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Negative Co-dominant Inhibition of recA Protein Function

Biochemical Properties of the recA1, recA13 and recA56 Proteins and the Effect of recA56 Protein on the Activities of the Wild-type recA Protein Function *in Vitro*

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We have investigated the biochemical properties of several *Escherichia coli* mutant recA proteins that display a null phenotype. These are the recA1, recA13 and recA56 proteins, each of which carries a single missense mutation. These proteins all share a common defect which is the inability to adopt the high affinity DNA binding state normally elicited by the nucleotide cofactor ATP. Consequently, other than the ability to bind ssDNA, they possess none of the *in vitro* enzymatic activities of recA protein. However, each protein has characteristics that are unique, leading to the conclusion that the observed mutant phenotypes arise through fundamentally different mechanisms. Despite the magnitude of these defects, the recA56 protein is able to differentially inhibit various activities of wild-type recA protein. Incorporation of recA56 protein into a presynaptic filament with the wild-type recA protein does not affect the ability of the wild-type protein to hydrolyze ATP, as judged by the turnover number (k_{cat}), provided that the ssDNA concentration is not limiting; however, the affinity of wild-type recA protein for ATP is lowered by the presence of recA56 protein. Similarly, the ability to cleave lexA protein is only modestly inhibited. However, both the ability to compete with SSB protein for ssDNA binding sites and the DNA strand exchange activity of wild-type recA protein are severely inhibited by the presence of recA56 protein. These results suggest that individual monomeric components of the recA protein-DNA filament are translated through protein-protein contacts to become macroscopic properties of the filament.

Keywords: recA protein; DNA binding proteins; ATP binding proteins; protein-protein interactions

1. Introduction

The recA protein of *Escherichia coli* has been the subject of intense scrutiny in the 27 years since a single mutation resulting in a recombination defective phenotype was first described by Clark & Margulies (1965). Genetic studies have shown that it is the central and essential gene product in the recombination and repair pathways responsible for maintaining the integrity of genetic material in the cell (for reviews see Radding, 1982; Smith *et al.*, 1987). It plays a role in conjugal recombination, post-replication recombinational repair, and SOS mutagenesis.

In addition to the detailed genetic studies on *recA* and the genetic pathways in which it is involved, an impressive volume of work has been done *in vitro* using purified recA protein. The 37,842 Da recA protein (Sancar & Rupp, 1979) possesses a number of fundamental activities which include: the ability

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to cooperatively bind both single- and double-stranded DNA; the binding and subsequent DNA-dependent hydrolysis of a number of nucleoside triphosphates (Weinstock *et al.*, 1981; McEntee *et al.*, 1981; Menetski *et al.*, 1988); and the ability to form regular helical filaments both in the presence and absence of DNA (Chrysogelos *et al.*, 1983). It also possesses more complex activities which can be viewed as being dependent on the aforementioned fundamental activities. These include: ATP-stimulated renaturation of complementary ssDNA† strands; the ATP-dependent exchange of complementary DNA strands; and the ATP and ssDNA-dependent cleavage of lexA protein, λ cI repressor, as well as the umuD protein (for reviews and comprehensive literature documentation see: Griffith & Harris, 1988; Stasiak *et al.*, 1991; Roca & Cox, 1990; Radding, 1991; Kowalczykowski, 1991*a*)

The binding of recA protein to ssDNA is modulated by the nucleotide cofactors ATP and ADP (Menetski & Kowalczykowski, 1985). Compared to the affinity of recA protein for ssDNA in the absence of any cofactors, the binding of ADP reduces the apparent DNA binding affinity. ATP binding, on the other hand, increases the affinity of the recA protein for ssDNA. By coupling these observations with the fact that recA protein hydrolyzes ATP to ADP one can see how recA protein can modulate its affinity for DNA.

In the presence of ATP, recA protein binds to ssDNA with both a high affinity and a characteristic extended filament structure adopting a state which has come to be referred to as the high affinity binding state. Both of these characteristics define this DNA binding state, which will be referred to frequently throughout this paper. In addition the ternary complex of recA protein, ssDNA and ATP is necessary for every activity of recA protein to date. Mutant recA proteins which show defects in recombination *in vivo* are, without exception, somehow incapable of adopting this high affinity state and, as a result, do not form a ternary complex competent for DNA strand exchange (Kowalczykowski, 1991*a*,*b*).

From the first report of a gene which affected the recombination frequency (Clark & Margulies, 1965) a remarkable range of mutant recA alleles have been characterized either genetically, biochemically or both. There are alleles which show enhanced activity over the wild-type level; the alleles of *recA441*, *recA730* (Lavery & Kowalczykowski, 1988, 1992*a*), and recA803 (Madiraju *et al.*, 1991). Some are modestly enfeebled like the *recA142* and *recA430* alleles (Clark, 1973; Morand *et al.*, 1977; Kowalczykowski *et al.*, 1989; Menetski & Kowalczykowski, 1990). There are those that show differential effects on particular functions, like the coprotease-constitutive and recombination-defective alleles of Wang & Tessman (1986) or the *recA1730* allele (Dutreix *et al.*, 1989) which shows differential repressor cleavage specificities. Finally, there are those alleles which show essentially no activity *in vivo* like *recA1*, *recA56* and *recA13* (Lloyd & Low, 1976; Csonka & Clark, 1979; Bagg *et al.*, 1981; Weisemann *et al.*, 1984). With the exception of *recA1* (Rusche *et al.*, 1985; Bryant, 1988), these null alleles have not been studied in any great detail *in vitro* perhaps due to the apprehension that they would not yield any significant information. However, they may offer more than one expects since the interpretation of the *in vivo* phenotype is straightforward and the severity of the defect dictates that the mutated residue will be important to recA function. Interestingly, both *recA1* and *recA56* show dominant, codominant, or recessive behavior to various recA protein wild-type activities when heterodiploid strains containing the null allele and the wild-type protein are examined (Kawashima *et al.*, 1984; Yancey & Porter, 1984; Ennis *et al.*, 1989). This indicates that these proteins retain at least the abilities to bind to DNA or to interact with the wild-type protein. If these dominant/recessive relationships hold for *in vitro* experiments, it implies that the observed behavior is relevant to the physiological interactions which occur in the cell and that there are not crucial additional components to the reaction pathway which are being missed in the purified *in vitro* system.

The results from these *in vivo* experiments suggest that these proteins may be employed *in vitro* in a manner like that discussed by Herskowitz (1987), where the effect of dominant negative mutations on the wild-type protein can be studied to learn more about the activities of the wild-type protein. There are potentially two ways defective alleles of DNA binding proteins can exert a negative effect on the wild-type protein. The first way an effect could be exerted is simply through competition for available DNA binding sites. The second is through the formation of mixed multimers which subsequently results in "poisoned" heteromultimers which are unable to catalyze all, or a subset of recA protein activities. In the case of recA protein both types should be considered; however, the distinction between these two types of inhibition is one which can probably only be made *in vitro*.

We have examined the *in vitro* properties of several mutant recA proteins that are encoded by the null alleles *recA1*, *recA13* and *recA56*. All of these are single missense mutants with the following substitutions: *recA1*, Gly160→Asp (Kawashima *et al.*, 1984); recA13, Leu51→Phe; and recA56, Arg60→Cys (Kowalczykowski, 1991*a*). These positions are strongly conserved evolutionarily in all the 23 recA-like proteins examined from other bacterial sources. Gly160 is invariant in all species, while Leu51 is replaced conservatively by a methionine in

† Abbreviations used: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; etheno M13 DNA, modified single-stranded M13 DNA containing 1,N^6-ethenoadenosine, and 3,N^4-ethenocytidine; ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); SSB protein, *E. coli* single-stranded DNA binding protein; DTT, dithiothreitol; PEP, phosphoenolpyruvate.

Synechoccus, and Arg60 is replaced by an alanine residue in the recA protein of *Bordetella pertussis*, otherwise these residues are also conserved in all the other species (Roca & Cox, 1990).

