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Activation of recA protein: the pitch of the helical complex with single-stranded DNA

Elizabeth A.Hewat, Rob W.H.Ruigrok[1] and Elisabeth DiCapua[1]

Laboratoire de Biologie Structurale, CEA and CNRS URA 1333, DBMS/DSV, CENG, 85 X, F-38041 Grenoble and [1]European Molecular Biology Laboratory, Grenoble Outstation, 156 X, F-38042 Grenoble, France

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The complex of recA protein with single-stranded DNA in the presence of ATP is the active species in the three enzymatic activities of recA: the initiation of strand exchange, the hydrolysis of ATP and the cleavage of repressors. Here we find by cryo-electron microscopy of unstained and unfixed samples that the helical structure of the protein coat in this complex differs slightly but significantly from the structure in the complex with double-stranded DNA. We discuss how the larger pitch of the complex with single strands (100 ± 2 Å compared with 95 ± 2 Å with double strands) could contribute to its higher enzymatic activity.

Key words: recA/helical complex/cryo EM

Introduction

recA of *Escherichia coli* is necessary for homologous recombination (Clark and Margulies, 1965) and the control of the SOS response of DNA repair following UV or drug treatment (Witkin, 1976). These effects have been shown *in vitro* to be produced by the activity of recA protein: the purified protein (M_r 37 800) can perform strand exchange between two DNA substrates (reviewed in Griffith and Harris, 1988) and it can induce the cleavage of lexA protein [the repressor of a family of ~20 genes, many of which are involved in DNA repair (Little and Mount, 1982)]. For both activities, recA requires the presence of both DNA and ATP. ATP is hydrolyzed at a rate of 20–30 ATP molecules per minute (reviewed in Roca and Cox, 1990), but the non-hydrolyzable analogue ATPγS supports both strand exchange (Menetski *et al.*, 1990; Rosselli and Stasiak, 1990) and repressor cleavage (Phisicky and Roberts, 1981).

The active form of recA, the complex with DNA and ATP, has been shown by EM to be a filament with the DNA covered by a helical protein coat of six recA units per turn of 95 Å pitch (reviewed in Egelman and Stasiak, 1986). The structure of the protein coat in these filaments appears indistinguishable whether formed on single-stranded (ss) or double-stranded (ds) DNA. However the level of enzymatic activity of the two complexes differs in strand exchange, ATP hydrolysis and lexA cleavage, the single strand usually being more active (Roca and Cox, 1990). Under well controlled conditions, however, the double strand can support both strand uptake (Müller *et al.*, 1990) and strand exchange (West *et al.*, 1981; Conley and West, 1989). ATP hydrolysis in the presence of dsDNA was shown to be controlled by the binding efficiency of dsDNA (Pugh and Cox, 1988a). But the lexA cleavage reaction is usually an order of magnitude slower in the presence of dsDNA (the rate relative to ssDNA was 20% in Craig and Roberts, 1980; <5% in Takahashi and Schnarr, 1989; 20% in our hands, where binding of the DNA is controlled, E.DiCapua, unpublished).

The basis of the difference may lie in the charges and hydrophobicity resulting from the occupancy of binding sites. The binding sites for ss and dsDNA can be predicted to be (i) existing as two entities, since both are occupied simultaneously during strand exchange, but also (ii) interchangeable to some extent, so as to be able to accommodate both the reactants and the products of strand exchange (albeit with different affinities in order to drive the reaction, reviewed by Stasiak *et al.*, 1991), and to explain the partial equivalence of ssDNA and dsDNA for lexA cleavage. A still puzzling difference between the binding of ds and ssDNA to recA is the binding stoichiometry: while in the double strand complex, precisely 3 base pairs (bp) are bound per recA unit, the stoichiometry to ssDNA varies between 6 and 3 nucleotides per recA, depending both on the method of analysis and on the model used for interpretation (reviewed in Roca and Cox, 1990). Biochemistry tends to suggest 6 nt/recA by titration with the fluorescent analogue etheno-DNA, while by enzymatic assays 3 nt/recA are found by titration of the ATPase and lexA cleavage activities. Direct visualization by EM shows a trend towards 3 nt/recA (calculated by combining the contour length of complexes, the known number of nucleotides in the DNA, and the established number of recA units per unit length); this stoichiometry, however, is seldom reached either by all the molecules in a population (Flory *et al.*, 1984), or even, at all (our own unpublished observations), although single experiments did achieve the expected stoichiometry (Koller *et al.*, 1983); this legitimates the structural models of Howard-Flanders *et al.*, (1984) where 3 nt/recA in the 'resident' complex 'assimilate' 3 bp/recA into the second site, ready for strand exchange. However, the biochemical observations suggest a rather 'fuzzy' binding site for ssDNA. This 'fuzzy' site may be a physical reality which might indeed enhance the reactivity of the complex, in contrast to a more static, well-filled binding site.

