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Interaction of recA Protein with Single-stranded DNA
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We have investigated quantitative molecular aspects of the interaction of recA protein with single-stranded DNA, by using a fluorescent modified-DNA referred to as etheno-M13 DNA. In addition, the effects of the nucleotide cofactors ATP and ADP, and the analogues ATP-γ-S, AMP-P-C-P, and AMP-P-N-P on this interaction have been studied. It is shown that ATP, AMP-P-N-P and, in particular, ATP-γ-S significantly increase the affinity of recA protein for single-stranded DNA, whereas ADP and, to a lesser degree, AMP-P-C-P decrease the affinity. Binding to etheno-M13 single-stranded DNA is co-operative, with the value of the co-operativity parameter, ω, being ≈ 50 under all conditions measured. The effect that ADP has on recA protein–DNA affinity is to lower the intrinsic binding constant, but it has no effect on the co-operativity of binding. In addition, the stability of the recA protein–DNA complex is very salt dependent (d log K/d log [NaCl] ≈ -10) and it is the intrinsic binding affinity rather than the co-operativity of binding that is affected; thus, under all conditions observed, recA protein binds single-stranded DNA co-operatively with a value of $\omega = 50 \pm 10$. The binding affinity is also influenced by the type of anion present, being $\approx$10,000-fold higher when acetate ion is present instead of chloride ion. These data have been interpreted to suggest that recA protein forms up to five ionic interactions when it binds to single-stranded DNA and that five to six anions are displaced upon binding. The modulation of recA protein–DNA complex stability by nucleotide cofactors suggests that these cofactors play a role in the cycling of recA protein on and off single-stranded DNA, with ATP being required for DNA binding under physiological conditions and ADP serving as a "release" factor. These results are discussed in terms of a model for the role of ATP hydrolysis in a recA protein–single-stranded DNA binding cycle.

1. Introduction

The *Escherichia coli* recA protein has been shown to play a key role in homologous genetic recombination and the induction of the SOS repair system (for reviews, see McEntee & Weinstock, 1981; Radding, 1982; Dressler & Potter, 1982; Little & Mount, 1982). *In vitro*, biochemical studies have demonstrated that recA protein possesses a variety of related enzymatic activities, which include: (1) the DNA-dependent hydrolysis of ATP (Roberts *et al.*, 1978; Ogawa *et al.*, 1978); (2) the catalysis of the renaturation of complementary single strands of DNA in an ATP hydrolysis-dependent reaction (Weinstock *et al.*, 1979); (3) the formation of D-loops and the subsequent strand exchange reaction between a duplex DNA molecule and a homologous single-stranded DNA molecule (Shibata *et al.*, 1979; McEntee *et al.*, 1979); (4) the reciprocal exchange of strands between gapped and linear (or nicked) duplex molecules (West *et al.*, 1981*a,b*; DasGupta *et al.*, 1981); and (5) the proteolytic cleavage of lexA protein and λ repressor in a reaction dependent on ATP and single-stranded DNA (Craig & Roberts, 1980; Little *et al.*, 1980).

The elements common to each of the above reactions are that recA protein must interact with both single-stranded and double-stranded DNA, in addition to ATP, and that the energy of either ATP hydrolysis or ATP binding is utilized by recA protein to catalyze these reactions. Previous studies have demonstrated that recA protein will bind to single-stranded and double-stranded DNA (West *et al.*, 1980; McEntee *et al.*, 1981*a,b*; Stasiak *et al.*, 1981; Flory & Radding, 1982; Dunn *et al.*, 1982; Silver & Fersht, 1982,1983) as well as to ATP (Cotterill *et al.*, 1982; Weinstock *et al.*, 1981*a,b*).

In order to more fully understand the molecular mechanism by which recA protein catalyzes the complex reactions mentioned above, it will be

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necessary to have a more quantitative understanding of how recA protein interacts with each of its various substrates and, in particular, how these interactions are affected by the presence of other substrates. The effect of nucleotide cofactors on DNA binding affinity is of specific interest, since it has been reported that both ATP and ADP stimulate the dissociation of recA protein from DNA (McEntee *et al.*, 1979,1981*a*). The detailed equilibrium studies described below should help to provide a molecular explanation of these effects as well as clarify their functional significance.

In this paper, we have focused on quantitative equilibrium properties (i.e. intrinsic binding affinity and degree of co-operativity) and the molecular aspects (i.e. number of ionic contacts involved) of the interaction of recA protein with single-stranded DNA, and have investigated how these parameters are affected by ATP, or its hydrolysis product, ADP. The approach we have used is to chemically modify M13 single-stranded DNA at the adenosine and cytidine bases by using chloroacetaldehyde to produce 1,N^6-etheno-adenosine and 3,N^4-etheno-cytidine, yielding the fluorescent DNA product referred to as etheno-M13 single-stranded DNA (εM13: Secrist *et al.*, 1972; Barrio *et al.*, 1972). Etheno-modified polynucleotides have been used successfully in previous DNA binding studies (Kowalczykowski *et al.*, 1981*b*) and it has been shown that recA protein will bind to etheno-calf thymus DNA, with a resultant increase in fluorescence upon complex formation (Silver & Fersht, 1982,1983). By using this fluorescence increase to monitor the binding of recA protein to the modified M13 single-stranded DNA, we have quantitatively investigated how these interactions are affected by nucleotide cofactors. Thus, to the extent that this modified single-stranded DNA represents a good analogue for normal single-stranded DNA (see Discussion), we have characterized the equilibrium aspects of these interactions and have shown that both ATP and ADP significantly modulate both the affinity of recA protein for single-stranded DNA, as well as the structure of the bound DNA. Based on these results, we suggest that one role of ATP hydrolysis is to allow recA protein to cycle on and off single-stranded DNA.

2. Materials and Methods

(a) *Chemicals and buffers*

All chemicals used were reagent grade and all solutions were made in glass-distilled water. Titrations were conducted in TMD buffer (20 mM-Tris·HCl (pH 7·5), 4 mM-$MgCl_2$, 0·1 mM-dithiothreitol) at the NaCl concentrations indicated. ATP and ADP were purchased from P-L Biochemicals, and ATP-γ-S, AMP-P-C-P and AMP-P-N-P from Boehringer-Mannheim. All were dissolved as concentrated stock solutions at pH 7.

(b) *Phage DNA*

M13 mp 7 phage were prepared as described by Messing (1983), followed by extraction of the purified phage with phenol to yield the single-stranded M13 DNA. The single-stranded DNA concentrations were determined by using a molar extinction coefficient (per nucleotide residue) of 8784 M^{-1} cm^{-1} at 260 nm in a buffer containing 10 mM-Tris·HCl (pH 7·5), 1 mM-EDTA.

