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Geometry of the DNA strands within the RecA nucleofilament : role in homologous recombination

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Abstract. Homologous recombination consists of exchanging DNA strands of identical or almost identical sequence. This process is important for both DNA repair and DNA segregation. In prokaryotes, it involves the formation of long helical filaments of the RecA protein on DNA. These filaments incorporate double-stranded DNA from the cell's genetic material, recognize sequence homology and promote strand exchange between the two DNA segments. DNA processing by these nucleofilaments is characterized by large amplitude deformations of the double helix, which is stretched by 50% and unwound by 40% with respect to B-DNA. In this article, information concerning the structure and interactions of the RecA, DNA and ATP molecules involved in DNA strand exchange is gathered and analyzed to present a view of their possible arrangement within the filament, their behavior during strand exchange and during ATP hydrolysis, the mechanism of RecA-promoted DNA deformation and the role of DNA deformation in the process of homologous recombination. In particular, the unusual characteristics of DNA within the RecA filament are compared to the DNA deformations locally induced by architectural proteins which bind in the DNA minor groove. The possible role and location of two flexible loops of RecA are discussed.

1. Introduction 430

2. Transformations of the RecA filament 431
2.1 The different forms of the RecA filament 431
2.2 Orientation and position of the RecA monomers in the active filament 433
2.3 Transmission of structural information along the filament 433

3. RecA-induced DNA deformations 435
3.1 Characteristics of RecA-bound DNA 435
3.2 Stretching properties of double-stranded DNA 436
3.3 DNA bound to architectural proteins 437
3.4 Implications for RecA-induced DNA deformations 438
3.5 Axial distribution of the DNA stretching deformation 438

4. Contacts between RecA and the DNA strands 440
4.1 The DNA-binding sites 440
4.2 Possible arrangement of loops L1 and L2 and the three bound strands of DNA 442

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5. Strand arrangement during pairing reorganization 444
5.1 Hypotheses for DNA strand association 444
5.2 Association via major or minor grooves 446
5.3 Post-strand exchange geometries 446

6. Conclusion 447

7. Acknowledgments 448

8. References 448

1. Introduction

Homologous recombination consists of exchanging DNA strands of identical sequence. This process is important for DNA repair, especially that of double-strand breaks (Kuzminov, 1999), and for DNA segregation (Campbell & Davis, 1999). It is essential for cellular viability in higher eukaryotes (Lim & Hasty, 1996; Tsuzuki *et al.* 1996) and also fundamental for the long-term survival of species, as it increases genomic diversity (see Haber, 1999). The central event in homologous recombination involves pairing exchange between three DNA strands. This process is remarkably faithful since the exchanged DNA strand is replaced by a segment of identical or almost perfectly identical sequence. Homologous recombination has been extensively studied over the last two decades and, notably, that promoted by the RecA protein of *E. coli* (Radding, 1991; Kowalczykowski & Eggleston, 1994; Takahashi & Nordén, 1994a; Camerini-Otero & Hsieh, 1995; Roca & Cox, 1997; Cox, 1999). More recently, the exchange promoted by human Rad51 has also been studied (Baumann & West, 1998; Gupta *et al.* 1999).

Both sequence recognition and strand exchange are performed within long helical polymeric filaments formed by monomers of the recombination proteins. The various steps of the reaction are now well defined for the RecA system: polymerization of the protein on DNA, initiated on a single-stranded fragment in the presence of ATP or one of its analogs, uptake and alignment of double-stranded DNA, checks for homology, strand exchange, release of the displaced strand and disassembly when ATP is hydrolyzed. The exact chronology and the causal links of the latter steps (between uptake of duplex DNA and disassembly) are less well known. In spite of a huge amount of published information, the detailed mechanism of both sequence recognition and strand exchange remains unclear. The reasons are essentially linked to the speed of the reaction and its dynamic propagation, which causes different steps to occur simultaneously along the nucleoprotein filament (Reddy *et al.* 1995) and makes it difficult to isolate and characterize the intermediate products of the reaction.

From a structural point of view, the nucleoprotein filament is remarkable in at least two aspects: the large amplitude deformations of incorporated DNA and the plasticity of the

Abbreviations: EM, electron microscopy; LD, linear dichroism; MtRecA, RecA protein from *Mycobacterium tuberculosis*; Mt-L1, Mt-L2, geometric folds of loops L1 and L2 of RecA as found in the crystal structure of MtRecA; NMR, nuclear magnetic resonance; PDB, Protein Data Bank (http://www.rcsb.org/pdb); B-DNA, biologically active form of DNA; R-DNA, parallel DNA triple helix, where the single strand interacts in the major groove of the duplex; S-DNA, stretched form of DNA, with a stretching factor of ~1·7; ST-DNA, stretched triplex DNA, where the single strand interacts in the minor groove of the duplex; TA-DNA, form of the DNA in the complex with the TATA-box binding protein; SANS, small-angle neutron scattering.

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filament itself. Within the active filament, the DNA is stretched by 50% with respect to canonical B-DNA (Stasiak *et al.* 1981) and unwound by 15° per base-pair step, more than 40% its standard helical twist (Stasiak & DiCapua, 1982). This is an unusual conformation for DNA, which can be attained only with the help of external forces (Hegner *et al.* 1999). In the case of strand exchange, these forces are provided by interactions with the RecA protein, possibly coupled to thermal fluctuations (Léger *et al.* 1998). The questions that immediately arise are whether there is a mechanistic reason for the DNA to be stretched and unwound and what is the molecular mechanism of this DNA deformation. Equally intriguing is the plasticity of the filament itself: upon ATP hydrolysis, it undergoes an allosteric transition which globally results in a 20% decrease in pitch (DiCapua *et al.* 1990b; Ellouze *et al.* 1995) and a 20% increase in diameter (Heuser & Griffith, 1989). It is tempting to assume that this filament plasticity is directly related to the strand-exchange mechanism (Kowalczykowski & Krupp, 1995), though no direct link has been established until now. These characteristics, DNA deformation and filament plasticity, are common to all recombination filaments, whether they are constituted by RecA or by Rad51 proteins, and have been conserved during evolution (Yu *et al.* 2001). Their relationship with the driving force for recognition and strand exchange may, therefore, be the key to understanding the mechanism of RecA/Rad51-induced homologous recombination.

To address this question, it is necessary to determine how the protein interacts with DNA at the atomic level, how this interaction evolves as the reaction advances and as ATP hydrolysis occurs and how the DNA strands recognize each other. All these questions are difficult to answer without the identification of any stable association intermediates. There is, however, a huge amount of partial and indirect structural information on the nucleoprotein filament. It is not always easy to interpret since it has been obtained in a variety of experimental conditions. Also, the stage of the reaction to which it refers is not always clear. The purpose of the present review is to organize this information and confront it with the different hypotheses proposed for the strand-exchange mechanism. We will limit this discussion to the data relative to RecA, as there are differences between eukaryotic and prokaryotic recombination (Ellouze *et al.* 1997a; Bianco *et al.* 1998; De Zutter & Knight, 1999; Kim *et al.* 2002). We will first review separately the structural and physical characteristics of the polymeric RecA filament and of stretched and unwound double-stranded DNA, before discussing their possible modes of interaction.