In this paper we show that the outer structure of the filament indeed reflects this state under some conditions of observation: in electron microscopy of samples in amorphous ice (unfixed, unstained, and not adsorbed to a surface), the pitch of the helical protein coat of the single strand complexes is found to be 100 ± 2 Å. while it is 95 ± 2 Å in the double strand complex. Both structures converge to ~95 Å in negatively stained specimens.

Results

The pitch of recA complexes with DNA in the presence of ATPγS

Figure 1 illustrates the appearance of the samples that were analyzed. The filaments, although never quite straight, were found to be rather regular and randomly oriented. Several preparations were analyzed and the results are shown in Table I: reproducibly, complexes with ssDNA have a pitch of 100 Å, as compared with 95 Å for the complex with dsDNA. The input stoichiometry of ssDNA did not have an influence on the pitch (Table I) [although the contour length of the complexes was very different, being short at 12 nt/recA (excess DNA) and long at 3 nt/recA (excess recA) where some of the protein was visible as self-polymer rods]. The same samples prepared with negative stain (uranyl acetate) showed a pitch of ~95 Å for both complexes, using the same microscope, at the same setting and with the same method of analysis. These specimens were also analyzed on micrographs from another microscope by optical diffraction, yielding the same result (not shown, see Materials and methods).

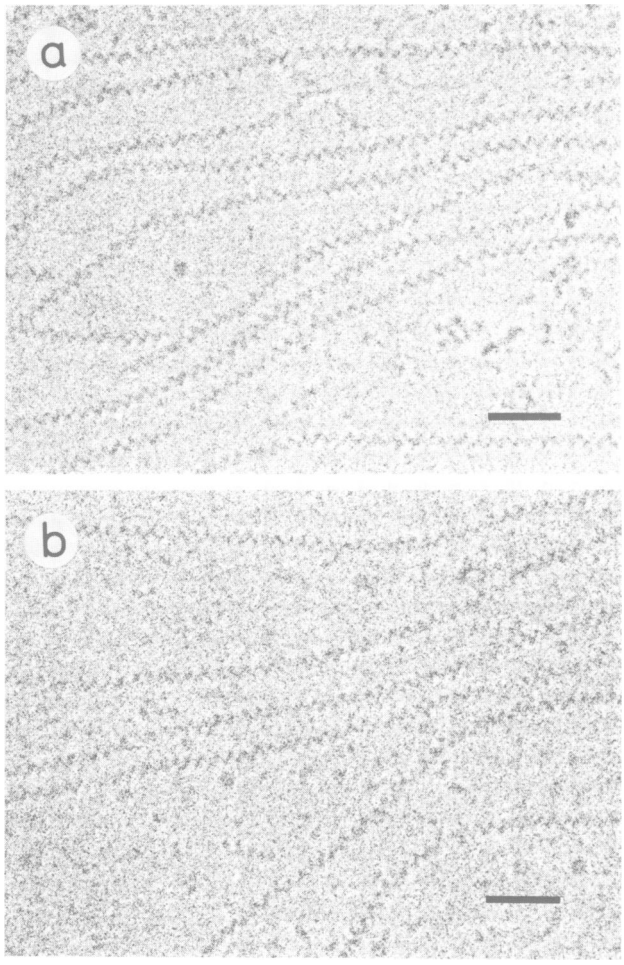

Fig. 1. Micrographs of recA complexes in amorphous ice in the presence of ATPγS. (**a**) Complex with ssDNA (M13 DNA, 6 nt/recA). (**b**) Complex with dsDNA (linear pUC8 plasmid DNA, 4.5 bp/recA). The bar represents 50 nm.