Each of these alleles has been defined genetically as being devoid of any of the typical recA activities; i.e. they are defective in SOS response, recombination defective, lexA cleavage defective, SOS mutagenesis defective (Bagg *et al.*, 1981; Weisemann *et al.*, 1984; Ennis *et al.*, 1989; Csonka & Clark, 1979). In addition to examining the *in vitro* properties of these defective alleles, we also extensively examined the effect that recA56 protein has on the various activities of the wild-type recA protein and we attempt to correlate these results with observations from *recA56/recA* wild-type heterodiploids. The results of these investigations are presented below.

2. Materials and Methods

(a) *Chemicals and buffers*

All chemicals were of reagent grade or better and solutions were prepared using either glass distilled or Nanopure (Barnsted) water. ATP, dATP and ADP were purchased from Pharmacia LKB, ATPγS was purchased from Boehringer Mannheim and were dissolved as concentrated stocks at pH 7.5. The extinction coefficient used for adenine nucleotides was $1{\cdot}54 \times 10^4\ M^{-1}\ cm^{-1}$.

(b) *Proteins*

RecA wild-type protein was purified from *E. coli* strain JC12772 (Uhlin & Clark, 1981) using a preparative protocol (S.C.K., unpublished results) based on spermidine precipitation (Griffith & Shores, 1985). RecA56 protein was prepared from *E. coli* strain BEU293 (Uhlin *et al.*, 1983) using a preparative procedure based on polymin P precipitation, as described by Cox *et al.* (1981). This strain contains the plasmid pBEU41, which is a derivative of pBEU2, a temperature sensitive runaway replication vector, into which a *Bam*H1 fragment bearing the *recA56* gene has been inserted. The background of the strains carrying *recA56*, *recA13* and wild-type *recA* genes is JC10287, a derivative of AB1157 from which the *recA* gene has been deleted. Both recA56 and wild-type recA proteins have been further purified to remove contaminating exonuclease activity using ion exchange chromatography. RecA13 protein was purified from *E. coli* strain JC15746 which carries pJC946, a derivative of pBR322, which has had the *Eco*RI and *Pst*I sites removed and the aforementioned *Bam*H1 fragment carrying the *recA13* gene inserted. The *recA13* gene is under the control of the *recA* promoter and therefore is still regulated by the *lexA* repressor. Since this strain does not undergo SOS induction only the basal level of recA13 protein in the cell can be purified, making substantial amounts of this protein difficult to recover. RecA13 protein was purified using the same procedure described for recA56 protein. RecA1 protein was purified from strain DM1415 (Mount, 1977) which carries a chromosomal copy of *recA1* as well as a *lexA51* allele, which is unable to repress the SOS system leading to constitutive recA1 protein synthesis. RecA1 protein was purified using the same protocol described for wild-type recA protein with the additional step of MONO Q chromatography after the ssDNA-cellulose column. The concentration of all the recA proteins were determined spectrophotometrically at 280 nm using an extinction coefficient of $2{\cdot}7 \times 10^4\ M^{-1}\ cm^{-1}$.

SSB protein was purified from strain RLM727 using a preparative protocol provided by Dr Roger McMacken of Johns Hopkins University (LeBowitz, 1985). The concentration of SSB protein was determined using an extinction coefficient of $3 \times 10^4\ M^{-1}\ cm^{-1}$ at 280 nm (Ruyechan & Wetmur, 1976). LexA protein was purified from strain JL652 (Little, 1984) using the procedure described by Schnarr *et al.* (1985). Protein concentration was determined using a molar extinction coefficient of $7300\ M^{-1}\ cm^{-1}$ at 280 nm.

Lactate dehydrogenase and pyruvate kinase were purchased from Sigma as ammonium sulfate suspensions. Working solutions were made by dissolving the protein suspension in the reaction buffer of choice.

(c) *Etheno M13 ssDNA*

Etheno M13 DNA was made as described by Menetski & Kowalczykowski (1985). The concentration of etheno M13 DNA was determined using an extinction coefficient of $7000\ M^{-1}\ cm^{-1}$ at 260 nm (Menetski & Kowalczykowski, 1987).

(d) *M13 DNA*

Single strand M13 ssDNA and replicative form dsDNA were isolated as described by Messing (1983). The replicative form was linearized using *Eco*RI restriction endonuclease. The concentrations of ssDNA and dsDNA were determined using extinction coefficients at 260 nm of 8784 and $6500\ M^{-1}\ cm^{-1}$, respectively.

(e) *NTP hydrolysis assay*

The rate of ATP hydrolysis was measured by following the oxidation of NADH spectrophotometrically at 340 nm, as described by Kowalczykowski & Krupp (1987). The buffer employed, unless otherwise indicated, was 25 mM Tris-acetate (pH 7·5), 10 mM magnesium acetate, 0·1 mM DTT, 1.5 mM phosphoenolpyruvate (PEP) and 12·5 units/ml each of lactate dehydrogenase and pyruvate kinase. The assays were all carried out at 37 °C and, where pertinent, the order of addition of reaction components is detailed in the Figure legends. The uncertainty associated with these experiments is approximately ±5% and the rates reported are averages of several experiments.

(f) *DNA binding assay*

RecA protein binding to etheno M13 was monitored fluorometrically as described by Menetski & Kowalczykowski (1985). All titrations were carried out at 25 °C. The concentrations of etheno M13, recA protein and nucleotide are detailed in the accompanying Figure legends. The buffer used unless otherwise indicated consisted of 25 mM Tris·HCl (pH 7·5), 4 mM magnesium chloride, and 0·1 mM DTT. NaCl was used to dissociate the complexes in the salt titration experiments. When ATP was added to the reactions, a regenerating system consisting of PEP and pyruvate kinase was present.

(g) *DNA strand exchange assay*

The DNA strand exchange assay was performed using the agarose gel method first described by Cox & Lehman (1981) except ethidium bromide was omitted during

electrophoresis. The assays were carried out at 37°C in reaction buffer consisting of 25 mM Tris-acetate (pH 7·5), 6 mM magnesium acetate, 0·1 mM DTT, 3 mM PEP, 1 mM ATP and 12·5 units ml^{-1} pyruvate kinase. The concentrations of M13 ss- and dsDNA were 5 μM and 10 μM, respectively. The concentrations of wild-type recA protein and SSB protein were 3 μM and 0·45 μM, respectively. The concentration of recA56 protein was varied as indicated in the Figure legends. The order of addition of components was as follows: to complete reaction buffer, the wild-type recA and recA56 proteins were added; M13 ssDNA was then added and, after 2 min SSB protein was added; 5 min later the reaction was initiated by the addition of M13 dsDNA. Portions (40 μl) were removed at various timepoints and stopped by the addition of 0·1 vol of a solution of 5% SDS and 0·25 M EDTA. The samples were then run on a 0·75% (w/v) agarose gel for 6 h at 1·6 V cm^{-1}. After staining the gel in a 2 $\mu g\ ml^{-1}$ ethidium bromide solution and destaining in distilled water, the extent of the reaction was measured using a Bio-Image (Millipore) image acquisition and analysis system. The amount of substrate remaining and the product present was compared to a zero timepoint and expressed as a percentage. The uncertainty in these experiments is approximately ±10% and the recA56 protein concentration dependences are derived from multiple experiments.

(h) *LexA protein cleavage*

The assay for lexA protein cleavage was conducted as described by Little *et al.* (1980). The buffering conditions were identical to those described above for the NTP hydrolysis assay with the addition of 50 mM NaCl. In this case the extent of cleavage was ascertained using the Bio-Image system described above. The amount of intact lexA protein remaining was compared to the zero timepoint and expressed as a percentage. If necessary, the data were corrected for gel loading inconsistencies using recA protein as a constant internal control within each assay. The uncertainty in these assays is approximately ±10% and the concentration dependence is derived from several experiments at each recA56 protein concentration. To ensure that the low rates of cleavage observed in the presence of recA56 protein were not due to either lexA protein autolysis or some residual activity of recA56 protein, 2 experiments were done: one with a high concentration of recA56 protein alone, and another with wild-type recA protein in the absence of ATP. In both cases the rate of cleavage was essentially zero (data not shown).