2. Transformations of the RecA filament

2.1 The different forms of the RecA filament

There are at least three types of RecA filament. The functional RecA filament, often termed the 'active' form (Fig. 1*a*), is composed of a right-handed helical assembly of RecA monomers on a single DNA strand, in the presence of ATP. It has been characterized by several complementary methods, including electron microscopy (EM) (Stasiak & DiCapua, 1982; Flory *et al.* 1984; Heuser & Griffith, 1989; Yu & Egelman, 1990, 1992a), small-angle neutron scattering (SANS) (DiCapua *et al.* 1990b; Ellouze *et al.* 1995) and linear dichroism (LD) (Nordén *et al.* 1992; Morimatsu *et al.* 2002). There are slightly more than six monomers per helix turn, the helical pitch varies between 90 and 100 Å and the diameter is 100 Å. The stoichiometry of association between DNA and RecA is three nucleotides per RecA monomer and each RecA filament can bind up to three DNA strands (Kubista *et al.* 1990; Muller *et al.* 1990; Takahashi *et al.* 1991). The DNA is situated inside the filament, near the helical axis in a coaxial arrangement (Egelman & Yu, 1989). In addition to its function in DNA recombination, the nucleofilament displays

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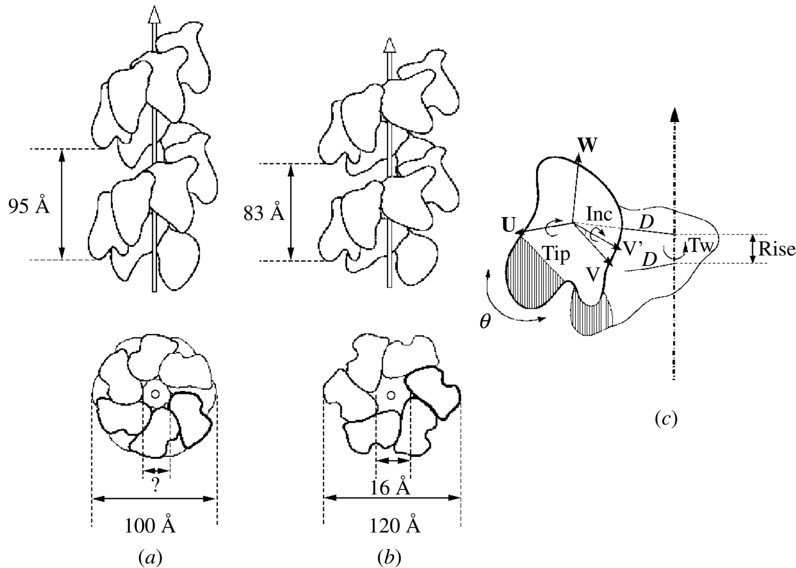

Fig. 1. Schematic representation of the organization of RecA monomers in (*a*) the active form of the RecA filament (RecA/ATP/DNA, the DNA is not represented) and (*b*) the crystal structure (RecA/ADP; PDB entry 2REB, Story *et al.* 1992). The top views in (*a*) and (*b*) are oriented perpendicular to the helical axis and the bottom views are along the helical axis. Image (*c*) gives the parameters that define the position of each monomer with respect to the helical axis (lateral displacement D, rotations Inc and Tip) and with respect to the preceding monomer (Rise, Tw). The helical pitch P is directly related to the latter parameters: $P = \text{Rise} \times 360^\circ/\text{Twist}$. Possible rotation by an angle θ of the C-terminal lobe (hatched zone) with respect to the central domain is indicated by a curved arrow (see Section 2.2). The crystal structure in (*b*) is characterized by Twist and Rise values respectively of 60° (6 monomers/turn) and 14 Å, for $P = 83$ Å. The corresponding values for the active form in (*a*) are 58° (6·2 monomers/turn) and 15 Å, for $P = 95$ Å. Note that the scheme in (*a*) only represents one possible organization of the monomers within the active filament. In particular, there is presently no indication on the displacement D value.

ATPase activity and stimulates the autocleavage of some proteins (coprotease activity) including the LexA repressor (Roberts *et al.* 1978; Little *et al.* 1980) and the UmuD protein (Burckhardt *et al.* 1988) (LexA cleavage induces the SOS response to DNA damage and that of UmuD induces translesional DNA replication). Note that both ATPase and coprotease activities have been observed in the absence of DNA, when the salt concentration is sufficiently high (Pugh & Cox, 1988; DiCapua *et al.* 1990a). Filaments with the same overall geometry can be observed with the weakly or non-hydrolysable analogs ATPγS and ADP-AlF_4 in the place of ATP and with a double-stranded DNA instead of single-strands.

Following hydrolysis of ATP to ADP, the RecA filament is globally characterized by a lower pitch (80–85 Å) and a weak DNA binding affinity (Fig. 1*b*). Recent high-resolution EM studies have allowed deconvolution of the RecA/ATP and RecA/ADP average forms into subgroups with varying pitch values (Yu *et al.* 2001), which questions the generally admitted choice of the pitch to distinguish the two forms.

In the absence of any cofactor, the pitch of the RecA filament decreases to 64 Å, only one DNA strand is accommodated and the stoichiometry of association increases to five nucleotides per RecA monomer (Yu & Egelman, 1992a; Ruigrok *et al.* 1993).

The 3D (three-dimensional) structure of *E. coli* RecA has been determined by X-ray diffraction in the form of a helical filament generated in the absence of DNA and without cofactor (Story

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et al. 1992; PDB entry 2REB). This filament accommodates ADP as a cofactor and its global helical characteristics correspond to those observed by SANS for the inactive RecA/ADP form (Ellouze *et al.* 1995). Very recently, RecA protein from *Mycobacterium tuberculosis* (MtRecA) has also been crystallized in the presence of ATPγS, dATP and ADP-AlF_4, although in a low pitch form (72 Å) (Datta *et al.* 2000, 2003; PDB entries 1MO3, 1MO4, 1MO5, 1MO6).

2.2 Orientation and position of the RecA monomers in the active filament

The transition between the different forms of the filament is highly cooperative upon ATP hydrolysis (see Roca & Cox, 1990; Yu & Egelman, 1992b). What modifications in the 3D atomic structure lead to these global changes is still unknown. No large internal modifications have been detected within each of the two major domains of RecA, namely the C-terminal domain (residues 270–328) and the ATP-binding domain (31–269). However, changes have been observed in their relative position (Yu *et al.* 2001; Morimatsu *et al.* 2002; VanLoock *et al.* 2003) and a global movement of rotation/translation of the monomers with respect to the helical axis can be expected (Fig. 1*c*).

Rotation of the C-terminal domain forming a protruding lobe outside the filament has been established from EM observations (Yu *et al.* 2001). This was confirmed by a recent study using site-specific LD, mainly based on information from residues in the C-terminal domain (Morimatsu *et al.* 2002). A 37° change in monomer orientation with respect to the helical axis was measured when passing from the RecA/ATPγS to the RecA/ADP form. Note that, due to its location outside the filament and its lack of interactions with neighboring monomers, rotation of the C-terminal domain is not likely to influence the overall geometry of the filament.

Movements of the central ATP-binding domain are more difficult to detect via EM since it is located in the smooth and continuous filament region. Whether the central domain rotates or not, its movement is probably different from that of the C terminal since analysis of urea-induced unfolding of RecA indicates some change in the contact between these two domains upon binding ATP or ADP (Yamazaki *et al.* 2003).

Rotation of the monomers, together with their possible displacement from the helix axis, can induce changes in their relative arrangement and interactions, in which case interaction with the bound DNA may also be affected. Modifications in the subunit–subunit interface have been found when comparing free RecA filaments and RecA/ATP/DNA filaments, by exploring the interface via disulfide bond formation (Logan *et al.* 2001) or by introducing selected mutations (Eldin *et al.* 2000). It cannot be excluded that changes of this nature also occur between RecA/ATP and RecA/ADP filaments. Finally, a recent reconstruction of the active filament from high-resolution EM data is consistent with differential rotations of the ATP-binding core and the C-terminal domain. This reconstruction implies important modifications at the monomer–monomer interface when going from the active to the inactive forms of the filament (VanLoock *et al.* 2003; PDB entry 1NO3). In particular, ATP would lie between neighboring monomers and would, therefore, directly participate in stabilizing the active form.

2.3 Transmission of structural information along the filament

Atomic level information on the mechanism of cooperative changes along the filament is still scarce and partial. A proposition based on the crystal structure of *E. coli* RecA involves the flexible loop L2 (residues 194–210) situated at the inner surface of the filament (Story *et al.* 1992).