Strand exchange and ATPase activity are much enhanced in the presence of 12 mM Mg^{2+} (Roca and Cox, 1990). Since these concentrations of Mg^{2+} lead to aggregation of filaments ('network formation', Tsang *et al.*, 1985), they are usually avoided in structural studies. We looked at a sample in 12 mM Mg acetate; indeed, most of the material was tangled, but isolated filaments at the edges showed a pitch of 100 ± 2 Å.

The difference in pitch between the ss complex and the ds complex is significant

As the difference between the two measured pitches is small and only just larger than the standard deviation, it is very important to exclude preparation artefacts and fluctuations, both at the level of the biochemistry (by using identical buffer conditions) and at the level of microscopy (by avoidance of flow and packing artefacts).

We therefore mixed the two complexes (which had been pre-formed in the same buffer, independently to avoid a hypothetical sequestration of the protein by the DNA with more affinity, or even a reaction between the two DNAs) and prepared the mixture for cryo-microscopy. The DNAs could be distinguished because the dsDNA was linear, and the ssDNA circular. The analysis of the pitch was as above; stretches of 10 helical turns were measured and the result is shown as a histogram (Figure 2); the hatched bars are from stretches belonging to circular molecules, the black bars from linear molecules. The histogram clearly shows two separate peaks for the two sorts of complexes. It also visualizes the width of the distribution expressed as standard deviation in Table I.

This experiment also addressed the question of whether the difference might arise from the difference in DNA sequence: in this experiment, the ssDNA was from phage φX174 whereas the experiments in Table I were performed with M13 ssDNA. While this choice was directed by the size of the circles (5386 nt are easier to identify as circles in a tangled mixture of filaments than are 7300 nt), the reproduction of the 100 Å pitch with a different DNA

Table I. Helical pitches of recA complexes with DNA in the presence of ATPγS

	In amorphous ice				In negative stain
Complex with ss M13 DNA	*3 nt/recA*	*6 nt/recA*	*12 nt/recA*	*6 nt/recA 12 mM mg^{2+}*	*6 nt/recA*
	100 ± 2	100 ± 2	100 ± 2	100 ± 2	96 ± 4
	n = 20	*n* = 107	*n* = 28	*n* = 32	*n* = 30
Complex with linear dsDNA	*all at 4.5 bp/recA*				
	95 ± 1	95 ± 2			94 ± 2
	n = 20	*n* = 9			*n* = 30

The values are in Å; *n* is the number of stretches 10 turns long that were measured.

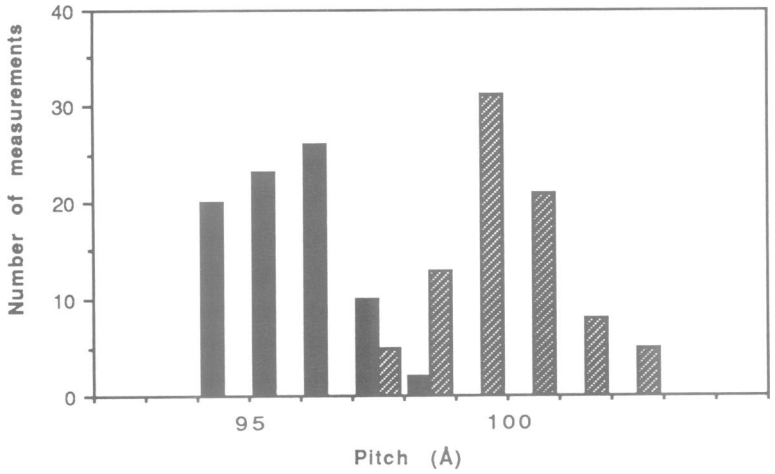

Fig. 2. Distribution of the pitches measured in a mixed population of complexes with circular ssDNA (hatched bars) and linear dsDNA (black bars). *Short* linear fragments were excluded from the analysis, as they may arise from breakage of either of the two DNAs.

suggests that, as expected, the sequence of the DNA does not play a role in binding to recA protein.

