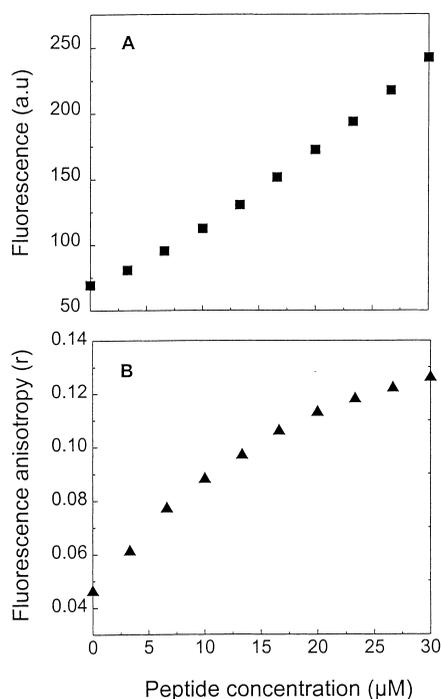


Fig. 3. Fluorescence changes of poly(dεA) upon the addition of peptide. L2 loop peptide was added in a step wise manner to a 4 μM poly(dεA) solution. Changes in the fluorescence intensity (A) and in the anisotropy index (B) were presented as a function of the peptide concentration.

shape of the binding curve, however, suggests that the process involves some cooperativity.

Further information about the DNA-peptide interactions was provided by LD measurements on poly(dT)-L2 loop peptide mixtures. Poly(dT) is a very flexible chain and cannot be significantly aligned by shear forces, thus it exhibits no LD signal when alone (Fig. 4A). However, upon addition of the L2 loop peptide, significant positive LD signals arise around 290 and 230 nm (Fig. 4A), demonstrating the formation of a

stiff, most likely filamentous [25] complex. That such a stiff complex is formed indicates that the peptides bind in large clusters to the DNA, i.e. cooperative binding. The absorption at 230 and 290 nm is dominated by $\pi \rightarrow \pi^*$ transitions in the plane of the tryptophan residue, so the positive LD signals should correspond to a rather parallel orientation of these tryptophan transitions relative to the main axis of the peptide-DNA complex.

The positive signal at 280 nm is weaker than that at 290 nm and almost vanishing at 260 nm, despite the fact that the peptide absorption is even larger at these wavelengths and, thus, anticipated to contribute to the LD. The vanishing of the LD signal at 260 nm can be ascribed to a balance between a negative contribution from the DNA bases, absorbing around 260 nm, and a positive contribution from the protein chromophores. The positive contribution around 290 nm is due to tryptophan, so the tryptophan residue and the DNA bases exhibit LD signals of opposite sign, indicating that the tryptophan orientation is parallel to the orientation axis of the L2-DNA complex whereas the DNA bases are oriented perpendicularly. This excludes intercalation of the tryptophan residue of L2 loop peptide between the DNA bases, as has been concluded for the tryptophan residue at the same position in the entire RecA [17]. Thus, the immobilization of the DNA bases by the binding of the peptide probably has an origin other than the presence of an intercalating moiety, as observed for the RecA-DNA complex [26].

3.3. Modification of the duplex DNA structure by the L2 loop peptide

Also the peptide complex with double-stranded DNA was examined by LD spectroscopy. Duplex DNA is stiffer than single-stranded DNA and exhibits a large negative LD signal (when free in solution), corresponding to a perpendicular orientation of nucleobases relative to the main axis of the DNA [27]. Upon binding of the L2 loop peptide to a double-stranded DNA, a positive signal appeared around 230 nm while the signal around 260 nm remained negative (Fig. 4B). This corresponds to a rather parallel orientation of the tryptophan