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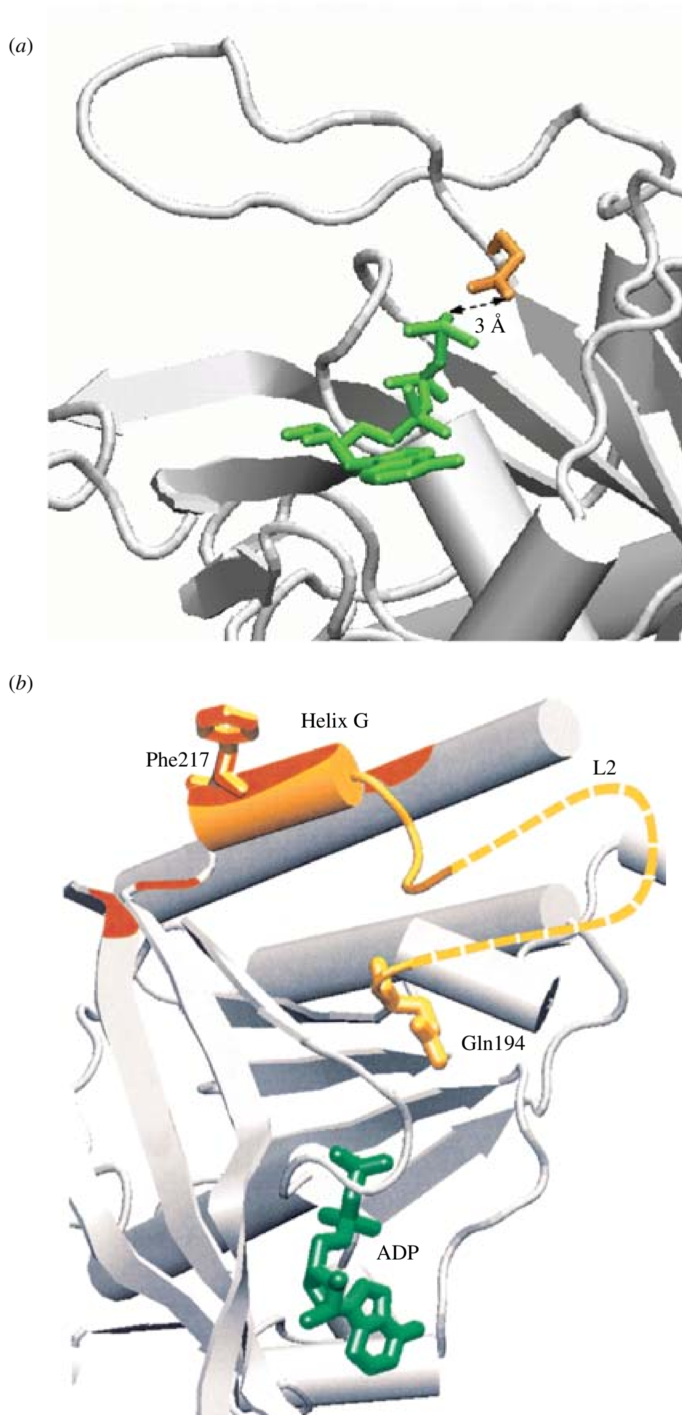

Fig. 2. (*a*) Relative position of the residue Gln195 of MtRecA (orange) with respect to the γ-phosphate of ATPγS (green), in the crystal structure of MtRecA/ATPγS (PDB entry 1MO4). The nitrogen of Gln195 is within a hydrogen-bonding distance of the terminal oxygens of ATPγS. (*b*) Details of the region of *E. coli* RecA (PDB entry 2REB) formed by loop L2 (dotted line) and the following helix G, which contains Phe217

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L2 is one of two loops (together with L1, residues 156–165) whose structure is unresolved in the crystallographic study of *E. coli* RecA or RecA/ADP in the absence of DNA. Information on these loops has, however, very recently become available in the crystal structures of MtRecA/ATPγS/Mg^{2+} for loop L1 (PDB entry 1MO5), MtRecA/ATPγS for loop L2 (1MO4) (Datta *et al.* 2003). Loops L1 and L2 of *E. coli* RecA respectively present 75 and 80% of sequence homology with the corresponding loops of MtRecA. Residue Gln194 situated at the junction between loop L2 and the rest of the protein has been proposed to be part of the ATP-binding site, based on binding-site analogies with other ATP-binding proteins (Story *et al.* 1992; Story & Steitz, 1992; Voloshin *et al.* 2000). It shows no interaction with ADP in the crystal of *E. coli* RecA, but is sufficiently close to the expected location of the γ-phosphate of ATP to contact it in the active form. Indeed, in the crystal structure of MtRecA/ATPγS (Datta *et al.* 2003), the corresponding Glu195 effectively has its terminal nitrogen at a distance of only 3 Å from a γ-phosphate oxygen (Fig. 2*a*). It has been proposed that such a Gln194-controlled switch may induce a change in the L2 loop conformation or position. Loop L2 is likely to be flexible given that it remained unresolved in the RecA crystal. Two highly conserved consecutive glycine residues (Karlin & Brocchieri, 1996), critical for RecA function (Hörtnagel *et al.* 1999) and situated at the C-terminal junction between the loop and the protein core may participate in this flexibility (see also De Zutter *et al.* 2001). Based on engineered disulfide cross-linking and mutation studies, residue Phe217 has been proposed to mediate a change in loop conformation via its insertion into a hydrophobic pocket, where it would bind more tightly than in its crystal position (Skiba *et al.* 1999; De Zutter *et al.* 2001). Phe217 belongs to the small helix G following loop L2 and close to the nucleotide-binding site (Fig. 2*b*). The authors of this study propose that its position may be affected by a conformational change of loop L2 driven by ATP binding.

3. RecA-induced DNA deformations

3.1 Characteristics of RecA-bound DNA

Association with RecA induces important DNA stretching and unwinding, respectively by 50 and 40% of the canonical values. These results come from studies of the DNA contained in the nucleofilament (Stasiak *et al.* 1981; Stasiak & DiCapua, 1982), but also of additional DNA incorporated into the preformed nucleofilament (Kiianitsa & Stasiak, 1997; Malkov *et al.* 2000). When it is part of the filament, the DNA is deformed during RecA polymerization. On the contrary, for the DNA incorporated into the nucleofilament, the change of state induced by the protein approach can be rapidly reversed via DNA release when the degree of homology between the incorporated and constitutive DNA is insufficient (Bazemore *et al.* 1997a; Ellouze *et al.* 1997b). Other studies have better characterized the DNA participating in the nucleofilament. LD experiments showed that the nucleic acid bases of DNA are, on average, roughly perpendicular to the filament axis (60°–80° for single-stranded DNA, 70°–90° for double-stranded DNA) (Nordén *et al.* 1992). In addition, the structure of a tetranucleotide DNA strand bound to a RecA dimer has been determined by NMR (Nishinaka *et al.* 1997). In this structure,

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(see Section 2.3). The interfacial residues interacting with the neighboring monomer are represented in brown. The pathway connecting Gln194 to Phe217 is in yellow. The views have been generated using the graphical program VMD (Humphrey *et al.* 1996).

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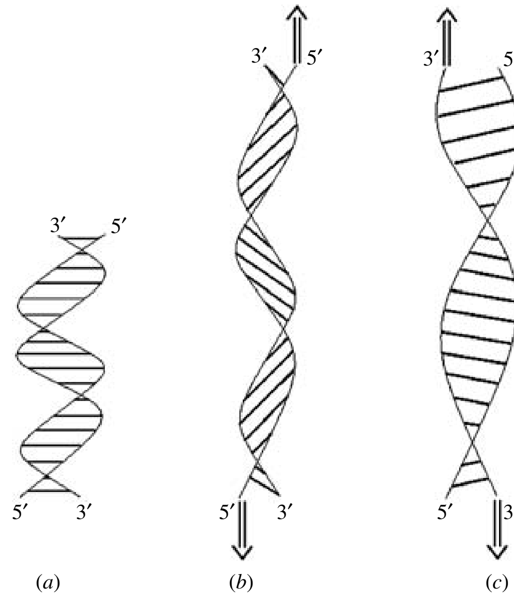

Fig. 3. The chemical structure and helicity of the B-DNA duplex (*a*) imply that it will react differently to stretching forces applied to its 5′ or 3′ extremities. Simulations suggest that 5′ stretching leads to base-pair inclination without unwinding (*b*), whereas 3′ stretching unwinds the duplex (*c*) (Lebrun & Lavery, 1996).

an average axial base separation of 5·1 Å is stabilized by stacking interactions between sugar C2′ methylene groups and the following 3′-base. However, the overall winding in this average structure is twice that observed in RecA-bound DNA.