(c) *Etheno-M13 DNA*

M13 phage single-stranded DNA was reacted with chloroacetaldehyde by a method similar to that described by Kryzosiak *et al.* (1981). Chloroacetaldehyde was obtained from Pfaultz and Bauer Inc. and redistilled; the fraction distilling at 88·5°C to 97·5°C was used for the chemical modification. Changes from the quoted method were as follows. The reaction mix, consisting of 2 M-chloroacetaldehyde, 20 mM-potassium acetate, 0·5 mg M13/ml, pH 5·5, was allowed to react for 6 h and was then dialyzed against 10 mM-Tris·HCl (pH 7·5), 1 mM-EDTA at 4°C overnight. The 4°C dialysis step was added to keep DNA degradation to a minimum. The etheno-M13 DNA was then dialyzed into 10 mM-Tris·HCl (pH 7·5), 1 mM-EDTA at 50°C for 7 h to mature the derivatized bases. The product typically had an absorption maximum at 264 nm and a minimum at 244 nm with $A_{270}/A_{260} = 0{\cdot}95$. The integrity of the εM13 DNA was verified by agarose gel electrophoresis. It was found to be essentially the size of the intact unmodified M13 DNA and, due to the elimination of secondary structure by the chemical modification, the etheno-DNA actually ran at a higher apparent molecular weight in the gel than the unmodified M13 single-stranded DNA. This preparation yields etheno-M13 DNA that is stable for many months.

We have found that slight differences in the extent of chemical modification of the M13 DNA caused minor changes in the absolute magnitude of recA protein binding properties. An increased extent of modification resulted in an increased affinity of recA protein for the DNA (i.e. the salt titration midpoint might vary by 50 mM-NaCl); however, the relative differences in the binding affinity due to the nucleotide cofactor effects that have been observed here remained the same. All of the results presented in this paper were performed with 1 preparation of εM13 DNA, with the exception of the data in Table 2. The εM13 DNA used in Table 2 had a salt-titration midpoint that was 10 mM-NaCl higher than the first preparation.

The εM13 DNA concentrations were determined by using an extinction coefficient (per nucleotide residue) of 16,268 M^{-1} cm^{-1} at 260 nm. This value is higher than that reported by Silver & Fersht (1982) for calf thymus DNA, and was determined empirically by comparing stoichiometric titrations (data not shown) of phage T4-coded gene 32 protein with both unmodified M13 single-stranded DNA and εM13 DNA. Since it has been shown that the stoichiometry of gene 32 protein binding is unaffected by etheno-derivatization (Kowalczykowski *et al.*, 1981*b*), the extinction coefficient of the εM13 DNA was adjusted so that the site size for gene 32 protein binding to both the derivatized and underivatized M13 DNA was identical and in agreement with published values (Kowalczykowski *et al.*, 1981*a*).

(d) *recA protein*

recA protein was purified from strain KM1842 using the procedure of Cox *et al.* (1981). The DNA cellulose used in the procedure was prepared by the method of Litman (1968), using heat-denatured calf thymus DNA (Sigma). Protein concentrations were determined by using a molar extinction coefficient of $2{\cdot}7 \times 10^4$ M^{-1} cm^{-1} at 280 nm.

(e) *Fluorescent titrations*

Spectra and titration data were obtained using a Perkin-Elmer MPF-44E fluorescence spectrophotometer. Titrations were temperature controlled at 25°C and titrant was added with a Hamilton Micro Lab P programmable pipettor. The titrant was either recA protein in the "forward titrations", or concentrated NaCl or sodium acetate solutions in the "salt titrations" (described by Kowalczykowski *et al.*, 1981*b*). Etheno-M13 DNA fluorescence was measured by exciting at 300 nm and monitoring at 405 nm. The A_{260} and A_{280} of the sample were typically below 0·05 and 0·07, respectively, corresponding to concentrations of 7·35 μM-εM13 and 2·6 μM-recA protein. Titrations in the presence of cofactors were done at 100 μM for ADP and ATP and 50 μM for ATP-γ-S unless otherwise indicated. In experiments with ATP, which is hydrolyzed rapidly in the presence of recA protein and single-stranded DNA, an ATP regenerating system consisting of 2·6 units pyruvate kinase/ml (Sigma) and 2 mM-phosphoenolpyruvate (Sigma) was added to the cuvette. Sample volumes were normally 1·5 ml.

K and ω values were determined as described by Kowalczykowski *et al.* (1981*b*), which was applied here as follows: (1) the fluorescence endpoint for each titration curve was determined; (2) the apparent affinity, $K\omega$, was determined from the midpoint of each titration ($K\omega = [\text{free recA protein}]^{-1}$); and (3) theoretical titration curves were calculated using a value of the site size, n, equal to 5 and varying K and ω, while keeping their product, $K\omega$, constant. Where the final fluorescence endpoint value was not experimentally attainable, an estimated value was determined based on the endpoints of other, tighter-binding, data sets and attempts were made to fit the curve using that estimated endpoint. If no theoretical curve could adequately fit the data, the endpoint value was adjusted slightly and new curves were calculated until a suitable fit was obtained.

(f) *ATP hydrolysis*

ATP hydrolysis was determined by measuring the conversion of [^{3}H]ATP (ICN Pharmaceuticals) to [^{3}H]ADP. Separation of the ATP and ADP was achieved on polyethyleneimine-cellulose thin-layer chromatography plates (Brinkman) using 0·6 M-NaH_2PO_4 (pH 4·0) to develop the plates. The spots corresponding to ATP and ADP were cut out and counted in 4a20 scintillation cocktail (RPI) in a Beckman LS7800 scintillation counter.

3. Results

(a) *Binding of recA protein to εM13 single-stranded DNA*

The result of adding recA protein to εM13 DNA is shown in Figure 1. The data presented in Figure 1 (and all subsequent titration curves) have been corrected for the linear increase in apparent fluorescence (or scattering) that occurs upon addition of recA protein to a cuvette containing buffer only. This correction was accomplished by performing a duplicate recA protein titration under buffer conditions identical to each of the εM13 DNA titrations, except that no DNA was present in the cuvette. The strictly linear fluorescence increase observed in these control titrations was subtracted from the raw fluorescence data obtained in the presence of DNA; this procedure produced titration curves that showed a distinct plateau fluorescence value when the titrations were performed under stoichiometric conditions. The uncorrected curves (not shown) were similar to those obtained by Silver & Fersht (1982,1983). By correcting data in this manner, we have been able to extract more quantitative information regarding the binding of recA protein to DNA and the effect of nucleotide cofactors on this affinity.