3. Results

(a) *The recA56, recA1 and recA13 proteins do not possess any enzymatic activities*

Aside from the ability to bind ssDNA, which was evident by their binding to ssDNA cellulose during purification, these proteins possess few if any other activities. RecA1 protein can hydrolyze ATP to a limited extent depending on the solution conditions, but has no DNA strand exchange activity (Rusche *et al.*, 1985; Bryant, 1988; Lauder, 1992). RecA56 protein does not possess any of the activities normally associated with recA protein, including ATP hydrolysis, lexA protein cleavage and DNA strand exchange (data not shown). RecA13 protein is unable to hydrolyze ATP but was not explicitly tested for the ability to catalyze the cleavage of lexA protein or the DNA strand exchange reaction (data not shown). The fact that all of these proteins bind ssDNA led us to examine their ssDNA binding properties using the etheno DNA binding assay to try and determine the nature of the defect that leads to the observed deficiencies.

(b) *RecA56, recA1 and recA13 proteins bind ssDNA and the nucleotide cofactors ATP and ADP*

The DNA binding assay we used to measure the affinity of these proteins for DNA was described in detail previously (Menetski & Kowalczykowski, 1985). It consists of measuring the increase in fluorescence upon recA protein binding to the chemically modified, fluorescent ssDNA substrate, etheno M13. The point of saturation in a protein titration defines the apparent ssDNA binding stoichiometry (site size, n). The subsequent dissociation of this complex by the addition of NaCl allows determination of two other important parameters. The first is the relative fluorescence increase (RFI), which here is derived from the fluorescence value before the addition of salt divided by the value at the end of the titration when the protein has been dissociated from the DNA. This increase in fluorescence is a manifestation of the extension and subsequent unstacking of the bases in the ssDNA-recA protein complex. When ATP, dATP or ATPγS is added to a recA protein-etheno M13 ssDNA complex the fluorescence signal is increased further. This increase in fluorescence is due to the further extension of the recA protein-DNA filament. This has been observed in electron micrographs of these complexes and is characterized by the change in the helical pitch of the recA-DNA filament from 75 Å to 95 Å (Egelman & Stasiak, 1986; DiCapua *et al.*, 1990). This parameter (i.e. the RFI in the presence of the appropriate NTP) can be used to determine whether the recA protein has undergone this conformation change. The second parameter is termed the salt titration midpoint (STMP), and is defined as the NaCl concentration at which one-half of the protein has been dissociated from the etheno M13 ssDNA. This parameter measures the apparent affinity of the recA protein for etheno M13 ssDNA.

Titrations of etheno M13 ssDNA with each of the mutant proteins showed that the DNA binding stoichiometry is the same as observed for wild-type recA protein ($n = 7(\pm 1)$ nucleotides/recA monomer; data not shown). In Table 1 the RFI and STMP values derived from salt titrations for the mutant and wild-type recA proteins are compared. It is evident that in the absence of cofactor the recA56 and recA1 proteins bind to etheno M13 ssDNA almost as well as the wild-type protein and that the binding of ADP elicits essentially the same decrease seen with the wild-type protein. RecA13 protein shows a general decrease in the affinity for ssDNA both in the presence and absence of ADP.

In contrast to the wild-type protein, when ATP is added to any of the mutant protein-ssDNA

Table 1

DNA binding parameters of mutant RecA and RecA wild-type proteins

	Salt titration mid-point (mM NaCl)				Relative fluorescence increase			
	56	1	13	wt	56	1	13	wt
No cofactor	230	235	65	270	1·6	1·4	1·8	1·8
ADP†	135	145	50	160	1·6	1·3	1·5	1·8
ATP	90†/50‡	130†/70‡	35§/0‡	700‡	1·5	1·4†/1·3‡	1·6§	2·2‡
ATPγS†	90	> 1·6 M	*ND*‖	> 1·5 M	1·5	1·8	*ND*	2·2

The concentration of recA protein and etheno M13 are 1 μM and 3 μM, respectively. The $MgCl_2$ concentration is 10 mM, otherwise conditions are as described in Materials and Methods. The uncertainty in these experiments is ±10 mM for NaCl for salt titration mid-points and ±10% for the RFI values.

† 100 μM nucleotide.

‡ 500 μM nucleotide.

§ 50 μM ATP.

‖ Not determined.

complexes, the characteristic high affinity state is not induced. This failing is manifest in two ways. The first is the absence of the further increase in fluorescence upon the addition of ATP which is correlated with the extended conformation associated with the induction of the high affinity state. The addition of ATP to all the mutant protein-etheno M13 DNA complexes in fact decreases the fluorescent signal. The second is the low STMP (Table 1) for all the mutant protein-ssDNA-ATP ternary complexes confirming that the DNA binding affinity is indeed quite low. RecA13 protein shows the most extreme sensitivity to ATP; at concentrations greater than 500 μM the protein-etheno M13 ssDNA complex dissociates completely before the addition of any salt. The cofactor ATPγS normally induces a very high affinity state of the wild-type recA protein that is resistant to dissociation by elevated concentrations of NaCl; ATPγS differentially affects the recA56 and recA1 proteins, eliciting the usual high affinity state in recA1 protein but having no effect on recA56 protein that is discernibly different from that seen with ATP (Table 1). Consistent with the increase in affinity of recA1 protein for DNA in the presence of ATPγS is the observation that dATP, which is a more potent effector than rATP, elicits a higher affinity of recA1 protein for DNA than for an identical concentration of rATP (Table 2). In addition, the DNA binding affinity is also increased when the pH of the buffer is reduced. This appears to be consistent with the observation that at this reduced pH, recA1 protein has much greater ATPase activity than is observed at higher pH (Bryant, 1988; Lauder, 1992). The fact that these proteins are defective in the allosteric change induced by ATP readily accounts for their lack of *in vitro* activities as well as their null phenotype *in vivo*.

(c) *RecA56 protein forms mixed filaments with the wild-type recA protein*

RecA56 protein has been reported to differentially affect various wild-type recA protein activities *in vivo*; it has no effect on the UV survivability of wild-type recA/recA56 heterodiploids and only modest effects on the lexA repressor cleavage activity, but it is codominant for λ cI cleavage and homologous recombination and completely dominant for SOS mutagenesis (Ennis *et al.*, 1989). RecA56 protein has always been proposed to exert its inhibitory effects through the formation of heteromultimers with the wild-type protein which are consequently unable to catalyze various activities (Ennis *et al.*, 1989). The fact that all of these defective recA proteins can still bind to DNA suggests that a possible explanation for the codominance observed *in vivo* is due to inhibition of wild-type recA protein through competition for ssDNA binding sites. However, the ability to bind to DNA is probably not required for inhibition of wild-type recA protein activities since Sedgwick & Yarranton (1982) showed that a fragment of only 78 amino acid residues (which is almost certainly unable to bind DNA under *in vivo* conditions) was able to inhibit the *in vivo* activities of the wild-type protein. In fact most studies have implicated fila-

Table 2

Etheno DNA binding parameters for RecA1 protein as a function of pH

	pH 6·5		pH 7·5	
	STMP (mM NaCl)	RFI	STMP (mM NaCl)	RFI
No cofactor	190	1·4	235	1·3
ADP	ND	ND	145†	1·4
ATP	≈400†	1·6	130†/70‡	1·4†/1·3‡
dATP	≈300†	1·8	175†/140‡	1·6†/1·7‡
ATPγS	ND§	ND§	> 1·6 M†	1·8

The concentrations of recA1 and etheno M13 DNA are 0·8 μM and 3 μM, respectively. In the presence of ATP or dATP a regenerating system was included consisting of 2 mM PEP and 5 units ml^{-1} pyruvate kinase. The $MgCl_2$ concentration was 10 mM. The buffer at both pH values was 20 mM Tris·HCl. The uncertainty in these experiments is ±10 mM NaCl for salt titration mid-points and ±10% for the RFI values.

† 100 μM nucleotide.

‡ 500 μM nucleotide.

§ Not determined.