3.2 Stretching properties of double-stranded DNA

The physics of duplex DNA stretching has been studied using single molecule manipulation techniques (Bustamante *et al.* 2000, 2003). DNA can be stretched by as much as 80% of its initial length. The force *versus* elongation curve clearly indicates a change of state when going from standard DNA to a completely stretched S-DNA form (Cluzel *et al.* 1996; Smith *et al.* 1996). After an initial 5% elongation which requires forces up to 55–65 pN, only a small additional force is required to increase the elongation to 70%. Theoretical simulation of the stretching process by Lebrun and colleagues (Cluzel *et al.* 1996; Lebrun & Lavery, 1996) showed two stretching modes, obtained when pulling the two antiparallel DNA strands either by their 5′ extremities or by their 3′ extremities (Fig. 3). The second stretching mode is accompanied by unwinding. At extreme deformations, this mode leads to a so-called 'ladder' form where the two backbones are almost parallel. Another study by Kosikov *et al.* (1999) where DNA is stretched starting from either B-DNA or A-DNA also led to different stretching regimes. Rouzina and Bloomfield rather interpret the change of state observed at 65 pN as resulting from partial melting of the duplex (Rouzina & Bloomfield, 2001; Williams *et al.* 2002; Wenner *et al.* 2002). It appears, however, that non-melted, locally over-stretched DNA can be found in biological complexes as discussed below (see Section 3.3).

With 70% elongation with respect to B-DNA, the S-DNA form obtained by external forces necessarily differs from the RecA-stretched form of DNA, which is elongated by 50%. Both forms are unwound, although to a different extent (Léger *et al.* 1999; Bryant *et al.* 2003). Léger *et al.*

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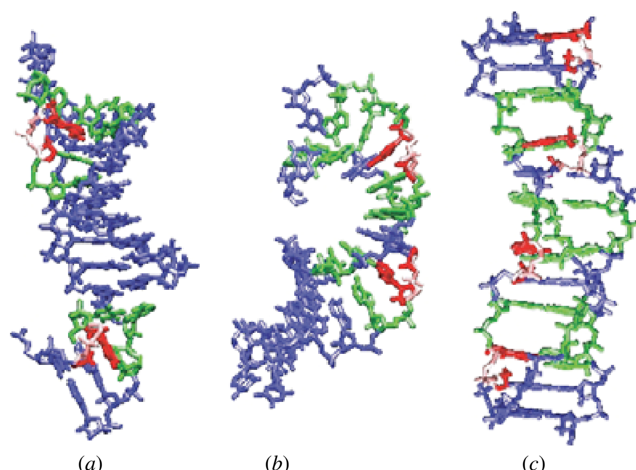

Fig. 4. Crystal structure of DNA bound to (*a*) IHF and (*b*) TBP, together with (*c*) the ST-DNA triple helix model. The structures represented in (*a*) and (*b*) come from PDB entries 1IHF (Rice *et al.* 1996) and 1YTB (Kim *et al.* 1993) respectively. Unstacked base pairs are represented in green, while the bases and the sugars presenting stacking interactions in the 3′-direction, via a sugar methylene group, are represented in red. In the three cases, the red steps precede a gap in stacking interactions in the 3′ direction. Analysis of the stacking interactions has been made on a geometric basis using the program ANUCLYZE (Gabb *et al.* 1996), modified to account for possible methylene/base-stacking interactions. The views have been generated using VMD (Humphrey *et al.* 1996).

(1999) have measured an helicity of 38 bp/turn for S-DNA, corresponding to a pitch of 220 Å. These numbers are 18·3 bp/turn and ~100 Å for RecA-bound DNA, 10·5 bp/turn and 36 Å for B-DNA. In fact, several experiments indicate that RecA-stretched DNA is likely to be an intermediate form between B-DNA and S-DNA, stabilized by its interactions with the filament. Léger *et al.* (1998) showed that RecA polymerizes at least 20 times faster on a double-stranded DNA in the S-form than on relaxed B-DNA. This was confirmed by the studies of Shivashankar *et al.* (1999) and Hegner *et al.* (1999). In the latter study, initiation of RecA polymerization did not occur within 10 min of RecA addition in the absence of external force, but took place in a few seconds when a 65 pN stretching force was applied.

3.3 DNA bound to architectural proteins

In an attempt to understand how the RecA filament can induce DNA deformation, reference can be made to the so-called architectural proteins including HMG, TBP, SRY, HU and IHF (Bewley *et al.* 1998). There are several indications that like these proteins, RecA interacts with the minor groove of double-stranded DNA, both in a filament built on double-stranded DNA (DiCapua & Muller, 1987; Tuite *et al.* 1997) and when DNA is incorporated into the RecA filament (Baliga *et al.* 1995; Podyminogin *et al.* 1996; Zhou & Adzuma, 1997; Singleton & Xiao, 2002). DNA bound to architectural proteins generally exhibits major deformations, often resulting in significant curvature (see, e.g. Fig. 4*a*, *b*). In such complexes, the double helical segment contacting the protein is unwound and its minor groove widened. Thus, the distance separating the phosphate groups situated at the 3′ extremities of the TATA-box bound by TBP (which measures the width of the minor groove) is twice that observed in B-DNA, while the phosphates at the 5′ extremities roughly conserve their initial distance. Lebrun *et al.* (1997) have shown that these induced

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deformations could be modeled in the absence of explicit protein, simply by pulling on the phosphate groups delimiting the bound DNA segment at the 3′ extremity of each strand. The asymmetry of the deformation is responsible for a strong curvature towards the major groove, which has also been interpreted as resulting from the junctions between a central segment in an unusual conformation, termed the TA form, and the rest of the DNA which remains in the B form (Guzikevich-Guerstein & Shakked, 1996). How exactly the protein induces such deformations has been discussed. It was suggested that the hydrophobic surface contacting the DNA can prompt the phosphate groups to pull apart in order to reach a more polar environment (Travers, 1995). This proposition is supported by a modeling study by Elcock & McCammon (1996), where a low dielectric volume filling the minor groove of DNA produced large DNA deformations characterized by minor groove widening.

3.4 Implications for RecA-induced DNA deformations

In the case of RecA filaments, DNA is unwound and stretched along its whole length. It is reasonable to assume that each RecA monomer produces local deformations equivalent to those induced by architectural proteins which, added together, result in an overall change of state of the double helix. The protein filament constitutes a dielectric medium lower than that of bulk water. The DNA face contacting the protein is essentially submitted to the effects of this medium, while the opposite face, situated in the large filament groove, is in contact with water.

Reproducing Lebrun *et al.*'s (1996) simulation, Bertucat and colleagues have observed that globally stretching the DNA by a factor of 1·5 by pulling its 3′ extremities led to exactly the same unwinding value as that observed in the RecA filament (Bertucat *et al.* 1998). Moreover, at, and only at, this exact degree of stretching, the DNA bases are found to be perpendicular to the helix axis. This stretched form can accommodate a DNA single strand in its enlarged minor groove, leading to a triple helix of a new type, termed 'stretched-triplex' or ST-DNA, which is, however, metastable (Bertucat *et al.* 1999; Figs. 4*c* and 5*c*). This structure can be related to the crystal structure of a RNA pseudoknot showing a stretched 6-residue loop segment interacting in the minor groove of a double helix (Su *et al.* 1999). In this case, however, interactions in the minor groove involve the 2′-OH groups and do not require sequence homology.

An alternative model for RecA-induced DNA stretching has been proposed by Nishinaka *et al.* (1998), based on their NMR structure determination of a tetranucleotide bound to RecA (Nishinaka *et al.* 1997). This model proposes two stretched DNA forms, obtained when the sugar puckers are all set to either C3′-endo or C2′-endo. The pitch of these model structures respectively correspond to those of the RecA/ATP and RecA/ADP filaments. This caused the authors to propose that strand exchange may be linked to sugar repuckering, while Bertucat *et al.* (1999) rather find that the link between sugar repuckering and pitch variation is independent of strand exchange. The models proposed by Nishinaka present a regular axial distribution of the stretching deformations, discussed below (Section 3·5).