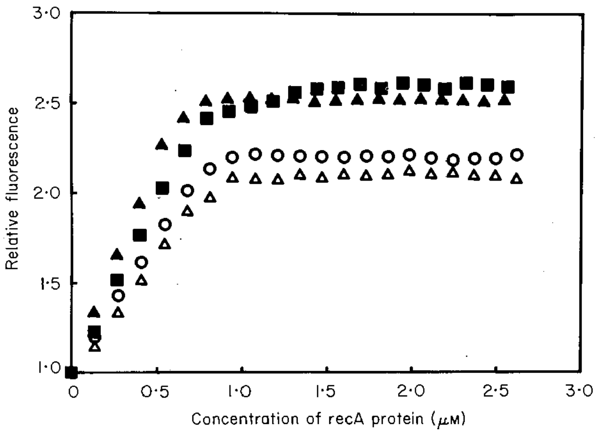

Figure 1. Titration of εM13 DNA by recA protein in the presence of different cofactors. Buffer is TMD; [DNA] = 3·7 μM: ■, 100 μM-ATP with regenerating system; ▲, 48 μM-ATP-γ-S; ○, no cofactor; and △, 100 μM-ADP.

Also shown in Figure 1 are titration curves performed in the presence of ATP, ATP-γ-S and ADP. All curves exhibit saturation behavior, with saturation of the DNA occurring at stoichiometries of 4·8, 4·8, 6·0 and 4·5 nucleotide residues per recA monomer, in the absence of nucleotide cofactor and in the presence of ATP, ATP-γ-S and ADP, respectively (Table 1); within the experimental uncertainty of ±0·7 nucleotide per recA protein molecule, these values are considered to be identical. These titrations, which were performed under tight binding conditions, are valuable in defining the recA protein binding site size, n

Table 1

Site sizes and the relative fluorescence increases for the binding of recA protein to εM13 DNA in the presence of nucleotide cofactors

Nucleotide cofactor	Relative fluorescence increase	n†
None	2·2	4·7
ATP-γ-S	2·5	5·2
ATP	2·5	5·5
ADP	2·2	4·7
AMP-P-C-P	2·2	4·8
AMP-P-N-P	2·2	5·3

Titrations were performed as for Fig. 1.

† Values of the site size represent an average based on several titrations, except for AMP-P-C-P and AMP-P-N-P.

(McGhee & von Hippel, 1974), which is the number of nucleotide residues covered by the protein when bound to single-stranded DNA. The average value of n is $5 \pm 0{\cdot}7$ nucleotides per recA protein molecule and is used in the quantitative analysis of the binding data in section (d), below. This value is in good agreement with values of the site size determined from the molar ratios of recA protein to DNA concentration required for maximal stimulation of enzymatic activities of the protein such as ATPase, protease and strand assimilation activities (see McEntee & Weinstock, 1981).

From the data in Figure 1 and Table 1, it is also clear that there is a greater fluorescence increase upon recA protein–εM13 DNA complex formation in the presence of ATP-γ-S or ATP than in their absence or in the presence of ADP. Similar observations have been reported by Silver & Fersht (1982). The fluorescence of etheno-modified nucleotides is quenched more in oligonucleotides and nucleic acids that have a greater degree of stacking (Tolman *et al.*, 1974) and it has been demonstrated that the unstacking of nucleotide bases (by, for example, heating or hydrolysis) results in an increase in etheno-nucleotide fluoresence. There is no contribution from intrinsic protein fluorescence or from energy transfer due to protein absorption because the excitation wavelength is 300 nm. Thus it appears that the binding of recA protein to εM13 single-stranded DNA results in an unstacking of the nucleotide bases and that the structure of the DNA within recA protein–ATP or recA protein–ATP-γ-S complexes is more unstacked and extended than it is within the complexes formed with ADP or with no cofactor. These results are consistent with electron microscopic observations, which have shown that recA protein–double-stranded DNA complexes formed in the presence of ATP-γ-S have a contour length much greater than the complexes formed in the absence of ATP-γ-S (Stasiak *et al.*, 1981; Flory & Radding, 1982; Dunn *et al.*, 1982), and that complexes of recA protein with single-stranded DNA are extended filamentous structures, in contrast to the collapsed structures of free single-stranded DNA (Flory & Radding, 1982; Dunn *et al.*, 1982).

(b) *Competition experiments*

To assess the effect of the etheno modification on the ability of recA protein to bind to the modified DNA, a competition titration was performed in which equimolar amounts of etheno-M13 and unmodified DNA were present in the cuvette. Upon the addition of recA protein, the titration curve obtained was identical to that observed when no native M13 single-stranded DNA was present and the salt-titration mid-point (see below) was also identical. This indicates that the apparent affinity of recA protein for the εM13 DNA is much greater than it is for the unmodified DNA. Although it may seem unusual that the modification of the adenine and cytosine bases by the addition of an extra ring would result in an increased binding affinity, similar results have been obtained for the binding of phage T4-coded gene 32 protein to poly(rA) and poly(εrA), i.e. gene 32 protein bound more tightly to the poly(εrA) (Kowalczykowski *et al.*, 1981*b*; Newport *et al.*, 1981) and for the binding of *E. coli* SSB protein to single-stranded M13 and εM13 DNA (S. C. Kowalczykowski, unpublished results).

Additional competition experiments were performed using either M13 replicative form DNA or linearized M13 duplex DNA as the competitor, with either no cofactor, ADP (100 μM) or ATP-γ-S (50 μM) present. In all cases, the data were identical to those obtained in the absence of M13 duplex DNA, again demonstrating a strong preferential binding for the εM13 single-stranded DNA over the duplex M13 DNA. A similar result was obtained with double-stranded poly(dA)·poly(dT) as a competitor, even when present at a tenfold excess over the εM13 DNA. Thus, in all cases, recA protein bound preferentially to single-stranded DNA. Only poly(dT) bound to recA protein more tightly than did εM13 DNA, which is in agreement with previously published results (McEntee *et al.*, 1981*a*; Silver & Fersht, 1983; Cazanave *et al.*, 1984).

The fact that recA protein shows this very distinct all-or-none binding behavior with competing DNA is consistent with its ability to bind single-stranded DNA co-operatively (see below). It has been shown that co-operative binding leads to an amplification of even subtle differences in intrinsic binding affinities, resulting in the complete binding of a protein to DNA for which it has only a slightly higher binding affinity before any binding to lower affinity DNA occurs (Newport *et al.*, 1981; von Hippel *et al.*, 1982). Due to this amplification effect, quantitative analysis of relative binding affinities from such competition experiments cannot be achieved.