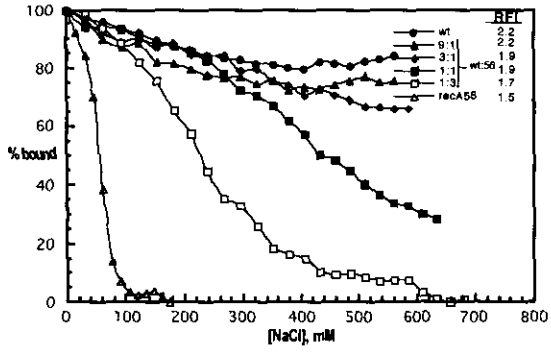

Figure 1. Etheno M13 DNA binding experiments of mixtures of wild-type recA protein and recA56 protein. DNA binding experiments were carried out as described in the Materials and Methods. The concentration of $MgCl_2$ in these experiments is 10 mM and the ATP concentration is 0·5 mM. The total concentration of recA protein was kept constant at 1·6 μM and is in excess (approx. 1·6 fold) of that required to saturate the etheno M13 ssDNA which is present at 3 μM. The concentrations of recA56 protein and wild-type recA proteins were varied to achieve the desired ratio. The RFI values from each experiment are indicated in the Figure inset. The data is normalized so the highest fluorescence value in each data set represents 100% binding.

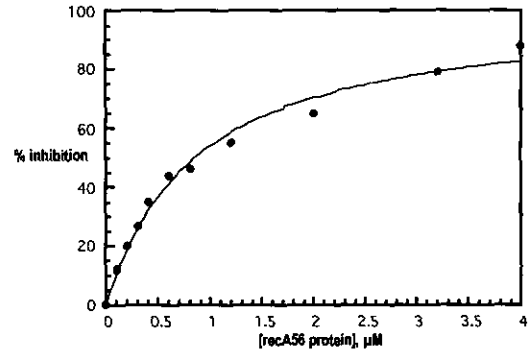

Figure 2. RecA56 dependent inhibition of the wild-type recA protein ATP hydrolysis activity. ATP hydrolysis was measured as described in the Materials and Methods. After allowing an assay mixture consisting of 0·8 μM wild-type recA protein and 3 μM etheno M13 ssDNA to reach a steady-state rate, a sample of recA56 protein was added and the rate was allowed to decay to a steady-state condition once more. The rate of this decay depends on the amount of recA56 protein added but generally occurs over a range of several minutes. This final rate was then used to calculate the extent of inhibition. The degree of inhibition is independent of the order of addition of recA56 and wild-type recA proteins indicating that the kinetics of filament mixing are fairly rapid.

ment "poisoning" through the formation of mixed multimers being the operative mechanism of inhibition (Kawashima *et al.*, 1984; Kowalczykowski & Krupp, 1989). Therefore, it was of interest to see whether we could observe the formation of mixed multimers using the etheno DNA binding assay to monitor the affinity state of the resultant mixed recA56/wild-type recA protein filament. These assays are done in the presence of ATP since the differences between recA56 protein and the wild-type protein are slight both in the presence of ADP and the absence of nucleotide cofactor.

In Figure 1 data from etheno DNA binding titrations of mixtures of recA56 protein and wild-type recA protein are shown. In these experiments, the proportion of recA56 protein was varied with respect to the wild-type recA protein. These data show that the addition of recA56 protein to the wild-type recA protein filament decreases the affinity of the resultant mixed filament and as the proportion of recA56 protein is increased the average affinity of the filament decreases. There is no indication of biphasic behavior indicating that the mixed filament is behaving as an average of the input amounts of recA56 protein and wild-type protein. This indicates that the recA56 protein interacts extensively with the wild-type recA protein producing a protein filament with reduced affinity for etheno M13 ssDNA.

(d) *RecA56 protein inhibits the wild-type recA protein ATPase activity*

Since recA56 protein has been shown to differentially inhibit the activities of wild-type recA protein *in vivo* (Ennis *et al.*, 1989), it was of interest to see what effect recA56 protein has on *in vitro* activities of the wild-type protein. The "simplest" activity of recA protein is DNA-dependent ATP hydrolysis. When recA56 protein is added to an ongoing wild-type recA protein ATPase assay, the rate of ATP hydrolysis decreases. As depicted in Figure 2, the extent of inhibition is dependent on the amount of recA56 protein added to the assay. At any given wild-type protein and ssDNA concentration, the degree of inhibition will increase as the amount of recA56 protein added is increased until a plateau is reached where further additions have little effect. The degree of inhibition observed shows hyperbolic behavior which is consistent with simple competition for ssDNA binding sites being the mechanism of recA56 protein inhibition of wild-type recA protein ATPase activity.

(e) *The degree of inhibition of the ATPase activity depends on the total protein to DNA ratio*

If the ratio of recA56 protein to wild-type recA protein as well as the total protein concentration are held constant and the amount of ssDNA is increased, the observed degree of inhibition of the ATPase rate decreases (Fig. 3). This implies that the inhibition of the wild-type recA protein ATPase activity arises solely through exclusion from DNA binding sites by recA56 protein and if sufficient ssDNA is present such that all the proteins can bind, no inhibition is observed. This indicates that in a filament which potentially could consist of both recA56 and wild-type recA proteins the ability of the wild-type protein to hydrolyze ATP is not

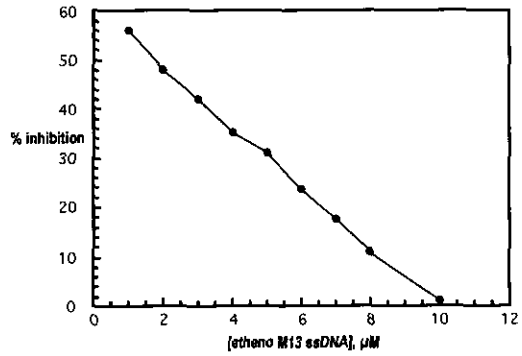

Figure 3. DNA dependence of recA56 protein inhibition of the wild-type recA protein ATP hydrolysis activity. ATPase assays were carried out as described in Materials and Methods. The concentrations of both recA56 and wild-type recA protein were maintained at 0·8 μM and the etheno M13 ssDNA concentration was varied. The rate before and after the addition of recA56 was used to determine the extent of inhibition.

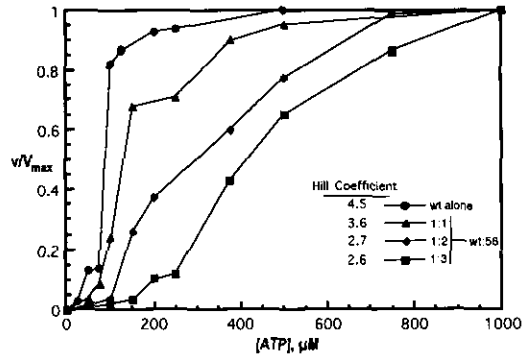

Figure 4. The effect of increasing amounts of recA56 protein on the apparent $S_{0.5}$ value for wild-type recA protein catalyzed ATP hydrolysis. The concentration of wild-type recA protein was constant at 0·5 μM and the amount of recA56 protein was increased to achieve the desired ratio. The amount of etheno M13 ssDNA was increased to maintain the total protein to ssDNA ratio constant at 1 recA monomer for every 8 nucleotides. The filled circles represent data obtained in the absence of recA56 protein; filled triangles represent data obtained at a 1:1, wt:56 ratio; filled diamonds represent data obtained at a 1:2, wt:56 ratio; and the filled squares represent data collected at a 1:3, wt:56 ratio. The inset gives the Hill coefficients obtained from Hill plots of the data above.

affected by the presence of recA56 protein. Conversely, recA56 protein is not "activated" for hydrolysis by the presence of wild-type recA protein, as the observed rate corresponds to that expected of wild-type recA protein alone. It is interesting to note that the stoichiometry derived from this experiment (i.e. the DNA concentration at which the inhibition is alleviated), corresponds to a site size (n) of 6·3 nucleotides per recA monomer. This result implies that at the stoichiometry of $n = 6$-7, all of the DNA binding sites are occupied and is consistent with the interpretation that the increase in ATPase activity between this point and the saturation of the ATPase activity which is normally observed at an apparent site size of $n = 3$-4 derives from recA protein associating with a recA protein-ssDNA filament rather than direct binding to free ssDNA sites (Lauder & Kowalczykowski, 1991). The alternate interpretation that the decrease in inhibition results from the sequential binding of a second ssDNA molecule to the filament is not consistent with the observed linear decrease of inhibition. Thus the inhibition of the ATPase activity of the wild-type recA protein by recA56 protein simply derives from the competition for DNA binding sites and the subsequent exclusion of wild-type recA protein from the ssDNA.