3.5 Axial distribution of the DNA stretching deformation

Determining the elongation distribution in a stretched DNA form is not straightforward (Zhurkin *et al.* 1993). Indeed, uniformly stretching a B-DNA by 50% with its bases perpendicular to the axis produces a 5·1 Å base separation and results in the base-stacking interactions being completely disrupted (see Zhurkin *et al.* 1994; Nishinaka *et al.* 1998). This is an unfavorable situation because of the loss of stacking energy and since the 1·7 Å space (5·1–3·4 Å) created

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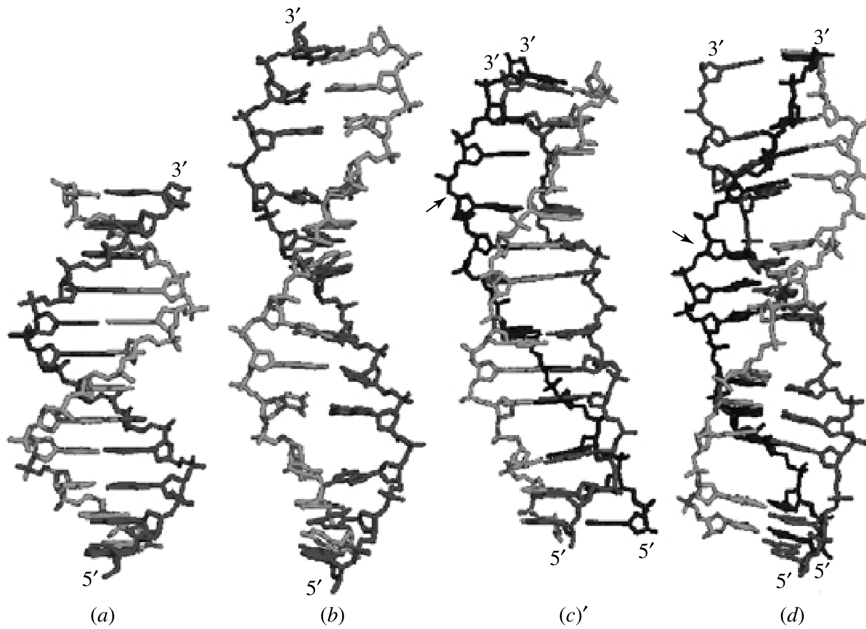

Fig. 5. Representation of (*a*) B-DNA, (*b*) B-DNA stretched by a factor of 1·5, (*c*) ST-DNA and (*d*) R-DNA. Stretching B-DNA by pulling on the 3′ extremities of each strand leads to an unwound structure where the minor groove is wide open. This allows insertion into this groove of a DNA single strand (arrow), identical in sequence to the first strand and oriented in the 5′–3′ direction, giving way to (*c*) the ST-DNA triple helix (Bertucat *et al.* 1998). Exchanging the base pairing within ST-DNA leads to (*d*) an R-DNA triple helix (Zhurkin *et al.* 1994; Bertucat *et al.* 1999), obtained here at constant stretching. The incoming DNA strand (arrow) is now paired with the complementary strand of the initial duplex (light gray).

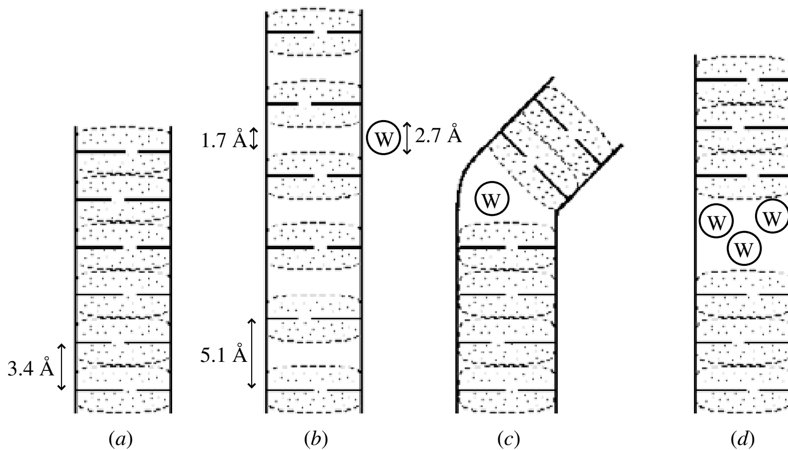

Fig. 6. Schematic representation of the stacking interactions within (*a*) B-DNA; bases are separated by 3·4 Å, (*b*) DNA uniformly stretched by 50%; the 1·7 Å wide space between two consecutive base pairs does not allow insertion of a water molecule (diameter 2·7 Å), (*c*) DNA locally kinked by minor groove binding proteins, (*d*) non-uniformly stretched B-DNA as found in the model ST-DNA triple helix. In cases (*c*) and (*d*) water molecules as well as amino-acid side-chains can intercalate into the gaps separating stacked base pairs.

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between the bases cannot accommodate a water molecule (Fig. 6*b*). Partial stacking recovery can be expected via sugar C2′ methylene/base-stacking interactions such as those found in the solution structure of single-stranded DNA bound to RecA (Nishinaka *et al*. 1997). Indeed, interactions of this sort are locally present in several cases of duplex DNA bound to architectural proteins (see Fig. 4*a*, *b*). They occur at unwound base-pair steps ($\sim 20^\circ$ twist) where roll deformations allow additional stacking recovery. However, the corresponding base separation does not exceed 4·2 Å, which is insufficient to account for the overall degree of stretching observed in these structures. Complete loss of stacking is thus required, at least locally. This is generally characterized by sharp kinks (Figs. 4*a*, *b* and 6*c*), where amino-acid intercalation often takes place. Thus, architectural proteins stretch DNA in a non-uniform manner which allows for a partial conservation of the stacking interactions.

In the case of RecA-bound DNA, stretching cannot generate curvature due to the stiffness of the filament (Nordén *et al*. 1992; Hegner *et al*. 1999). Base separation is nevertheless likely to be non-uniform, with consecutive groups of stacked base pairs interrupted by gaps in the stacking interactions (Fig. 6*d*). This situation is observed in the ST-DNA triplex model of Bertucat *et al*. (1998). Here, gaps in stacking interactions between consecutive base triplets reach 8 Å and coexist, like in TBP or IHF, with only slightly stretched (20%) base-pair stacks stabilized by C2′ methylene/base-stacking interactions or with completely unstretched stacks (Fig. 4*c*). Currently, there is no experimental evidence for such a non-uniform stretching and several attempts to find a residue that would intercalate into a possible gap in RecA-bound single-stranded or double-stranded DNA proved unsuccessful (Eriksson *et al*. 1993; Takahashi & Nordén, 1994b; Maraboeuf *et al*. 1995). Volodin *et al*. (1997) have shown that local differences in environment exist for the bases of a RecA-bound single-stranded DNA, with repeats every three bases. It is, however, not known whether this effect is related to stacking modifications or rather to a non-uniform winding or to specific interactions with protein side-chains.

4. Contacts between RecA and the DNA strands

4.1 The DNA-binding sites

RecA can bind three independent DNA strands, with sequences which are not necessarily related. It has been established that there are at least two distinct DNA-binding sites within RecA, although they may overlap to some extent. Detailed information on these sites can be found in two previous review articles (Takahashi *et al*. 1996; Rehrauer & Kowalczykowski, 1996). We briefly recall their characteristics, restricting our discussion to RecA–DNA complexes formed in the presence of ATP or its analogs.