(c) *Salt-stability of the recA protein–DNA complex*

Under the tight binding conditions of Figure 1, it is impossible to extract quantitative binding information from the titration data, because the binding constant is too large and it is difficult to determine whether the curvature near the saturation point is due to non-stoichiometric binding or to finite DNA lattice size effects (Epstein, 1978). Since, in general, the stability (i.e. affinity) of most protein–nucleic acid complexes decreases with increasing salt concentration (Record *et al.*, 1978), the stability of recA protein–εM13 DNA complexes was determined as a function of increasing NaCl concentrations. The results are displayed in Figure 2, and it is readily apparent that there are distinct differences in the stability (i.e. affinity) of recA protein–εM13 DNA complexes in the presence of various nucleotide cofactors. The approximate mid-point for dissociation of the recA–εM13 DNA (no cofactor) complex is 255 mM-NaCl, and for the ADP–recA–εM13 DNA complex it

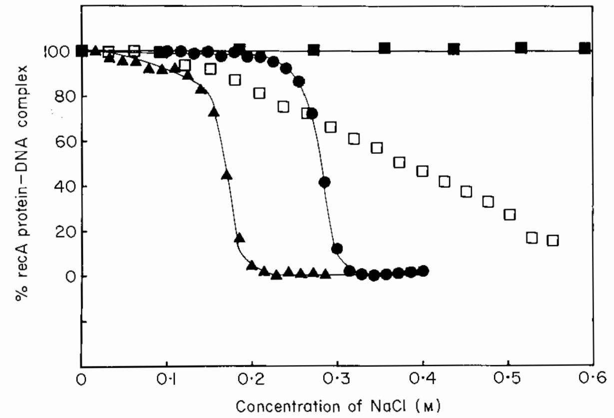

Figure 2. Effect of cofactors on the stability of recA protein–εM13 DNA complexes to dissociation by NaCl. Buffer is TMD; [recA protein] = 1 μM; [DNA] = 5 μM: ■, 48 μM-ATP-γ-S; ●, no cofactor; □, 100 μM-ATP in the presence of ATP regenerating system; and △, 100 μM-ADP.

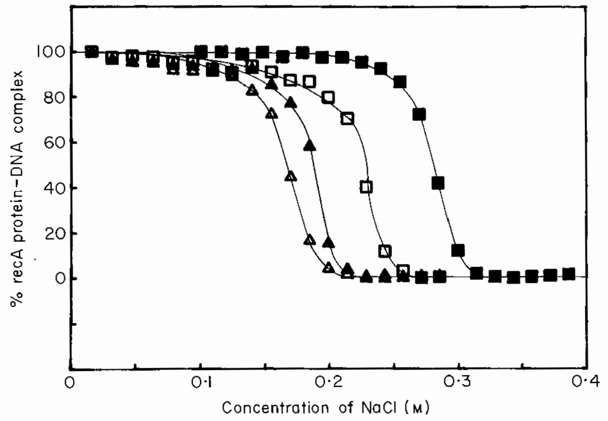

Figure 3. Effect of varying concentrations of ADP on the stability of recA protein–εM13 DNA complexes to dissociation by NaCl. Buffer is TMD; [recA protein] = 2·4 μM; [DNA] = 3·7 μM: ■, 0 μM-ADP; □, 24 μM-ADP; ▲, 48 μM-ADP; and △, 100 μM-ADP.

decreases to 165 mM-NaCl (see Table 2). The ATP-γ-S–recA–εM13 DNA complex is extremely stable and could not be dissociated either by NaCl concentrations as high as 2·5 M, by EDTA (44 mM) or by ADP (240 μM). The results obtained with ATP-γ-S and no cofactor are in general agreement with previously published results obtained from filter binding experiments (McEntee *et al.*, 1981*a*) and further confirm the usefulness of the etheno derivative of M13 DNA in DNA binding studies.

Interestingly, Figure 2 demonstrates that the stability of the ADP–recA–εM13 DNA complex is *less* than that of the recA–εM13 complex formed in the absence of cofactor. Thus, in contrast to the effect of ATP-γ-S, which is to *increase* the stability of the recA protein–εM13 DNA complex, ADP causes the affinity of recA protein for single-stranded DNA to *decrease*. The magnitude of this ADP-mediated destabilization of the recA–εM13 DNA complex is dependent on the ADP concentration, with the result that increasing the ADP concentration causes the stability of the recA–εM13 complexes to progressively decrease (Fig. 3). If the midpoint of each of the salt-titration curves in Figure 3 is plotted as a function of the ADP concentration, the resultant plot has the appearance of a hyperbolic binding curve and an apparent ADP dissociation constant of ≈25 μM for the inhibition of single-stranded DNA binding can be obtained (data not shown). This value is of the same order of magnitude as that obtained from direct equilibrium dialysis experiments (K_d = 98 μM; Cotterill *et al.*, 1982) and therefore appears to reflect a coupled equilibrium between ADP binding and (decreased) DNA binding. These results provide a plausible explanation for the inhibitory effect that ADP has on the recA protein-catalyzed strand assimilation reaction (Shibata *et al.*, 1979; McEntee *et al.*, 1979) and suggest that the inhibition is due to the decrease in affinity of recA protein for single-stranded DNA caused by the increased ADP concentration due to ATP hydrolysis. This would result in the dissociation of recA protein from the pre-synaptic complex and subsequent inhibition of strand assimilation (see Discussion).

Table 2

Salt titration midpoints for the dissociations of recA protein–εM13 DNA complexes in the presence of nucleotide cofactors

Cofactor	Salt	Salt titration midpoint† (mM)
None	NaCl	255
ADP	NaCl	165
AMP-P-C-P	NaCl	200
AMP-P-N-P (500 μM)	NaCl	590
(375 μM)		480
None	Sodium acetate	840
ADP	Sodium acetate	480

Titrations were performed as for Fig. 2, except that εM13 DNA preparation 2 was used; all nucleotide concentrations were 100 μM, except as indicated.

† Salt titration midpoints for the NaCl titrations were obtained from experiments in which the concentrations of recA protein and εM13 DNA were 1 μM and 5 μM, respectively; for the sodium acetate titrations, the concentrations were 0·735 μM and 3·7 μM, respectively.

Figure 2 also shows the effect of ATP on the stability of the recA protein–εM13 DNA complex. These data cannot be considered equilibrium results, due to the continual hydrolysis of ATP; however, the data must represent pseudo-equilibrium conditions, in that the steady-state concentrations of DNA-bound recA protein and unbound recA protein at each salt concentration are independent of time. The relative amounts of bound and free recA protein at the higher salt concentrations depend on the efficiency of the ATP regenerating system in converting ADP to ATP; for the data shown in Figure 2, the steady-state concentrations of ATP and ADP were approximately 85 μM and 15 μM, respectively. Thus, due to ATP hydrolysis, a rigorous interpretation of such data is made difficult by the presence of various bound forms of recA protein (i.e. ATP-, ADP- and no cofactor) and by the rates of interconversion between them. However, they are presented here so that at least a qualitative comparison of the effect of ATP on the stability of recA protein–DNA complexes can be made, rather than relying solely on studies involving ATP analogues.