(f) *RecA56 protein is incorporated into the wild-type protein-ssDNA filament*

The previous experiments which used the ATPase activity of wild-type recA protein to gauge the effect of recA56 protein addition examined the inhibition due to the exclusion of wild-type recA protein from the ssDNA through competition with recA56 protein. It was also shown that if sufficient ssDNA is provided, such that all of the protein added can bind, then no inhibition due to recA56 protein addition is observed (Fig. 3). However, under these conditions where the DNA is in excess, we wished to determine whether recA56 protein forms a mixed filament with the wild-type protein or whether both proteins are completely segregated. We reasoned that an indicator of such behavior might be the affinity of the wild-type protein for ATP, as measured by the ATP concentration dependence of ATP hydrolysis (i.e. $S_{0.5}$ value; Neet, 1983), which is defined as the substrate concentration that results in one-half the maximal observed rate for enzyme-substrate systems that do not display classic Michaelis-Menten behavior). The data in Figure 4 show that at concentrations of ssDNA where no inhibition is observed, increasing amounts of recA56 protein cause the apparent $S_{0.5}$, for ATP to increase. Thus the affinity of the mixed recA protein filament for ATP, is decreased by the presence of recA56 protein. In addition the resultant binding curves broaden as the amount of recA56 protein is increased, indicating a decrease in the co-operativity of ATP binding and hydrolysis between recA protein monomers in the mixed filament. Hill plots of these data confirm these observations and the Hill coefficients derived from the slopes of these plots are given in the inset to Figure 4. Although the recA56 protein decreases the affinity of the filament for ATP, the k_{cat} (turnover number) of wild-type recA protein for ATP hydrolysis is unaffected. The observation that the presence of recA56 protein decreases the $S_{0.5}$ of the wild-type recA protein also identifies a second way in which recA56 protein is able to exert an inhibitory effect on the activities of wild-type recA protein: not through competition and subsequent

exclusion from binding to ssDNA but by enfeeblement of the resultant mixed filament for the fundamental attributes of DNA and nucleotide binding affinity.

(g) *RecA56 protein inhibits the lexA repressor coprotease activity of wild-type recA protein*

Expression of the SOS response requires cleavage of the lexA repressor by the recA protein (Little & Mount, 1982). The *in vitro* reaction requires the formation of a ternary complex comprised of recA protein, ssDNA, and ATP. Thus it was of interest to see what effect the presence of recA56 in the recA protein filament would have on the rate and extent of lexA cleavage. The assay conditions employed are essentially identical to the conditions above, namely, a stoichiometric amount of DNA was present so that no competitive inhibition would occur. These data show that the addition of recA56 protein inhibits the rate of lexA cleavage catalyzed by the wild-type protein (Fig. 5). The extent of the reaction is not affected by the addition of recA56 protein (data not shown). The cleavage reaction cannot be completely inhibited even at a 6:1 ratio of recA56 protein to wild-type recA protein where the rate of cleavage is still 20% of that observed in the absence of recA56 protein.

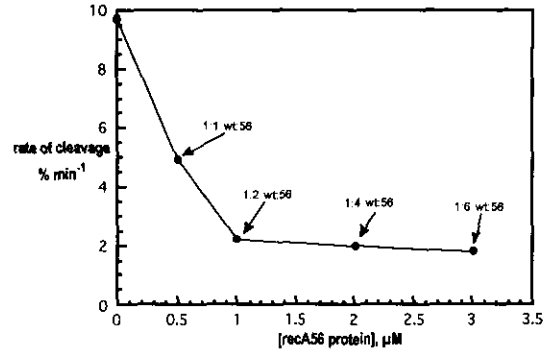

Figure 5. The effect of recA56 protein on the ability of wild-type recA protein to catalyze the cleavage of lexA protein. The assay for lexA protein cleavage was carried out as described in Materials and Methods. The concentration of wild-type recA protein was constant at 0·5 μM and the amount of recA56 protein was increased to achieve the desired ratio. The amount of etheno M13 ssDNA was increased to maintain the total protein to ssDNA ratio constant at 1 recA monomer for every 8 nucleotides. Time courses of the amount of lexA protein cleaved were plotted and the rates of cleavage at each recA56 protein concentration calculated. The values derived from these calculations are plotted against the amount of recA56 protein present in the reaction.

(h) *Adding recA56 protein to a wild-type recA protein filament decreases the ability of the filament to compete with SSB protein*

SSB protein is required for efficient pairing and exchange in the single-stranded by double-stranded DNA strand exchange reaction primarily to allow recA protein to form a saturated ssDNA-recA protein filament (Cox & Lehman, 1982). The function of SSB protein is to disrupt DNA secondary structure that impedes the binding of recA protein (Muniyappa *et al.*, 1984; Kowalczykowski & Krupp, 1987). The ability to facilitate recA protein binding to ssDNA is readily measured by monitoring the ATPase activity of recA protein on ssM13 DNA (or any other DNA substrate which has secondary structure) before and after the addition of SSB protein. Since we wanted to investigate the effect of recA56 protein on the DNA strand exchange activity, it was important to first establish what effect recA56 protein has on the ability of the wild-type protein to compete with SSB protein. Therefore the following experiments were carried out under the same conditions that are used in the DNA strand exchange assay except that dsDNA is not added.

The stimulation of the ATPase activity by SSB protein requires that recA protein be able to displace the SSB protein from the DNA formerly involved in secondary structure (Lavery & Kowalczykowski, 1992*b*). Since displacement of SSB protein requires the high affinity state of recA protein (Kowalczykowski *et al.*, 1989; Kowalczykowski, 1991*a*,*b*), it was expected that the presence of recA56 protein in the filament would have a deleterious effect on the ability of recA protein to compete with SSB protein. In agreement, the data in Figure 6 show that while recA56 protein has only a modest inhibitory effect on the wild-type recA protein ATPase rate in the absence of SSB protein, when SSB protein is added, inhibition reaches 100%. Inhibition of the wild-type ATPase activity is

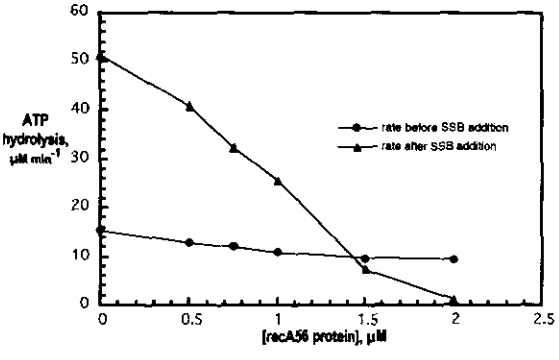

Figure 6. The effect of recA56 protein on the ability of the mixed presynaptic filament to compete with SSB protein. The ATP hydrolysis assay was carried out as described except that the buffer conditions and protein and DNA concentrations were identical to those described for the DNA strand exchange assay in Materials and Methods. The order of addition described therein is also the same as that employed in this assay except that dsDNA was not added to these reactions. The actual rates obtained before the addition of SSB protein (filled circles) and 5 min after the addition of SSB protein (filled triangles) are shown.