Complexes in the presence of ATP

The experiments described so far were performed using ATPγS. The use of the non-hydrolyzable analogue is legitimate since *in vitro*, both strand exchange and lexA cleavage can be performed in the absence of ATP hydrolysis. However, we were curious to see whether we could analyze the structure in the presence of the more physiological ATP.

As samples for cryo-EM need to be relatively concentrated in recA, the ATP present in the mixture will be used up rapidly. This was circumvented by incubating the complexes for only short periods, during which only a few percent of the ATP molecules were hydrolyzed (see Materials and methods). Such samples were analyzed as before. Table II shows that complexes with dsDNA had a pitch of 96 ± 2 Å. Complexes with ssDNA were found to have a pitch of 100 ± 2 Å. In this limited study, we did not find irregular filaments, as might have been expected from recA subunits in the process of hydrolyzing ATP. It would be interesting to study the structure of a filament hydrolyzing ATP with time but this is technically rather difficult. The method of blotting the grid to obtain a thin film for fast freezing possibly removes the substrate, which could locally result in high concentrations of ADP. As it stands, the structure of the filament in the process of hydrolyzing ATP rather supports the allosteric model where those units that contain uncleaved ATP determine the structure of their neighbors (Roca and Cox, 1990).

Table II. Helical pitch (Å) in the presence of ATP

	In amorphous ice		In negative stain
Complex with ss M13 DNA	100 ± 2.5 *n* = 20		94 ± 4 *n* = 15
Complex with linear dsDNA	96 ± 2 *n* = 17	96 ± 2 *n* = 7	93 ± 2 *n* = 19

The input stoichiometry was 6 nt/recA or 4.5 bp/recA (but the micrographs show that not all recA was bound in either sample, not shown).

Discussion

This paper shows that the pitch of the enzymatically more active complex of recA with ssDNA is slightly larger than that of the complex with dsDNA when measured in amorphous ice.

A pitch of 95 ± 5 Å for both complexes is found by solution scattering (DiCapua *et al.*, 1990b); the resolution of those measurements is, however, not sufficient to establish small differences. EM in amorphous ice is close to solution conditions while it has the advantage of direct visualization of local structure. Indeed, a pitch of 102 ± 1 Å has been measured previously for the complex with ssDNA in ice (Chang *et al.*, 1988). These authors concluded that the complex shrinks during the procedure of negative staining. In addition to their observation, we have now observed that the complex with dsDNA does not appear to shrink significantly in conventional preparation. Could it be that in fact the opposite effect takes place in ice, that is, might the structure get slightly stretched, be it by flow of liquid during blotting, or by surface tension? In this case the larger pitch would be induced preferentially on the ss complex rather than the ds complex, from which the conclusion would be that the ss complex is either more flexible or more deformable. We cannot exlude this interpretation. However, the mixed sample experiment, the small standard deviation of the pitch values and the reproducibility on different regions of the grid and between different specimens allow us to speculate that the difference does lie in the pitch rather than in the flexibility, and that this may have biological consequences.

Both the strand exchange model (Howard-Flanders *et al.*, 1984) and models for lexA cleavage (M.Schnarr and E.DiCapua, unpublished) postulate a primordial role for the wide groove in the helical coat around DNA: strand exchange uses the cleft to assimilate the second DNA partner, and in repressor cleavage, lexA seems to bind into the groove in order to get cleaved. This was observed by EM of mixtures with non-cleavable mutant lexA (E.H.Egelman, personal communication), and was predicted from the location in the crystal structure of recA mutations that inactivate its role in the cleavage of some repressors (R.Story and T.Steitz, personal communication).

We therefore suggest that the wider groove may account for the higher rates of lexA cleavage by ss complexes than by ds complexes. The wider groove might also contribute to ss complexes being a better substrate for the initiation of the strand exchange reaction than the ds complex. The ATPase activity is probably less sensitive to the groove dimensions; the size of the ATP molecule and its turnover as a substrate (30 hydrolyzed per minute (Roca and Cox, 1990) as compared to 0.5 lexA cleaved per minute (E.DiCapua, unpublished), or in the order of one strand exchanged per 30 min (Roca and Cox, 1990) make it likely that chemistry (e.g. binding affinities due to charges) will play a more important role than steric considerations.