The results in Figure 2 show that the stability of the recA–εM13 DNA complex in the presence of ATP is intermediate to that of the complexes formed in the presence of ADP, and those formed in the presence of ATP-γ-S. Particularly significant is the result that, in the presence of ATP, approximately 70% of the recA protein–εM13 DNA complexes are more stable than those formed in the absence of nucleotide cofactors, and that all of the complexes formed in the presence of ATP are more stable than those formed in the presence of ADP. The stability of the complexes formed in the presence of ATP can be extended to even higher NaCl concentrations, if the ATP concentration is increased to 500 μM (data not shown). Under these conditions, the ATP regenerating system maintains the steady-state concentrations of ATP and ADP at 490 μM and 10 μM, respectively. Only the data at 100 μM-ATP are plotted in Figure 2, in order to allow direct comparison with the ADP data, also at 100 μM. Since a large fraction of the recA protein–DNA complexes formed in the presence of ATP are more stable than both the ADP–recA protein–DNA complexes and the (no cofactor) recA protein–DNA complexes, this implies that the effect of ATP is to increase the affinity of recA protein for single-stranded DNA. In this regard, the effect of ATP is more like that of ATP-γ-S (i.e. an increase in affinity relative to no cofactor) rather than that of ADP (decrease in affinity).

A second interesting result from Figure 2 is that, although some of the recA protein–εM13 DNA complexes in the presence of ATP dissociate at salt concentrations lower than the (no cofactor) recA protein–DNA complexes, no dissociation is observed until the salt concentration required for the dissociation of the ADP–recA protein complexes is exceeded. This result suggests that this dissociation is a result of the formation of ADP–recA protein–DNA complexes due to ATP hydrolysis, and that these complexes then dissociate due to their intrinsically lower stability. These two observations taken together strongly suggest that ATP *binding* to recA protein results in the formation of stable recA protein–DNA complexes, and that ATP *hydrolysis* results in the formation of unstable recA protein–DNA complexes due to the production of ADP–recA protein–DNA complexes upon hydrolysis. Thus, in any interpretation of the role of ATP on recA protein–single-stranded DNA binding affinity, the effects of ATP binding *versus* ATP hydrolysis (resulting in ADP binding) must be discriminated (see Discussion). A rigorous understanding of these effects must await the results of detailed kinetic studies, which are in progress.

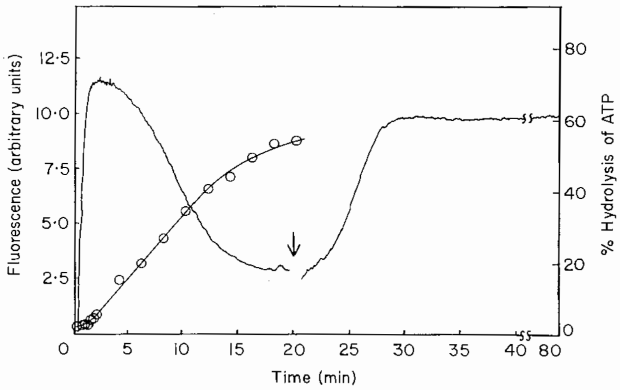

Figure 4. Effects of ATP hydrolysis and ATP regeneration of the stability of recA protein–εM13 DNA complexes. recA protein was added at 0 min and allowed to hydrolyze ATP in the absence of ATP regeneration. The left ordinate represents etheno-DNA fluorescence and the right ordinate represents percentage hydrolysis. ATP regeneration is then initiated (at the arrow) by the addition of phosphoenol pyruvate (4 mM). Buffer is TMD with 240 mM-NaCl; [recA] = 1 μM; [DNA] = 5 μM; and [pyruvate kinase] = 2·6 units/ml.

An additional demonstration of the fact that ADP and ATP significantly modulate the affinity of recA protein for single-stranded εM13 DNA and that ADP accumulation results in dissociation of the recA protein–DNA complex is provided by the following experiment. recA protein was added to a cuvette containing εM13 DNA and 100 μM-ATP, in the absence of an ATP regenerating system, at a NaCl concentration of 240 mM. At this salt concentration both the (no cofactor) recA protein–DNA complex and the ATP–recA protein–DNA complex are stable, while the ADP–recA protein–DNA complex is not. Upon addition of recA protein, the fluorescence increases to a level typical of the ATP–recA protein–εM13 complex (Fig. 4). Due to continual ATP hydrolysis by recA protein, the ADP produced induces the dissociation of recA protein from the εM13 DNA resulting in a progressive decrease in fluorescence to that which is typical of the uncomplexed εM13 DNA alone. Also shown in Figure 4, is the extent of ATP hydrolysis during the time-course; however, ATP turnover numbers obtained from such an experiment cannot be related to the dissociation rate because the dissociation of recA protein in this experiment is an equilibrium effect resulting from the accumulation of ADP. Under these conditions, an initial lag is seen in ATPase activity as well as slow DNA binding kinetics requiring several minutes for completion. This observation appears consistent with data presented by Kahn & Radding (1984) on a time-dependent lag in the pre-synaptic (single-stranded DNA binding) step of the strand assimilation reaction. To show that the dissociation is reversible by simply restoring the ATP concentration to nearly the original level, phosphoenol pyruvate was added (pyruvate kinase is already in the cuvette) to initiate ATP regeneration (arrow) and the fluorescence returned to a level typical of the ATP–recA protein–εM13 DNA complex. The final fluorescence value is not identical to that of the initial ATP complex due to the inability of the ATP regenerating system to quantitatively convert all of the ADP to ATP; instead, the fluorescence returns to a value typical of the presence of 18% ADP, which is the final steady-state level achieved by the ATP regenerating system under these conditions.

In addition to ATP-γ-S, the effects of two other non-hydrolyzable ATP analogues (AMP-P-N-P and AMP-P-C-P) on recA protein–εM13 DNA binding affinity were tested and the results are also summarized in Table 2. Each of these analogues has a different effect. The AMP-P-N-P analogue behaves more like ATP or ATP-γ-S in that it stabilizes the recA protein–DNA complex against dissociation by salt; concentrations of NaCl as high as 590 mM are required for dissociation. In contrast, the AMP-P-C-P analogue causes a decrease in the stability of the recA protein–εM13 complex relative to the no cofactor complexes. Thus this analogue surprisingly behaves more like ADP rather than ATP. The AMP-P-N-P analogue, in particular, may prove to be a very interesting substrate to use in further studies of recA protein–DNA interactions because this analogue behaves like ATP-γ-S by increasing the stability of the recA–εM13 complex, but the stabilization is not so large as to prevent dissociation by high salt concentration. This will allow for a quantitative characterization (see section (e), below) of the equilibrium properties of the high DNA-binding affinity state of an "ATP-activated" recA protein–DNA complex, in the absence of the difficulties introduced by ATP hydrolysis. However, due to unusual kinetic properties of the complex formed in the presence of this analogue (unpublished results), the quantitative effects of this analogue on the properties of the recA protein–DNA complex will be discussed in a subsequent publication. Although we cannot provide a molecular explanation for the opposite effects elicited by these two analogues, it has been shown that the bond angles and lengths of the P-C-P- and P-N-P-analogues are different from those of ATP (Yount, 1975). This may suggest that the binding of recA protein to the terminal phosphate of these ATP analogues is different and that recA protein can interact with the terminal phosphate of AMP-P-N-P, which is therefore ATP-like in its properties, but that recA protein cannot interact with the terminal phosphate of AMP-P-C-P, which is consequently more ADP-like in its effect on the recA–εM13 DNA complex.