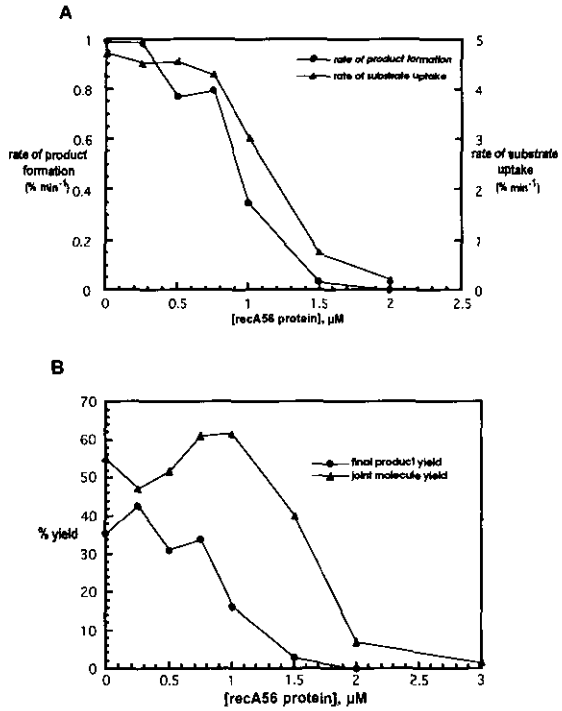

Figure 7. Inhibition of wild-type recA protein dependent DNA strand exchange activity by recA56 protein. Strand exchange assays were performed as described in Materials and Methods. The concentration of wild-type recA protein in these reactions is 3 μM. In A the initial rates of product formation (filled circles) and substrate uptake (filled triangles) are plotted against the amount of recA56 protein present. In B the total yield of product molecules at 80 min (filled circles) and the yield of joint molecules at 80 min (filled triangles) is plotted against the recA56 protein concentration.

always seen, when compared to that observed in the absence of recA56 protein, even though the rate is still increased by the addition of SSB protein at lower concentrations of recA56 protein. The observation that the addition of SSB protein exacerbates the inhibition by recA56 protein indicates that the affinity of the protein filament for ssDNA must be greater to compete with SSB protein for DNA binding sites than that required to observe ATP hydrolysis. This finding is consistent with the results from the study of the mutant recA142 protein which can hydrolyze ATP in the absence of SSB protein but not in its presence; this defect results from an inability of recA142 protein to resist displacement by SSB protein due to a lower affinity for DNA induced by ATP than that seen with the wild-type protein (Kowalczykowski *et al.*, 1989; Menetski & Kowalczykowski, 1990).

(i) *RecA56 protein inhibits the DNA strand exchange activity of the wild-type recA protein*

Given that the recA56 protein is able to affect various activities of the wild-type recA protein *in vivo* it was of considerable interest to see what effect the recA56 protein had on the *in vitro* activity commonly thought to best mirror the *in vivo* behavior of recA protein, that being its DNA strand exchange activity. The assay employed has been described previously (Cox & Lehman, 1981) and measures the conversion of homologous linear duplex DNA and circular ssDNA to nicked circular duplex DNA and linear ssDNA. This assay has been shown to require the action of SSB protein both presynaptically (McEntee *et al.*, 1980; Cox *et al.*, 1983), to remove the secondary structure from the ssDNA which impedes formation of the recA protein-DNA filament necessary for efficient DNA strand exchange (Muniyappa *et al.*, 1984; Kowalczykowski & Krupp, 1987; Kowalczykowski *et al.*, 1987) and postsynaptically, to bind the strand displaced from the heteroduplex molecule and allow efficient branch migration and subsequent product formation (Lavery & Kowalczykowski, 1992*b*). The data in Figure 7A show the effect that recA56 protein has on the rate of substrate uptake (linear duplex M13 DNA), and the subsequent rate of conversion of the joint molecule intermediates to product molecules (gapped circular duplex M13 DNA). In Figure 7B it can be seen that a significant effect on either substrate uptake or product formation is not manifest until a concentration of 1 μM recA56 protein is reached (wild-type recA protein concentration = 3 μM). However, after this point the DNA strand exchange activity of wild-type recA protein is quickly inhibited, with the ability to resolve intermediates into product molecules being affected to a greater extent. At any particular concentration of recA56 protein, differential effects can be seen in the inhibition of wild-type recA protein DNA strand exchange activity. At 1·5 μM recA56 protein, substantial amounts of substrate molecules are taken up and converted into heteroduplex intermediate species (Fig. 7B), whereas at this same concentration these species cannot be resolved into product molecules. This probably results from both functional and actual gaps in the recA protein-ssDNA filament which have little effect on initial pairing and limited exchange but which prevent branch migration and the subsequent resolution of the intermediates into product molecules. It is interesting to note that even in the assays where 2 μM recA56 protein is present there are still some substrate molecules taken up into intermediates. This observation is somewhat surprising in view of the results of the SSB protein inhibition experiments shown in Figure 6 where the inhibition by SSB protein is approaching 100% when this concentration of recA56 protein is present in the reaction, suggesting that pairing between substrates such as these requires only a very limited amount of contiguously bound recA protein.

4. Discussion

We have reported the quantitative DNA binding behavior of three mutant recA proteins that show null phenotypes *in vivo*. Consistent with this pheno-

type, each of these proteins is unable to promote both cleavage of lexA repressor and DNA strand exchange. We find that the molecular defect responsible for these failings is their inability to undergo the allosteric change induced by ATP which promotes a high affinity DNA binding form of recA protein. Instead, in each case, the addition of ATP lowers the apparent affinity for ssDNA below that seen with ADP, which normally induces a low affinity DNA binding state. Somewhat unexpectedly these mutant proteins, aside from the common attribute described above, can be distinguished from one another on a number of different accounts. Even though they all share the defect seen with ATP-dependent ssDNA binding, there are still differences in the degree of the defect in ssDNA binding both in the presence and absence of nucleotide cofactors.

RecA13 protein is by far the most defective of this group, at least as judged by the affinity it displays for ssDNA. In contrast to the recA1 and recA56 proteins, which show almost wild-type affinity for DNA in the absence of nucleotide cofactor and in the presence of ADP, recA13 protein shows much lower affinity under all conditions tested. This implies that the substitution of phenylalanine for the leucine at amino acid residue 51 results in a severe perturbation of the overall recA protein structure. Upon first examination this substitution seems a conservative one, however this residue, although not implicated directly in either DNA binding, ATP binding, or ATP hydrolysis (Story & Steitz, 1992) lies in the interior of recA protein and probably is a constituent of the hydrophobic core of recA protein. The hydrophobic core is those aliphatic and aromatic residues that pack tightly in the interior of a protein to remain solvent inaccessible and is the scaffold upon which the hydrophilic solvent-accessible residues rest (Lim *et al.*, 1992). Recent work by Lim *et al.* (1992) showed that mutations engineered into the core of the lambda repressor protein that changed the volume of the interior by ± 3 to 6 methylene groups (often through substitution of aromatic residues for aliphatic residues) resulted in proteins that had no biological activity and were poorly recognized by antibodies to the wild-type protein. They attributed these results to large global changes in the protein structure as a result of major core packing perturbations. Thus the substitution of the aliphatic side-chain of leucine with the considerably more bulky aromatic phenylalanine side-chain in the recA13 protein may result in significant structural perturbations in regions relatively distant from the mutation in both primary and secondary structure, leading to the defects in DNA binding both in the presence and absence of nucleotide cofactors. Consistent with these potentially large perturbations in the recA13 protein structure is the observation that the recA13 protein has no effect on the activity of the wild-type protein both *in vitro* and *in vivo* implying that the interface required for protein-protein interactions is somehow defective. Since the interface for interaction between recA proteins is quite extensive, the perturbations must be significant to disrupt these interactions. The possibility that recA13 protein is simply too defective a DNA binding protein to inhibit the wild-type protein is mitigated by the results of experiments by Sedgwick & Yarranton (1982) which showed that a truncated recA protein of only 78 aa (22%), which almost certainly does not possess the DNA binding domain, was able to inhibit the activities of wild-type recA protein *in vivo*, suggesting that the ability to interact with the wild-type protein is sufficient for inhibition to occur.

The recA56 protein shows a major defect only in the presence of ATP. However, the addition of other adenine nucleoside triphosphates such as ATPγS and dATP, which are more potent inducers of the high affinity DNA binding state in wild-type recA protein as well as recA1 protein, show no effect on the ssDNA binding affinity of recA56 protein. RecA56 protein, in contrast to recA1 protein, is unable to hydrolyze ATP under any conditions tested, including the low pH conditions which significantly enhance the ATP hydrolysis activity of recA1 protein (Bryant, 1988; Lauder, 1992). These observations along with the fact that the ssDNA binding affinity of recA56 protein in the presence of ADP (Table 1) is almost the same as that displayed by the wild-type recA protein implies that the defect in recA56 protein does not reside in the ssDNA binding site of the low affinity state, nor in a failure to bind nucleotide cofactor. Instead, the defect must reside in the interaction with the γ-phosphate moiety of nucleoside triphosphates, the conformation change which is normally elicited by the presence of this phosphate group or the DNA binding site of the high-affinity state. The arginine residue at amino acid position 60, which is changed to a cysteine residue in recA56 protein has not been implicated directly in ATP binding, but in the crystal structure it lies close to the ATP binding site. This result also implies that the DNA contacts made by recA protein in the presence of ADP and in the absence of nucleotide cofactor are significantly different from those made in the presence of ATP. This statement is supported further by the results from DNA binding studies of recA1 protein.