The first binding site concerns the DNA strand on which the filament forms. DNA is tightly bound to this site and cannot be displaced or removed even in the presence of high salt concentrations (Gourves *et al*. 2001), unless ATP is hydrolyzed or an electric field is applied (Svingen *et al*. 2001). The second site is the binding site of an incoming DNA which will be incorporated into the nucleoprotein filament. This site shows a higher affinity for single-stranded DNA than for duplex DNA (Mazin & Kowalczykowski, 1996). When the cofactor is ATPγS, it binds the DNA strand displaced after strand exchange (Mazin & Kowalczykowski, 1998). Under these conditions, the displaced strand remains attached to the filament, contrary to what happens when the cofactor is ATP. Binding to this site is much looser than to the first site and the DNA strand can be removed by SSB (single-strand binding protein) or displaced by another DNA strand

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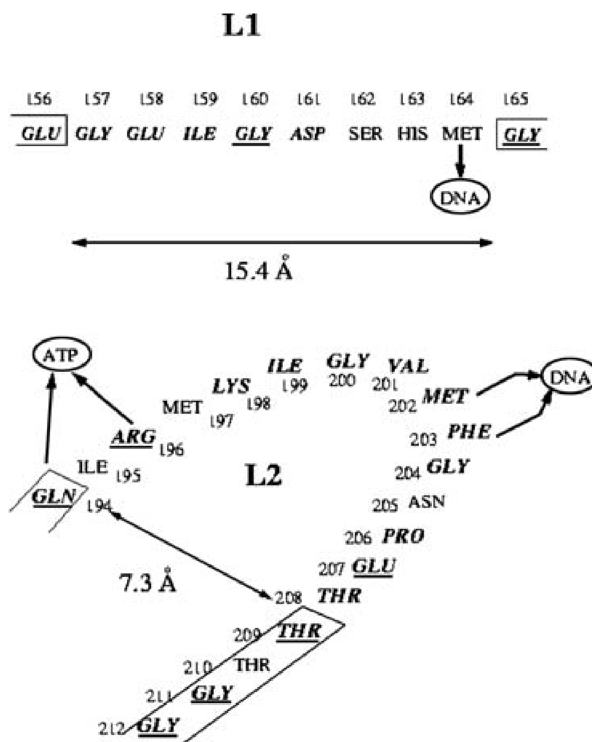

Fig. 7. Schematic representation of loops L1 and L2 of *E. coli* RecA, indicating the amino-acid sequence. Residues proposed to interact with ATP are indicated. Residues shown as pointing towards DNA are close to the constitutive DNA single strand of the RecA filament, according to cross-linking experiments or fluorescence spectroscopy. Residues represented in bold and italics are conserved among the RecA proteins of bacteria (Karlin & Brocchieri, 1996) and underlined residues are critical for RecA function (see Section 4·1).

with more affinity for RecA (the affinity being inversely proportional to its propensity to form a secondary structure; Biet *et al.* 1999).

Site location has been studied via cross-linking between amino-acid residues of RecA and DNA (Malkov & Camerini-Otero, 1995; Morimatsu & Horii, 1995; Rehrauer & Kowalczykowski, 1996; Wang & Adzuma, 1996), fluorescence spectroscopy of tryptophan inserted as a fluorescent probe for DNA binding (Maraboeuf *et al.* 1995) and studies based on mutagenesis or amino-acid substitutions (Tessman & Peterson, 1985; Nastri & Knight, 1994; Ishimori *et al.* 1996; Cazaux *et al.* 1998; Hörtnagel *et al.* 1999). These studies have been able to define several RecA regions as candidate binding sites. We will focus here on two regions situated on the inner surface of the filament, formed by the two loops L1 (156–165) and L2 (194–210) which are disordered in the crystal structure of *E. coli* RecA (Fig. 7).

Loops L1 and L2 have been shown to cross-link the constitutive DNA single strand of the filament. Met164 of loop L1 and Met202 of L2 form covalent bonds with 5-iodouracil residues (Malkov & Camerini-Otero, 1995; Wang & Adzuma, 1996), which means that the functionalized bases and the reactive amino acids are apparently close to one another, at least temporarily (the distance required is typically 4–5 Å for 5-bromouracil; Dong *et al.* 1994; Wang & Adzuma, 1996). By introducing single or multiple amino-acid substitutions in residues 152–159 which overlap the loop L1, Nastri & Knight (1984) have identified residue Glu154 as critical to RecA function. While not systematically investigated, Gly160 and Gly165 also appeared to be important for

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repair activity. Saturation mutagenesis of loop L2 identified residues Asn193, Gln194, Arg196, Glu207, Thr209, Gly211 and Gly212 as essential for recombinational activity (Hörtnagel *et al.* 1999). In addition, the fluorescence of a tryptophan replacing Phe203 of loop L2 was reported to be quenched upon DNA binding, indicating proximity to the DNA bases without intercalation (Maraboeuf *et al.* 1995). It was also shown that replacement of the negatively charged Glu207 by a neutral glutamine residue inhibits recombination, but neither DNA binding nor ATP hydrolysis (with, however, a reduced rate of hydrolysis), while replacement with a positively charged lysine residue slowed the strand-exchange mechanism (Cazaux *et al.* 1998). These observations make L2 a good candidate for the second DNA-binding site, although it has also been proposed to play a role in the first binding site, based on the annealing and recombinational activities of an isolated L2 peptide (Voloshin *et al.* 2000). Loops L1 and L2 have, in fact, both been proposed to participate in either the first or the second binding site and it was recently suggested that L1 indeed participates in both sites (Mirshad & Kowalczykowski, 2003). We remark, however, that while the activity of the second binding site can be investigated separately from that of the first one (Cazaux *et al.* 1998), the contrary is not easy to achieve since DNA binding to the first site is a prerequisite to recombination activity. In what concerns strand recognition, the DNA bound in the first binding site can be considered as part of the second binding site. In any case, it is probable that the two binding sites are very close to one another since the fluorescence of tryptophan replacing Phe203 of loop L2 is modified when adding either the first or the second DNA strand (Maraboeuf *et al.* 1995). Finally, residue Arg169 situated near the junction between helix F and loop L1 has been shown to be essential for recombination activity (Tessman & Peterson, 1985). There is evidence that this residue binds the first DNA strand (Ishimori *et al.* 1996). We discuss below the structural implications of DNA interacting with loops L1 and L2.

4.2 Possible arrangement of loops L1 and L2 and the three bound strands of DNA

A first remark is that there is not much space inside the RecA nucleofilament. The radius of the central cavity appearing in a top view of the crystal structure of *E. coli* RecA (in the absence of the disordered loops) is roughly 16 Å (see Hegner *et al.* 1999 and Fig. 1*b*), except for two residues at one extremity of loop L1 which reach a distance 12 Å from the filament axis. If the backbones of loops L1 and L2 followed the same folds as in MtRecA (here called the Mt-L1 and Mt-L2 folds, see Fig. 8*a*), they would respectively reach distances of 9 and 13 Å from the filament axis. Note that there is little chance that the cavity will be any larger in the RecA/ATP filament than in the RecA/ADP crystal structure, due to a smaller diameter of the filament (100 Å against 120 Å in the crystal structure). It has been noted that a stretched DNA strand situated within the filament interior, coaxial with the protein, could lie up to 15–17 Å from the filament axis (Egelman & Stasiak, 1986; Egelman & Yu, 1989; Malkov *et al.* 2000). Indeed, recent work based on fluorescence energy transfer is compatible with a position for the incoming DNA single strand at 16 Å from the filament axis (Singleton & Xiao, 2002; Xiao & Singleton, 2002). This leaves little space between the DNA and the protein to accommodate the L1 and L2 loops and suggests that part of these loops may lie in the grooves of incorporated duplex DNA.