The data presented in this section suggest a model in which the role of nucleotide cofactors is to modulate the affinity of recA protein for single-stranded DNA, with ATP inducing a tight binding form of recA protein and ADP inducing a weak binding form of recA protein. The interconversion of these forms is determined by the actual hydrolytic event that converts the ATP–recA–εM13 complex to an ADP–recA–εM13 DNA complex, which then dissociates from the single-stranded DNA. This effect of ADP on recA protein–single-stranded DNA complexes suggests that one role of ADP is to serve as a "release" factor to stimulate dissociation of the protein from the DNA and thereby allow the protein to recycle (see Discussion).

(d) *recA protein titrations at increasing NaCl concentrations*

The data from the NaCl salt titration experiments indicate that the affinity of recA protein for εM13 DNA is substantially reduced at higher salt concentrations and suggest the range of NaCl concentration in which to carry out additional titrations. Since it is not possible to extract quantitative binding information from stoichiometric titrations such as those in Figure 1, titrations of εM13 DNA with recA protein were carried out at various increasing NaCl concentrations. Such titrations are shown in Figure 5, and it is clear that as the NaCl concentration is increased: (1) the affinity of recA protein for εM13 DNA decreases, as expected from the salt-titration data; (2) the curves become sigmoidal, which is characteristic of co-operative recA protein binding; (3) the "lag" that occurs before the binding region increases; and (4) the relative fluorescence increase in the plateau region decreases. Observations (1) to (3) are good qualitative indications that the binding of recA protein to εM13 single-stranded DNA, in the absence of any nucleotide cofactors, is co-operative over the range of NaCl concentrations that are experimentally accessible (e.g. see Kowalczykowski *et al.*, 1981*b*).

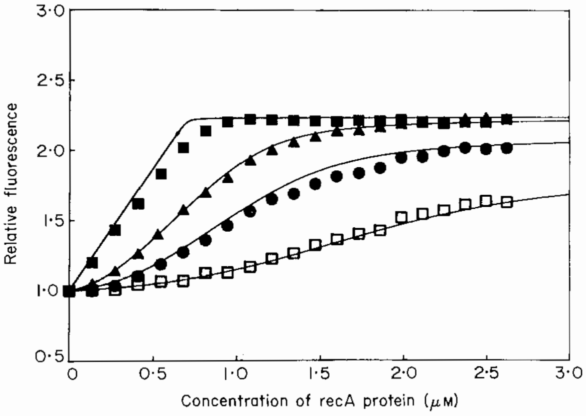

Figure 5. Protein titration curves at different NaCl concentrations in the absence of cofactors. Buffer is TMD; [DNA] = 3·7 μM: ■, 0 mM-NaCl; ▲, 260 mM-NaCl; ●, 280 mM-NaCl; and □, 295 mM-NaCl. The curves represent the best fits to the data using the values in Table 3.

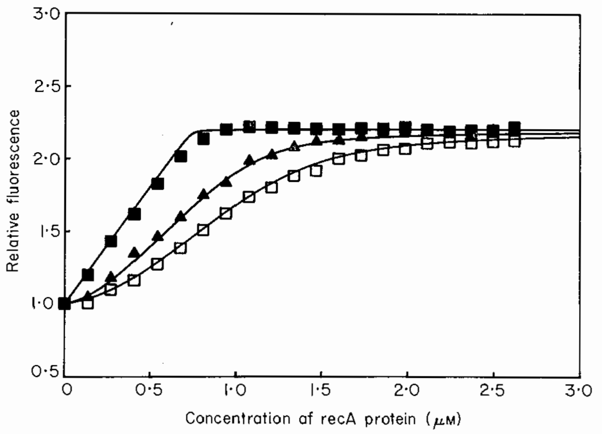

Figure 6. Protein titration curves at different NaCl concentrations in the presence of 100 μM-ADP. Buffer is TMD; [DNA] = 3·7 μM; ■, 0 mM-NaCl; ▲, 150 mM-NaCl; and □, 170 mM-NaCl. The curves represent the best fits to the data using the values in Table 3.

Before proceeding with the quantitative analysis of these binding curves, it was of interest to see whether the effect that ADP had on the affinity of recA protein for DNA was on the degree of binding co-operativity or on the intrinsic binding affinity. The results of titrations performed in the presence of ADP are shown in Figure 6 and, qualitatively, they are similar to those obtained in the absence of ADP, i.e. a decrease in affinity at higher salt concentrations and sigmoid (co-operative) binding. The only difference in these two sets of data is the range of NaCl concentrations in which the data can be acquired, due to the lower stability of the ADP–recA–εM13 DNA complex. In the next section, it will be demonstrated quantitatively that the co-operativity of recA protein binding to εM13 DNA is unchanged by the presence of ADP.

(e) *Determination of* K *and* ω

The data presented in Figures 5 and 6 can be analyzed quantitatively to determine the intrinsic binding affinity, K, and co-operativity parameter, ω (McGhee & von Hippel, 1974) using the analysis procedure described by (Kowalczykowski *et al.*, 1981*b*). Figure 7 shows an example of the calculated curves, together with the experimental data. The "best-fit" curve for each titration was selected by visual inspection. Emphasis was placed on the ability of the curve to more accurately fit the lower one-half to three-quarters of the data to minimize the possibility of error being introduced by "end-effects" due to the finite size of the DNA lattice (Epstein, 1978; Kowalczykowski *et al.*, 1981*b*). From Figure 7, it is clear that a value of $\omega = 10$ is too low to fit the data, whereas values greater than 100 are too "steep". The best fit of the data occurs when $\omega = 50$, which means that the intrinsic binding constant, K, is equal to $5{\cdot}1 \times 10^4$ M^{-1} with the apparent affinity, $K\omega$, being equal to $2{\cdot}55 \times 10^6$ M^{-1}.