RecA1 protein is the least defective of the three proteins studied, but this is a distinction which can only be made *in vitro* since all three recA alleles show essentially the same phenotype *in vivo*. As mentioned above, the DNA binding defect in recA1 protein is most severe in the presence of ATP. When dATP is used as the nucleotide cofactor, the resultant ssDNA binding affinity is increased over that seen with rATP, although the affinity for ssDNA that is induced by dATP is still lower than that observed in the absence of cofactor. However, when ATPγS is present the affinity of the recA1 protein for DNA is essentially the same as that seen with the wild-type protein in the presence of ATPγS. These observations imply that the interaction of recA1 protein with nucleotide cofactors is not defec-

tive and that induction of the high affinity state is possible. Furthermore, the recA1 protein retains some ability to hydrolyze ATP. At pH 7·5, the pH which can be considered standard for recA protein assays, recA1 protein hydrolyzes ATP at a rate approximately 10 to 20% of that observed for wild-type recA protein (Bryant, 1988; Lauder, 1992). The mutation in recA1 protein lies in a disordered region in the three-dimensional crystal structure, referred to as loop 1. Examination of the crystal structure of recA protein has implicated this loop in DNA binding (Story *et al.*, 1992). Thus it is possible that the observed defect lies not with an intrinsic inability to undergo the necessary conformation change induced by ATP but perhaps this change is hindered by the mutant DNA binding site being unable to bind ssDNA in the correct fashion. It is possible that the change of the glycine residue at amino acid position 160 to an aspartate residue might prevent the approach of the region involved in DNA binding due to electrostatic repulsion between the charged aspartate side-chain and the phosphodiester backbone of the DNA molecule. The fact that the affinity for etheno M13 ssDNA increases when the pH is lowered suggests that this is a plausible explanation (Table 2). At this lower pH, recA1 protein also exhibits considerable ATP hydrolysis activity (60 to 80% of the wild-type activity) although it is still unable to compete with SSB protein (Lauder, 1992; Bryant, 1988). This result agrees with the observation that the apparent affinity at the lower pH is still lower than that observed with wild-type recA protein (Table 2). If the aspartic acid is substituted with an isosteric but non-ionizing asparagine residue, the mutant protein now has ATPase activity at both pH 7·5 and 6·2 and can compete with SSB protein to some extent at this lower pH, suggesting that the charge on the aspartate is indeed detrimental (Bryant, 1988). These observations suggest that the site of ATP binding and interaction is unperturbed and that the observed phenotype derives from an aberrant DNA binding site. In addition, this particular site for DNA binding must be specific to the ATP-induced high affinity state and different from that used with ADP or in the absence of nucleotide cofactor.

It may appear that the induction of a low DNA binding affinity by ATP is an aberration particular to these mutants. However, this is also observed with the wild-type protein at low concentrations of ATP (<100 μM); at these concentrations of ATP the affinity for ssDNA is actually lowered relative to that seen in the absence of ATP (Menetski *et al.*, 1988). At concentrations of ATP beyond 100 μM the affinity for DNA is increased. This is not due to ATP hydrolysis since this phenomenon takes place at concentrations where very little hydrolysis occurs and is also observed when ATPγS is used as a cofactor (Menetski *et al.*, 1988, see Fig. 2). Thus as a result of their respective mutations, the equilibrium constants for the ATP-dependent transition from a low to high DNA binding affinity state for these mutants may be so high, that the transition will not occur at realistic ATP concentrations. This is definitely true of recA56 and recA13 proteins which can be dissociated from ssDNA at ATP concentrations of 1 mM and above. The equilibrium constant defect for recA1 protein may not be as severe, since the high affinity state is reached when the potent effector ATPγS is used, unlike the recA56 and recA13 proteins. This is similar to the results obtained from studies on the mutant recA142 protein which showed a much broader dependence on ATP, not achieving a high affinity DNA binding conformation until concentrations of ATP well over 1 mM were employed (Kowalczykowski *et al.*, 1989).

Despite the serious DNA binding defect, a defective mutant recA protein, recA56 protein, forms mixed filaments *in vitro* with the wild-type recA protein and these two proteins combine to form a filament that displays an affinity for DNA which is a function of the input amounts of each protein. This provides quantitative evidence of the heretofore qualitative assumptions that the mechanism of inhibition by mutant recA proteins is through the formation of heteromultimers which are then "poisoned" for various activities (Sedgwick & Yarranton, 1982; Yancey & Porter, 1984; Larminat & Defais, 1989). These experiments show that the incorporation of recA56 protein into a wild-type recA protein filament results in a structure which subsequently displays a lower affinity for DNA. Other assays which measure the affinity of the filament either directly or indirectly also show that extensive mixing occurs and these mixed filaments behave as the sum of their component parts and not as two distinct populations of recA protein molecules.

The presence of recA56 protein in the protein-DNA filament has no intrinsic inhibitory effect on the ability of wild-type recA protein to hydrolyze ATP. Inhibition is observed only when the ssDNA substrate is limiting, resulting in competition for binding sites, or when the ATP concentration is sufficiently low to detect the increase in the $S_{0.5}$ values that the presence of recA56 protein causes. Secondly, although the affinity of the filament does not affect the V_{max}, the $S_{0.5}$ is increased, indicating that the filament affinity for ATP is being decreased by the presence of recA56 protein. Since the recA56 protein is not hydrolyzing ATP in these reactions, the $S_{0.5}$ value is a reflection of the affinity of the wild-type protein for ATP. Since the $S_{0.5}$ is increasing as recA56 protein is added one must conclude that the microscopic properties of the recA-DNA filament (monomer components) are being transmitted *via* protein-protein contacts to become macroscopic properties of the filament. This "averaging" of filament components also explains why one sees a homogeneous filament which is hydrolysing ATP and not a mixture of the conformations induced by ATP and ADP; a similar conclusion was derived by Lee & Cox, (1990*a*,*b*) based on the antagonistic effects of ADP and ATPγS on the ATP hydrolysis by the wild-type recA protein. Concomitant with the increase in the

$S_{0.5}$ is the broadening of the transition of the ATP titration curves to yield lower Hill coefficients, demonstrating that as the recA56 protein content of the filament increases, the co-operativity of the filament decreases. Thus the $S_{0.5}$ and the co-operativity reflect properties of the filament as a whole. This notion is consistent with the fact that ATP binding by recA is hyperbolic, with a K_d at least 20-fold less than the apparent $S_{0.5}$ value for ATP hydrolysis, which is clearly sigmoidal (Kowalczykowski, 1986). It was proposed that this difference could reflect a requirement for a cluster of several recA molecules on the DNA that become active for ATP hydrolysis; this would result in an $S_{0.5}$ value greater than the K_d and the sigmoidal behavior of the ATP hydrolysis activity. The presence of recA56 protein in the filament must either destabilize this "cluster" or somehow interfere with its formation at low ATP concentrations, resulting in an increase in the ATP concentration required to initiate hydrolysis.