High salt concentrations have been found to induce ATP hydrolysis by recA in the absence of polynucleotide (Pugh and Cox, 1988b), and this was related to a structural change that brings the pitch of the self-polymer recA filament from ~75 Å (the 'compact', inactive form of recA, DiCapua *et al.*, 1990b) to ~95–100 Å (by solution scattering, DiCapua *et al.*, 1990a). We find by cryo EM (to be published elsewhere) that the pitch at high salt concentrations is 101 ± 3 Å, which explains that these conditions also promote lexA cleavage (DiCapua *et al.*, 1990a). Similarly, preliminary results (M.Cuillel and E.DiCapua, in preparation) reveal that heparin and RNA both induce the high pitch as well as cleavage of lexA by recA at rates comparable to those of ssDNA.

Materials and methods

Samples

Samples were prepared as in DiCapua *et al.* (1990b). The buffer was 20 mM potassium phosphate pH 6.8, 4% glycerol and 2 mM Mg acetate, conditions in which ATPγS-stabilized binding of both ssDNA (phage M13 or phage φX174 DNA) and dsDNA (linearized plasmid pUC8) have been demonstrated previously (DiCapua, 1986; DiCapua *et al.*, 1990b). recA was 1–4 mg/ml, DNA was 4.5 bp/recA for ds, and 3–12 nt/recA for ss (see text). ATPγS was 1 mM, and incubation was for 1 h at 37°C, followed by up to a day at room temperature. High Mg^{2+} samples were obtained by incubation in 12 mM Mg acetate instead of the usual 2 mM.

In ATP samples, the recA concentration was 1 mg/ml, and the ATP concentration was 10 mM for ds complexes, (i.e. 400 ATP substrates per recA unit) and 50 mM for ss complexes (enough for potentially 60 min digestion at 37°C). recA–ssDNA complexes were incubated for 2 min at 37°C (thin layer chromatography showed that only a few percent of the ATP had been hydrolyzed after 2 min). After longer incubation times, aggregation of the filaments prevented visualisation by EM. dsDNA complexes were incubated for 10 min because the covering of dsDNA by recA is a time-dependent reaction (even after 10 min incubation only ~20% of the recA was bound to the dsDNA with the rest of the protein in the background as self-polymers). Long incubation in this case is possible because the ATPase activity of recA in a dsDNA complex under these conditions is much lower than with ssDNA (as confirmed by thin layer chromatography that showed that <10% of ATP was digested after 10 min incubation of the ds complex).

Electron microscopy

Cryo-microscopy. Frozen hydrated specimens were prepared on holey carbon films as described by Dubochet *et al.* (1985). A Zeiss cryo-box was employed since it allows manipulation of the specimen grid in gaseous rather than in liquid nitrogen. The holey carbon films, supported on 400 mesh copper grids, were glow-discharged immediately prior to use; 4 μl of undiluted sample was applied to the grid, blotted with filter paper and rapidly plunged into liquid ethane cooled by nitrogen gas close to −175°C. The time between taking the sample from the tube and freezing the grid was ~15 s.

Specimens were observed at temperatures of −165 to −175°C in a Zeiss 10C microscope equipped with a top entry cold stage and a fixed specimen holder. The microscope was operated at 100 kV and images were recorded at a dose of $<10\ e^-/Å^2$ and a magnification setting of 20 K (some images taken at 31.5 K gave the same results, not shown). The magnification was calibrated using the 40 Å first layer line in computed Fourier transforms of microtubule images (Amos and Klug, 1974; Chrétien, 1991).

Negative staining: This was as in DiCapua *et al.* (1990b). Samples were diluted to 50 μg/ml protein for adsorption onto carbon films and were stained with 1% uranyl acetate. These samples were analyzed both on the Zeiss cryo-microscope described above and on a Jeol 100 CXII microscope calibrated with catalase crystals (Wrigley *et al.*, 1968), providing a control for calibration; the results were identical within statistical error.

Data processing

The micrographs were digitized using a CCD TV camera coupled to a PC with an ADC card. Digitized images were viewed on a high resolution TV monitor. A pixel size of 10.46 μm on the micrograph was employed. For a magnification of ×20 000 this corresponds to a pixel size of 5.23 Å at the specimen. The helical pitch of the complexes was estimated by taking the average over 10 turns, which were measured on nearly straight stretches.

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