As noted elsewhere (Takahashi *et al.* 1996), loop L1 presents extremities too far apart to be compatible with its folding into a regular secondary structure, while loop L2 may potentially be folded into a β-hairpin (Fig. 7). The propensity of the isolated peptide L2 to form a β-hairpin has been established by the group of Camerini-Otero (Wang *et al.* 1998) and by Sugimoto (2001). The first group showed that a β-hairpin structure is induced upon DNA binding, while Sugimoto

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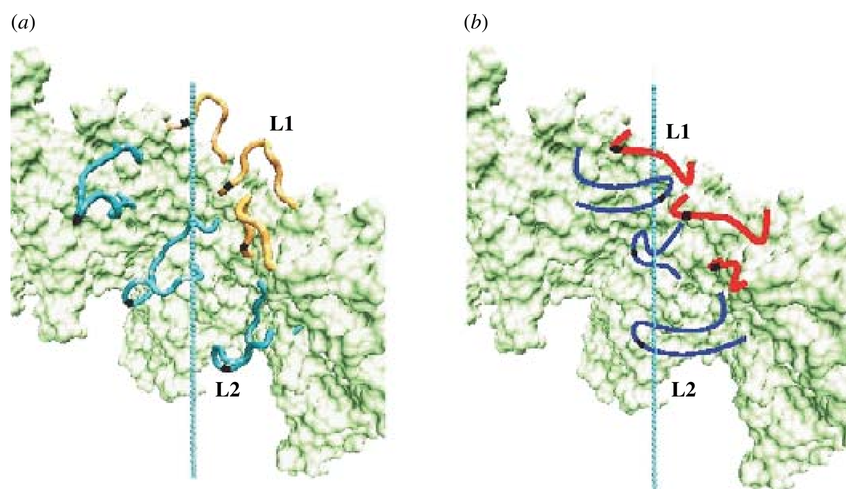

Fig. 8. Possible folds of loops L1 and L2 within the *E. coli* RecA protein. (*a*) The loop folds are taken from MtRecA crystal structures, PDB entries respectively 1MO5 for loop L1 (yellow) and 1MO4 for loop L2 (cyan). (*b*) Schematic representation of model folds for loops L1 (red) and L2 (blue). Loop L2 has been modeled as a β-hairpin structure. In both views, three monomers are represented, viewed from inside the filament of RecA crystal. The locations of residues Met164 for loop L1, Met202 for L2 are indicated by black dots. The filament axis is represented in cyan. The views have been generated using VMD (Humphrey *et al.* 1996).

showed that folding can occur even in the absence of DNA, provided that the small helix G following loop L2 is incorporated to the peptide (see Fig. 2*b*). While the Mt-L2 fold recently crystallized shows almost no β-hairpin character, it cannot account for the cross-linking results of Wang & Adzuma (1996). Applying this fold to the *E. coli* RecA/ADP crystal structure together with the Mt-L1 fold for loop L1 (Fig. 8*a*) would place Met202 at a distance of 29 Å from the closest Met164 of L1. Since that distance is about twice the maximum distance between bases bound to a same monomer (~12–15 Å), this would not allow these residues to simultaneously cross-link the same DNA single-strand bound in the first site, as observed experimentally. We certainly cannot exclude that the geometric change in the monomer orientations and positions accompanying the passage from the inactive to the active form could bring these two residues closer to the axis and to each other. However, it is rather probable that the structure of loop L2 is modified in the presence of DNA, as strongly suggested by the conformational changes observed in isolated L2 peptides upon DNA binding. There are documented examples, among which the case of methyl transferase is most spectacular, where loops change both their position and conformation upon binding DNA (Kilmasauskas *et al.* 1994). We can thus speculate which zones within the filament can be reached by the extremity of a flexible loop L2. In a model β-hairpin conformation, fully extended from residue Arg196 to residue Thr209, loop L2 has a length of 25 Å and can cross the filament interior (Fig. 8*b*) (Bastard, Thureau & Prévost, unpublished observations). We have explored the regions which can be scanned by such a loop and found that Met202, situated at its extremity, could easily come close to Met164 in a neighboring monomer. With the restriction that the crystal structure of RecA is not the active form, the arrangement shown in Fig. 8*b* is consistent with the first DNA strand being clamped between the L1 and L2 loops, with its phosphodiester backbone contacting the protein interior. It agrees with

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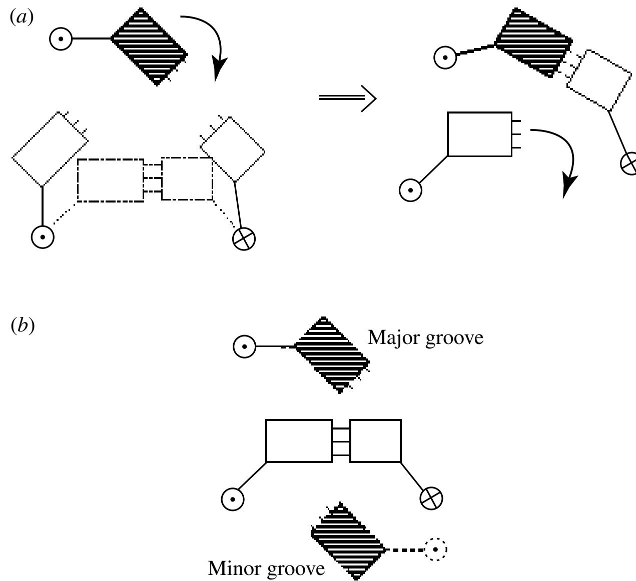

Fig. 9. Two possibilities for strand association: (*a*) the base pairs of the double-stranded DNA can open before its association with the invading strand; in this hypothesis, the side of introduction of the invading strand is not relevant; (*b*) the invading strand can associate the duplex DNA before exchange, via either the minor or the major groove. The representation in (*a*) and (*b*) corresponds to a view perpendicular to the base-pair plane. The bases are represented by rectangles. The base of the incoming strand is cross-hatched. The phosphodiester backbone is represented by a dot for the strands in a 5′–3′ orientation and by a cross for the 3′–5′ strands.

the arrangement proposed by Wang and Adzuma based on their cross-linking results (see Fig. 6 of Wang & Adzuma, 1996).

Additional movement of loop L2 could also occur during the steps of strand exchange. By separately cross-linking two independent DNA strands in the presence of ATPγS, Adzuma's group showed that upon introduction of the second strand, Met202 of L2 looses its contact with the strand situated in the first site and cross-links the second strand (Wang & Adzuma, 1996). This may indicate a position or conformational change for loop L2, displacing Met202 away from the first strand.

5. Strand arrangement during pairing reorganization

5.1 Hypotheses for DNA strand association

The relative position of the three DNA strands within the RecA filament necessarily varies as strand exchange occurs, although no details of this process are known. Two situations have been envisioned and discussed (Fig. 9) (Howard-Flanders *et al.* 1984; Stasiak, 1992; Camerini-Otero & Hsieh, 1993; Kurumizaka & Shibata, 1996). In the first model, the double-stranded DNA opens prior to single-strand association, in which case sequence recognition would rely on the RecA-bound single strand directly reading the Watson–Crick functional groups of the complementary strand of the incoming duplex DNA. It has, however, been objected that strand separation is not observed in the absence of strand exchange (Hsieh & Camerini-Otero, 1989).

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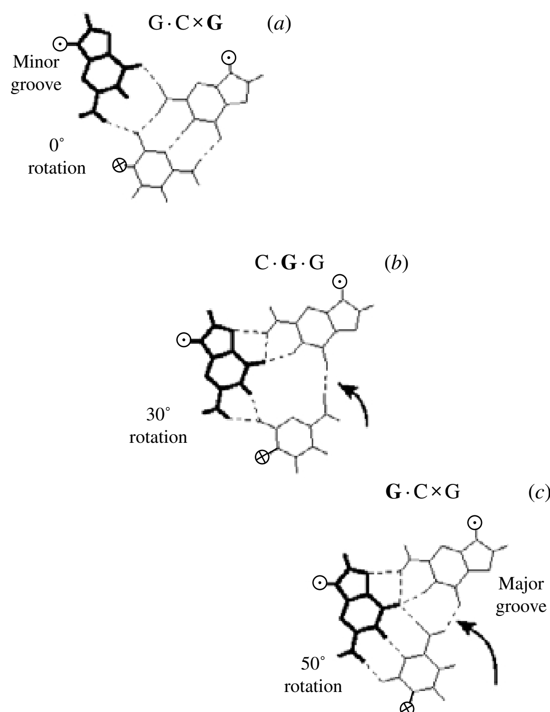

Fig. 10. Three steps in the simulation of pairing exchange within a G · C × G base triplet belonging to a ST-DNA triple helix (Bertucat *et al.* 1999). (*a*) Within the ST-DNA triplet, the incoming guanine (bold line) interacts in the minor groove side of base pair G · C via its Watson–Crick functional groups. Rotation by 30° of the cytosine around its phosphodiester backbone, accompanied by relaxation of the rest of the triplex, leads to an intermediate structure of the base triplet where each base interacts with the other two (*b*). A 50° rotation leads to complete pairing exchange (*c*). The incoming guanine now is paired with the cytosine of the original duplex and the displaced guanine is situated on the major groove side of the newly formed base pair. Exchanging all the base triplets of a ST-DNA leads to an R-DNA triple helix.