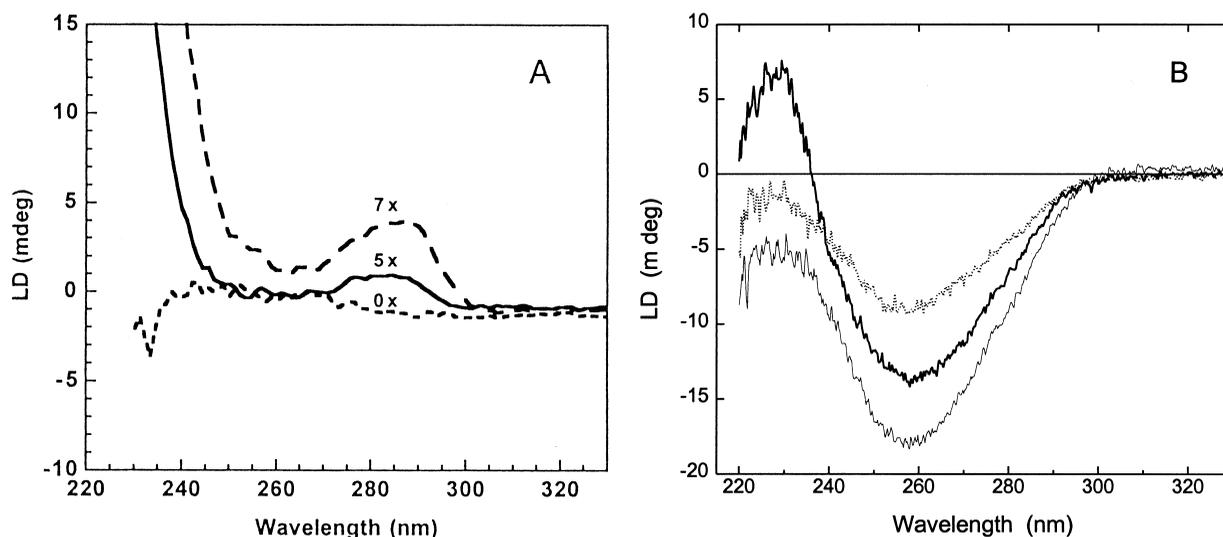


Fig. 4. LD of poly(dT)- and calf thymus duplex DNA-L2 loop peptide complex. (A) Flow LD of 10 μM poly(dT) was measured in the absence (dots) and presence of 50 (continuous) and 70 μM (broken line) peptide. Shear was 1800/s. (B) Flow LD signal of 10 μM (in bp) calf thymus DNA in the absence (thin line) and presence of 40 μM L2 loop peptide (thick line) or scrambled peptide (dotted line). Shear was 300/s.

tophan molecular plane and a perpendicular orientation of DNA bases in the double-stranded DNA-peptide complex, and thus again indicates that the tryptophan residue may not be intercalated between the DNA bases. Binding of the control peptide also altered the LD signal but without giving rise to any positive signal around 230 nm (Fig. 4B), indicating a different binding mode. Surprisingly, the magnitude of the LD signal of the double-stranded DNA decreased upon binding of the L2 loop in contrast to the case with single-stranded DNA (Fig. 4B). When poly(dA):poly(dT) was used as a double-stranded DNA, the decrease in LD at 260 nm upon addition of L2 loop peptide was even larger (not shown).

A decrease of the LD signal indicates either a change in the local orientation of the chromophore (tilt of DNA bases) or a change in the orientability of the whole complex. Since we observed a larger decrease for shorter DNA (poly(dA):poly(dT)) than for larger DNA (calf thymus DNA) a changed orientability may be the explanation. The orientability could be reduced upon peptide binding either by bending the DNA or by increasing the flexibility of the DNA. We have searched for possible bending (or kink) of DNA by measuring the fluorescence energy transfer from fluorescein attached at one end of DNA to rhodamine attached at the other end. Bending should decrease the distance between the two extremities of DNA and, hence, increase the energy transfer. The emission spectrum of the dye-labelled DNA upon excitation of fluorescein exhibited a slight shoulder around 580 nm (rhodamine fluorescence), indicating some energy transfer. Addition of the peptide only slightly changed the shape of the emission spectrum, which indicates that there is a very weak increase in energy transfer. Although the change was larger at higher peptide concentration (not shown), the peptide only faintly bends DNA or makes it (dynamically) more flexible. By contrast, binding of the control peptide increased the magnitude of this energy transfer signal around 580 nm with a decrease of fluorescein fluorescence around 520 nm (Fig. 5). The control peptide certainly promotes DNA bending in accordance with the correspondingly observed marked decrease of the flow LD signal.