Experiments very similar to those described above were carried out to ascertain the effect of recA56 protein on the ability of the wild-type protein to catalyze the cleavage of lexA protein. The presence of recA56 protein in the recA protein-ssDNA filament lowered the rate of lexA cleavage by the wild-type protein. This could be due to the decrease in the affinity of recA protein filament for DNA and thus a lower K_m for lexA protein binding, or simply due to sequestering of lexA protein at regions of the filament that can bind lexA protein but cannot catalyze lexA cleavage. Since the amount of wild-type protein was held constant and the amount of recA56 protein and ssDNA was increased to achieve the desired ratio, the number of potential lexA binding sites is significantly increased, assuming that even though recA56 protein cannot cleave lexA protein it is able to bind lexA protein. An unexpected result is the relative insensitivity of the rate of cleavage to further additions of recA56 protein beyond a 1:2 ratio of wild-type recA protein to recA56 protein (Fig. 5). In fact significant cleavage still occurs at a 1:6 ratio of wild-type recA protein to recA56 protein. This result suggests that an extensive contiguous filament of wild-type recA protein is not required for lexA protein cleavage. If this is indeed the case then perhaps all that is required for lexA protein cleavage is two adjacent recA protein monomers that have ATP bound and the makeup of the rest of the recA filament is less important. The idea that short tracts of recA protein on ssDNA are able to catalyze lexA cleavage makes sense when one considers the teleological role of lexA cleavage in the cell, to induce the SOS system. To require extensive filament formation as a prerequisite would not be desirable and furthermore may be impossible if only short regions or fragments of ssDNA are the signal that DNA damage has occurred (Sassanfar & Roberts, 1990).

The ability to compete with SSB protein and proficiency in the DNA strand exchange activity share the same underlying requirements of recA protein. Therefore the effect that recA56 protein has on these activities of the wild-type protein can be discussed simultaneously. Since it has been shown previously that the high affinity state is absolutely required for these activities (Kowalczykowski, 1991*b*) it was expected that recA56 protein would have a severe effect on the ability of the wild-type protein to catalyze these reactions. Aside from the marked sensitivity that both of these activities show to the presence of recA56 protein the most notable feature they share is the sharp transition from little to almost complete inhibition as a function of recA56 protein concentration (Figures 10A and B of Lauder, 1992). This suggests that there may be a "critical" concentration of recA56 protein, or more precisely a critical proportion, which when reached results in the filament being significantly disabled in its ability to catalyze DNA strand exchange and resist displacement by SSB protein. This type of behavior is consistent with the induction of the high affinity state by ATP which shows a co-operative transition from low to high affinity as the ATP concentration is increased (Menetski *et al.*, 1988). Addition of recA56 protein to the wild-type protein-DNA filament is tolerated until the point is reached where the high affinity form shifts to the low affinity form over a relatively short concentration range leading to a filament in the low affinity state and the subsequent inhibition by SSB protein. This is another example of the microscopic properties of the recA protein-DNA filament effecting a change in the macroscopic properties of the filament. The marked inhibition of these activities by recA56 protein is also consistent with the results from experiments done with *recA56*/wild-type *recA* heterodiploids which showed significant reductions in the level of homologous recombination and SOS mutagenesis (Ennis *et al.*, 1989), activities which the *in vitro* DNA strand exchange assay is thought to represent well in its requirements of recA protein function (Kowalczykowski, 1991*a*).

By examining the effect recA56 protein has on the numerous and varied activities which can be assayed *in vitro* we have been able to establish a hierarchy of sensitivities to the addition of recA56 protein. We have also been able to correlate our observations *in vitro* with those previously made *in vivo*. The coexpression of recA56 in a heterodiploid with the wild-type allele shows only very modest effects on the ability of the strain to cleave lexA protein and induce the SOS response (Ennis *et al.*, 1989). Similarly, the heterodiploids were not sensitized to UV radiation, which has as a large part the ability to induce the SOS genes responsible for excision repair of UV produced photoproducts through lexA cleavage and inactivation (Ennis *et al.*, 1989). These observations are consistent with our results showing that at a 1:1 ratio of recA56 protein to wild-type recA protein the rate of lexA cleavage is still 50% of that seen in the absence of recA56 protein. Although this could be termed "codominant" behavior, the requirements *in vivo*

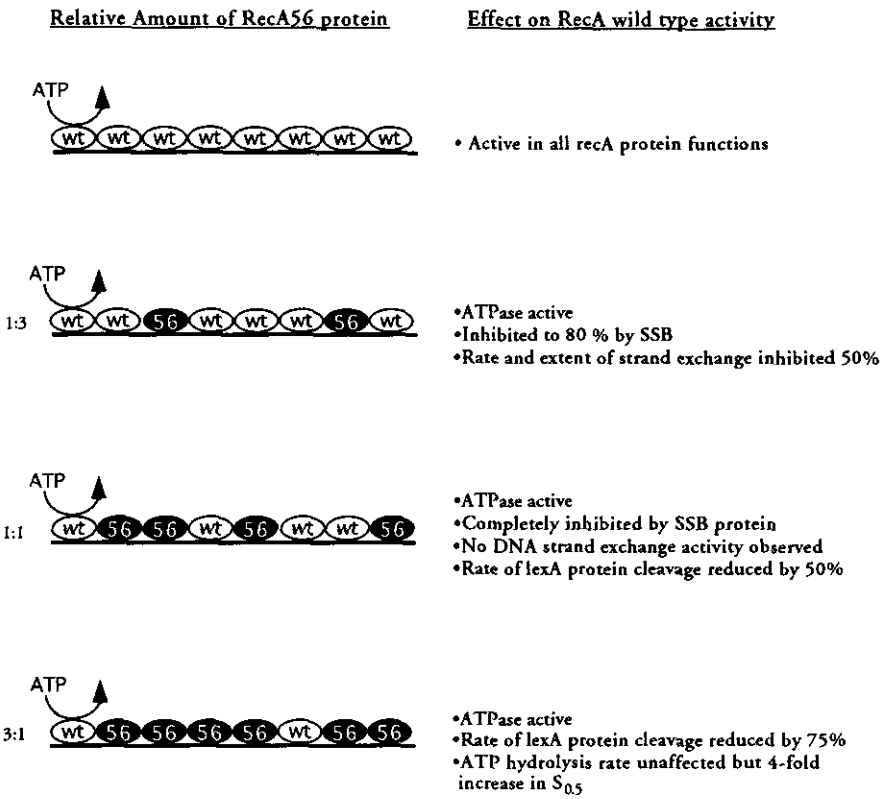

Figure 8. A summary of the effects of recA56 protein incorporation into the wild-type recA filament. In all cases the effects are due only to the addition of recA56 protein to the filament rather than the exclusion of wild-type recA protein from binding ssDNA (see the text for further discussion).

will determine the observed phenotype, suggesting that this amount of cleavage is sufficient for SOS induction. *In vivo* recA56 protein also impairs the *ability of wild-type recA protein to carry out homo*logous recombination and SOS mutagenesis, two activities of which the former requires the DNA pairing abilities of recA protein and the latter, which probably requires this activity since no recombination defective, SOS mutagenesis competent recA allele has been isolated. Both the ability of the wild-type recA protein to compete with SSB protein and the ability to catalyze DNA strand exchange were severely impaired by the addition of relatively small amounts of recA56 protein. We observed complete inhibition well before a 1 : 1 ratio was reached. Since the capacity to catalyze these reactions relies on the ability of the recA-ssDNA filament to assume a high affinity state it is not surprising that recA56 protein, which lowers the affinity of the filament, inhibits these activities. Consistent with the notion that the mechanism of recA56 protein inhibition of the wild-type protein is *through filament enfeeblement is the observation* that heterodiploids in which the wild-type allele is replaced with an allele which has been shown to be *more proficient than the wild-type allele (recA730)* show that the degree of co-dominance shifts in favor of the more proficient allele (Ennis *et al.*, 1989).

The results of these mixture experiments and the hierarchy derived are summarized in Figure 8. In *this Figure three different ratios of wild-type recA* protein to recA56 protein have been illustrated, along with the observed effects that this amount of recA56 protein has on the wild-type protein activities. In each case the effects of recA56 protein addition represent only those effects due to the addition of recA56 protein to the wild-type protein filament and not to exclusion through competitive binding phenomena. The hierarchy of sensitivity of the wild-type protein to addition of recA56 protein is not unique, as similar results are seen with the recA142 protein (Kowalczykowski & Krupp, 1989; Kowalczykowski *et al.*, 1989). In general this hierarchy can also be applied to single proteins that have mutations rendering them defective to some extent. The most severe effects are seen in the DNA strand exchange activity while the least affected is the ATPase activity. This is true of both the recA142 and recA430 proteins (Kowalczykowski *et al., 1989*; Menetski & Kowalczykowski, *1990*) as well as the recA1 protein discussed earlier.

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