Alternatively, association may involve an intact duplex, which implies sequence reading via weaker interactions in either the major or the minor grooves (Howard-Flanders *et al.* 1984). Interactions in either groove of a stretched/unwound DNA are theoretically possible and the corresponding triple helices, respectively called R-DNA (Zhurkin *et al.* 1994) and ST-DNA (Bertucat *et al.* 1998), have been modeled. The R-DNA triple helix allows faithful sequence recognition and has been independently synthesized (Shchyolkina *et al.* 1994) and structurally characterized (Chiu *et al.* 1993; Dagneaux *et al.* 1995a, b). However, the arrangement of the three strands within the model ST-DNA triplex (Fig. 5*c*) appears appropriate for strand exchange to occur via base-pairing exchange (Bertucat *et al.* 1999) (Fig. 10). This process was simulated by simply rotating the complementary strand of incoming duplex DNA and gave way to a triplex of the R-form (Fig. 5*d*), with the displaced strand positioned in the major groove of the newly formed heteroduplex. Such a conversion from ST-DNA to R-DNA triplexes via pairing exchange appeared to be highly exothermic. While the ST-DNA triple helix is metastable and only allows limited sequence recognition, its characteristics make it a credible transient intermediate of association (Bertucat *et al.* 2000). This model is consistent with two-step recognition process occurring partly during association and partly during strand exchange, in accord with experimental kinetic observations (Bazemore *et al.* 1997a).

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5.2 Association via major or minor grooves

Experimentally, the face of association of the incoming double helix to the RecA-coated single-stranded DNA has not been fully established but as already noted, a consensus has emerged favoring the minor groove side (Adzuma, 1992; Baliga *et al.* 1995; Podyminogin *et al.* 1996; Tuite *et al.* 1997; Zhou & Adzuma, 1997; Singleton & Xiao, 2002). A noticeable exception can be found in a radio-probing study of the strand-exchange product (Malkov *et al.* 2000). However, interpretation of this experiment relies on a model DNA position with base pairs centered at the helix axis (with no lateral displacement). This assumption conflicts with a recently published characterization of post-strand exchange geometry for the three DNA strands, using fluorescence energy transfer (Singleton & Xiao, 2002; Xiao & Singleton, 2002), which establishes a lateral duplex displacement from the helix axis. This study also confirms a shift of the displaced strand by $\sim$3 bp along the axis as observed in the radio-probing experiment, and furnishes precise information on the relative strand locations.

5.3 Post-strand exchange geometries

It has not been determined whether a triple helix conformation appears in the course of the strand-exchange process. Although a number of elements support this hypothesis (Hsieh *et al.* 1990; Rao *et al.* 1991; Muller *et al.* 1992; Chiu *et al.* 1993) others argue against it (Adzuma, 1992; Podyminogin *et al.* 1996; Malkov *et al.* 2000; Singleton & Xiao, 2002). These contrasting results may be due to different experimental conditions, the most striking difference being undoubtedly the cofactor present. Each time a stable triple helix was observed, identified with the R-form DNA, the cofactor was ATP. Conversely, studies which concluded in a disconnection of the displaced strand from the heteroduplex, including the radio-probing and fluorescence studies already mentioned, used ATPγS as the cofactor (Adzuma, 1992; Mazin & Kowalczykowski, 1998; Malkov *et al.* 2000; Xiao & Singleton, 2002). Note that in the former studies, the observation of a triple helix which was stable after deproteinization required the release of the 5′-end of the displaced single strand to be impeded. This argues in favor of the triple helix being a strand-exchange product, like the three-stranded intermediate observed when using ATPγS, rather than an association intermediate as initially proposed. The importance of the cofactor has been further highlighted in a study by Jain *et al.* (1995) on psoralen photocross-linked strand exchange RecA complexes. In this study, all three DNA strands could be cross-linked only if strand exchange had taken place in the presence of ATP and if ATP had been hydrolyzed. When the cofactor was ATPγS, only the strands of the newly formed duplex could be cross-linked.

It is not surprising to observe different post-exchange arrangements for the three strands when using ATP or ATPγS, in the light of what is known about the role of ATP hydrolysis in RecA function (see, e.g. Cox, 1994). ATP hydrolysis is not required for DNA strand exchange (Menetski *et al.* 1990; Rehrauer & Kowalczykowski, 1993; Kowalczykowski & Krupp, 1995), but it is a determinant for the reaction directionality (Jain *et al.* 1994; Shan *et al.* 1996) and for bypassing zones of sequence heterology (Rosselli & Stasiak, 1991; Kim *et al.* 1992; Shan *et al.* 1996; Bucka & Stasiak, 2001). It has been established that ATP hydrolysis is coupled to the process of strand exchange (Nayak & Bryant, 1999) and there is strong evidence that this coupling essentially affects the last step of the reaction, i.e. strand release (Bedale & Cox, 1996). Further, a kinetic study by Gumbs & Shaner (1998), performed in the presence of either ATP or

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ATPγS, argues in favor of identical processes up to the stage where base-pairing exchange has taken place (see also Bazemore, 1997b).

As seen earlier, ATP hydrolysis and the resulting filament plasticity are intrinsically linked to possible modifications of the binding sites, situated near the monomer interface and comprising the two flexible loops of RecA. A known consequence is a modulation of DNA-binding affinity. Given the proximity of loop L2 to both DNA-binding sites and its involvement in DNA binding, the proposed conformational change of this loop upon ATP hydrolysis is likely to produce an arrangement of the DNA strands which is completely different from that observed in the absence of hydrolysis. The following scenario can be proposed in order to interpret the observed differences. Without ATP hydrolysis, loop L2 may impede the displaced DNA strand from closely interacting with the duplex DNA. During its conformational change or movement driven by ATP hydrolysis, it may drag the displaced strand along, favoring strand release. When the strand movement is impeded, by attachment of its 5′-end, loop L2 could reorganize its position, refolding onto the three strands structured as a triple helix.

6. Conclusion

The mechanism of RecA-induced strand exchange between single-stranded DNA and homologous double-stranded DNA is still far from being unraveled. The principal reasons explaining why a model has not emerged were clearly established over 10 years ago by Egelman (1993), who pointed out: (i) the fact that the active form differs from the crystal form of the filament; (ii) the lack of information on the atomic structure of stretched/unwound DNA in the presence of RecA; (iii) the lack of precise knowledge concerning the RecA residues directly interacting with DNA; (iv) the missing electronic densities in the crystal structure of RecA, corresponding to two flexible and disordered loops. These questions unfortunately remain unanswered. However, new information has been obtained for each of these four points, some opening promising ways to fill-in the missing data.

Structural characterization of the monomer orientations and positions within the active form is progressing rapidly (Morimatsu *et al.* 2002; VanLoock *et al.* 2003), which should allow a more precise identification of the residues on the internal surface of the active filament. The arrangement of the three DNA strands within the post-strand exchange RecA/ATPγS filament has also been characterized (Malkov *et al.* 2000; Singleton & Xiao, 2002). The stretched form of DNA has been the subject of physical studies and models are now available which compare favorably with the structure of DNA stretched by architectural proteins (Cluzel *et al.* 1996; Lebrun & Lavery, 1996; Lebrun *et al.* 1997). The specific properties of stretched DNA may contribute substantially to RecA-induced strand exchange, as indicated by the model of Bertucat and colleagues bearing on the nucleic acid part of the process (Bertucat *et al.* 1999, 2000). Finally, particular conformations for loops L1 and L2 have been solved within MtRecA crystal structures (Datta *et al.* 2003). They undoubtedly represent a new reference although they may greatly differ from the forms directly involved in DNA binding (Wang *et al.* 1998; Sugimoto, 2001).

Building a model for RecA-induced strand exchange still requires a characterization of the arrangement of the three DNA strands within the association complex and knowledge of the interactions of each strand with specific binding regions of the protein. In addition, such a model will necessarily have to include the dynamical aspects of the reaction, notably coordinating strand

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exchange with the reorganization of the DNA-binding sites and ATP-driven conformational rearrangements of the filament with conformational changes of loop L2.

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