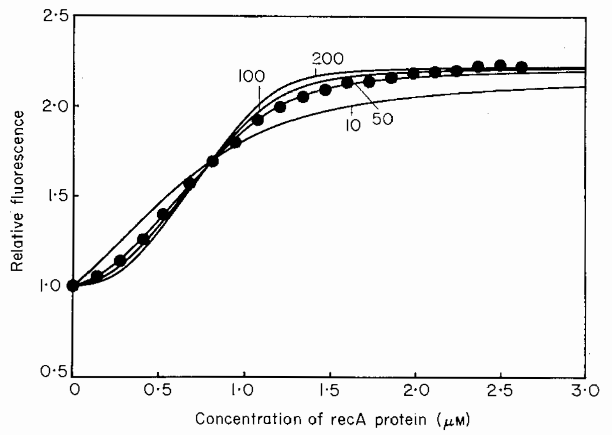

Figure 7. Example of fitting of experimental data to values of intrinsic binding constant, K, and co-operativity parameter ω. Buffer is TMD plus 260 mM-NaCl; [DNA] = 3·7 μM. The product, $K\omega$, is determined as described in the text and held constant at $2{\cdot}55 \times 10^6$ M^{-1}. The theoretical curves represent different ω values of 10, 50, 100 and 200, and the corresponding values of K are $2{\cdot}55 \times 10^5$ M^{-1}, $5{\cdot}10 \times 10^4$ M^{-1}, $2{\cdot}55 \times 10^4$ M^{-1} and $1{\cdot}28 \times 10^4$ M^{-1}, respectively.

By applying this data fitting approach to the titrations shown in Figures 5 and 6, values of the binding constant and the co-operativity parameter were determined for recA protein binding to εM13 DNA, both in the presence and absence of ADP. These values are summarized in Table 3, and the results clearly show that: (1) binding of recA protein to εM13 DNA is co-operative at all salt concentrations studied with $\omega = 50$ (± 10); (2) the co-operativity of binding is identical both in the presence or absence of ADP; and (3) since the value of the co-operativity parameter is independent of salt concentration, all the salt dependence of binding resides in an effect on the intrinsic affinity of binding. The co-operativity parameter has been

Table 3

Values of K *and* ω *for recA protein binding to εM13 DNA*

[NaCl] (mM)	$K\omega$(M^{-1})	K(M^{-1})	ω	K_{Mg} corrected (M^{-1})‡
With ADP†				
130	$4{\cdot}35 \times 10^6$	$8{\cdot}7 \times 10^4$	50	$8{\cdot}9 \times 10^4$
150	$3{\cdot}0 \times 10^6$	$6{\cdot}0 \times 10^4$	50	$6{\cdot}2 \times 10^4$
160	$2{\cdot}0 \times 10^6$	$4{\cdot}0 \times 10^4$	50	$4{\cdot}1 \times 10^4$
170	$1{\cdot}8 \times 10^6$	$3{\cdot}6 \times 10^4$	50	$3{\cdot}6 \times 10^4$
No cofactor				
260	$2{\cdot}55 \times 10^6$	$5{\cdot}1 \times 10^4$	50	$5{\cdot}2 \times 10^4$
280	$1{\cdot}5 \times 10^6$	$3{\cdot}0 \times 10^4$	50	$3{\cdot}0 \times 10^4$
285	$1{\cdot}05 \times 10^6$	$2{\cdot}1 \times 10^4$	50	$2{\cdot}2 \times 10^4$
295	$6{\cdot}5 \times 10^5$	$1{\cdot}3 \times 10^4$	50	$1{\cdot}3 \times 10^4$

Values were determined from individual titration curves as shown in Fig. 7 and described in the text.

† The ADP concentration was 100 μM.

‡ The corresponding values of K in column 2 were corrected for the magnesium ion concentration as described in the text.

determined for several single-stranded DNA binding proteins, and for comparison to recA protein–single-stranded DNA binding, ω has been shown to be $\simeq 1000$ for T4-coded gene 32 protein (Kowalczykowski *et al.*, 1981*b*; Newport *et al.*, 1981), 50 to 300 for fd phage-coded gene 5 protein (Alma *et al.*, 1983), and estimated to range between 50 and 10^5 for *E. coli* SSB protein (see Kowalczykowski *et al.*, 1981*a*).

One use of the co-operativity parameter is that it is related to the average size and the size distribution of recA protein clusters that are co-operatively bound to the DNA. A value of $\omega = 50$ corresponds to an average cluster size of four co-operatively bound recA protein molecules at 50% saturation of the single-stranded DNA, and increases to an average cluster size of 11 protein molecules at 90% saturation (Schellman, 1975; Kowalczykowski *et al.*, unpublished results). If recA protein function depends on a certain critical cluster size (McEntee *et al.*, 1981*a*), then correlation of function with cluster size will require accurate knowledge of ω under a variety of experimental conditions such as salt and cofactor concentrations.

(f) *Ionic interactions involved in recA protein–εM13 DNA complex formation*

The data presented in Table 3, i.e. values of K and ω as a function of NaCl concentration, are valuable with regards to two additional molecular aspects of the recA protein–single-stranded DNA complexes. First, the data can be used to determine the number of ionic interactions, m', formed in the recA protein–DNA complex (Record *et al.*, 1978) and second, the data can be extrapolated to ionic conditions that reflect more accurately the effective physiological salt concentrations found in *E. coli* (see Discussion), i.e. approximately 220 mM-NaCl (Kao-Huang *et al.*, 1977; von Hippel *et al.*, 1982).

The number of ionic interactions formed between recA protein and single-stranded DNA can be obtained from analysis of the salt dependence of the binding constant by plotting the data in the form of log K *versus* log [NaCl] (Record *et al.*, 1976,1977; de Haseth *et al.*, 1976). In the absence of competitive effects due to magnesium ions, the slope of such a plot is given by:

$$\mathrm{d}\log K/\mathrm{d}\log[\mathrm{NaCl}] = -m'\psi - \frac{aK_a[\mathrm{X}^-]}{1+K_a[\mathrm{X}^-]},$$

where m' is the number of ionic interactions formed between the protein and the DNA phosphates, ψ is the fraction of ions thermodynamically bound to the DNA and is equal to 0·71, a is the number of anions displaced by binding, K_a is their affinity, and $[\mathrm{X}^-]$ is their concentration. Note that if $K_a[\mathrm{X}^-] > 1$, the above equation can be simplified to (Newport *et al.*, 1981):

$$\mathrm{d}\log K/\mathrm{d}\log[\mathrm{Na}^+] = -m'\psi - a.$$

This simplified equation can be used if there is no curvature in the log-log plot, and it allows for a more straightforward interpretation of the slope, because only two terms need to be considered: $m'\psi$, which is due to the ionic interactions formed between the protein and DNA, and a, which is due to anion release upon complex formation.