Since denaturation of DNA increases the flexibility and decreases the magnitude of the LD signal, we have searched

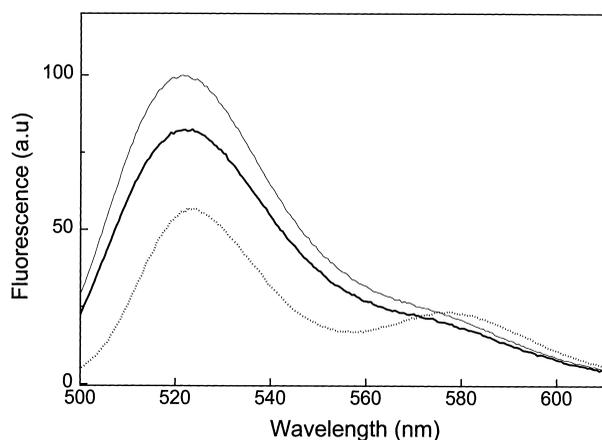


Fig. 5. No strong bending of DNA by L2 loop binding. Fluorescence energy transfer from fluorescein to rhodamine at the two extremities of oligonucleotide (3 μ M in bp) was measured by the excitation of fluorescein in the absence (thin line) and presence of 15 μ M L2 loop (thick line) or scrambled peptide (dotted line).

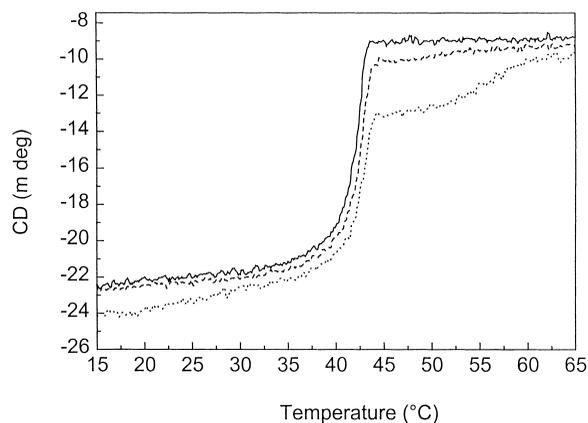


Fig. 6. The effect of L2 loop peptide on thermal denaturation of the poly(dA):poly(dT) duplex. Denaturation of 33 μ M, in bp, of poly(dA):poly(dT) by temperature increase was studied in the presence of 0 (—), 11 (---) or 33 (····) μ M peptide by monitoring changes of the CD signal at 248 nm.

for indications of strand separation of the duplex DNA upon binding of the L2 loop. The CD change of duplex DNA upon peptide binding was very small (not shown), as also reported previously [18], showing that no strand separation occurs. By contrast, thermal denaturation of the poly(dA):poly(dT) duplex showed that the L2 loop peptide can prevent this to some extent (Fig. 6). In the absence of peptide, poly(dA):poly(dT) denatures around 42°C with high cooperativity, but in the presence of peptide, the DNA was partly denatured at higher temperatures. Adding more peptide, a larger part of DNA was denatured at the higher temperature, showing that the peptide indeed did not bind only to single-stranded parts of duplex DNA but really bound to intact double-stranded DNA. In a likewise manner, binding of RecA does not cause strand separation of duplex DNA [8,28]. DNA strand separation, which is necessary for strand exchange to proceed, may occur only after the pairing of homologous DNA molecules has taken place in the RecA filament.

4. Discussion

The present work shows structural similarity between DNA complexes with the L2 loop peptide and with the intact RecA protein and supports the validity of further structural analysis of the complex with the peptide in order to understand the mechanism of the homologous recombination reaction.

The analysis also indicates a significant restriction of mobility and a rather perpendicular orientation of the DNA bases, both properties that may be important for the search for homology by facilitating base-base contact between the two DNA strands in the RecA filament. We have observed similar structural features for the DNA complex with a RecA homologue, *Xenopus* Rad51 protein [29]. Our analysis also indicates changes in the L2 loop peptide upon interaction with DNA. A change in the fluorescence of tryptophan indicates either a conformational change of the peptide upon binding to DNA, or an effect of a direct contact of the residue with DNA. Preliminary results indicate that fluorescence changes induced by other factors (SDS and salt) than DNA, which change the conformation of the peptide into β -sheet like DNA [18,25], is smaller than those induced by DNA (MT unpublished data). There may be some direct contact

between the tryptophan residue and DNA. This could explain that substitution of the residue at position 203 in the peptide and the peptide with another amino acid affects the DNA binding [18].

5. Conclusion

The L2 loop peptide has previously been demonstrated to have several of the biochemical activities of the whole RecA protein, including binding to DNA, unstacking single-stranded DNA, forming filaments and pairing homologous DNAs. In this paper we show that the peptide recapitulates still another activity of the intact protein, the binding of the peptide restricts the local motion of DNA bases of single-stranded DNA.

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