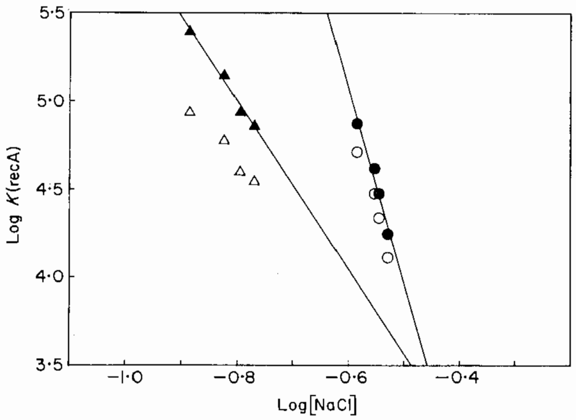

Figure 8. Log-log plot of the intrinsic binding constant, K, (M^{-1}) *versus* concentration of NaCl(M). △, 100 μM-ADP; ▲, 100 μM-ADP (Mg^{2+} corrected); ○, no cofactor; and ●, no cofactor (Mg^{2+} corrected). The least-squares lines through the data are: $y = 1{\cdot}84 - 3{\cdot}15\,x$, for ADP; $y = 1{\cdot}24 - 4{\cdot}74\,x$ for ADP, (Mg^{2+} corrected); $y = 1{\cdot}41 - 10{\cdot}5\,x$ for no cofactor; and $y = 1{\cdot}60 - 11{\cdot}11\,x$ for no cofactor (Mg^{2+} corrected).

In Figure 8, we have plotted log K *versus* log [NaCl] for the data presented in Table 3, as well as those data corrected for magnesium ion concentration. They were corrected by using the published values for K_{Mg} at different NaCl concentrations and equations (3) and (4) of Record *et al.* (1977) and by using the maximum value that m' can have, which is 5 (since $n = 5$). In this way, the greatest amount of magnesium-correction would be introduced and the log-log slopes in Figure 8 represent minimum and maximum values†. The slopes of these lines are −3·15 and −4·7 for the data in the presence of ADP (uncorrected and magnesium corrected, respectively), and −10·5 and −11·1 for the data in the absence of ADP. If anion effects were absent (i.e. if $a = 0$), then the maximum value of these slopes would be −3·5. Clearly, in the case of the no cofactor data, the slope is very much greater, suggesting that anion effects are involved in complex formation.

To determine whether anion effects played a role

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† Unfortunately, it is not possible to obtain equivalent data for the recA protein–single-stranded DNA complexes in the absence of magnesium, because the specific nucleotide effects on DNA binding affinity that we have demonstrated are dependent on the presence of magnesium ions; i.e. in the absence of Mg^{2+}, neither ATP nor ATP-γ-S stabilize the recA protein–DNA complex; instead, they destabilize the complex (unpublished results). This effect is likely to be the basis of the preparative procedure utilizing ATP elution of recA protein from single-stranded DNA cellulose described by Cox *et al.* (1981).

in the formation of recA protein–εM13 DNA complexes, salt titrations were performed using sodium acetate as the titrant instead of NaCl, and those results are also presented in Table 2. As can be seen from the values of the salt titration midpoints, a much greater concentration of sodium acetate than of NaCl is required to dissociate the recA protein–εM13 DNA complex, indicating that the complex is much more stable in sodium acetate buffer. This is the expected result if anions are released upon complex formation and if acetate ions bind more weakly to recA protein, as might be expected by the Hofmeister series (see von Hippel & Schleich, 1969).

As mentioned earlier, this anion effect contributes to the slope of the log *K versus* log [Na^+] plot, which makes the interpretation of this slope in terms of the number of ionic interactions formed between recA protein and single-stranded DNA difficult. However, despite this additional complication, the following qualitative conclusions can be made from the salt dependence data. First, there is a difference between the slopes of the log-log plots for the data obtained in the presence of ADP (slope $= -3{\cdot}15$) compared to the data in the absence of ADP (slope $= -10{\cdot}5$). This suggests that there are differences between the (no cofactor) recA protein–DNA complexes and the ADP–recA–DNA complexes in either the number of ionic interactions (m') formed between recA protein and the DNA, and/or the number of anions released from the protein upon binding. Second, there must be anion release upon DNA binding, because the values of m' derived from the magnesium-corrected data, in the presence and absence of ADP, would be 6·6 and 15·6, respectively. Since the value of m' cannot exceed the value of n ($= 5 \pm 1$), then the difference between those values and the maximum value of 5 must be due to anion release upon binding. Third, the type of anion present in the buffer has an effect on the absolute magnitude of the binding constant. As indicated in Table 2, the affinity of recA protein for εM13 DNA is much higher in the sodium acetate buffer than in NaCl, and similar observations have been made in the case of *lac* repressor protein (de Haseth *et al.*, 1976) and gene 32 protein (Kowalczykowski *et al.*, 1981*a*,*b*). The consequence of this higher DNA binding affinity in acetate buffers may be quite significant with regard to recA protein function and is mentioned in the Discussion.

4. Discussion

The major conclusions that can be drawn from the experimental observations described in this paper are as follows:

First, the binding of recA protein to single-stranded DNA is greatly modulated by the binding of nucleotide cofactors. The nucleotides ATP, ATP-γ-S, and AMP-P-N-P increase the affinity of recA protein for single-stranded DNA relative to the affinity when no cofactor is present, whereas the nucleotides ADP and AMP-P-C-P decrease the affinity. The extent to which DNA binding affinity is decreased by ADP is dependent on the ADP concentration, which suggests that a coupled competitive equilibrium exists between ADP binding and single-stranded DNA binding.

Second, the εM13 DNA fluorescence increase obtained upon complex formation with recA protein is dependent on the nucleotide cofactor present; the increase is greater for complexes formed in the presence of ATP or ATP-γ-S than it is for those formed in the presence of ADP or no cofactor. Since the fluorescence increase is a measure of the extent to which the nucleotide bases are unstacked, this implies that the binding of recA protein to single-stranded DNA results in an unstacking of the DNA, and that the recA protein–single-stranded DNA complexes are more unstacked in the presence of ATP or ATP-γ-S than with ADP or no cofactor.

Third, quantitative analysis of the titration curves shows that recA protein binding to single-stranded DNA is co-operative ($\omega = 50$) and that the co-operativity parameter is unaffected by NaCl or ADP concentration. The fact that the co-operativity of binding is unchanged by these variables means that their effects on recA protein–single-stranded DNA affinity are mediated through changes in the intrinsic binding affinity of the complex rather than through changes in the co-operativity of binding. This result, that recA protein binding is co-operative under all conditions investigated, suggests that co-operativity is important to the function of this protein.

Fourth, analysis of the salt dependence of the intrinsic binding affinity of the recA protein–single-stranded DNA complex demonstrates that there is a large ionic component to the free energy of binding. The binding affinity decreases with increasing salt concentration and is also effected by the type of anion present in the buffer. At a comparable salt concentration, the affinity in the presence of acetate anions is estimated to be approximately 10,000-fold greater than in the presence of chloride. Although this specific anion effect complicates the detailed analysis of ionic interactions involved in the recA protein–single-stranded DNA complex, the data presented here suggest that in the protein–DNA complex, recA protein both occludes five nucleotide residues ($n = 5$) when bound, and forms up to five ionic contacts with DNA phosphates. In addition, at least five to six anions are displaced from the protein when complex formation occurs. Preliminary studies on the recA protein-catalyzed strand assimilation reaction have shown that this activity is much enhanced in acetate buffers (L. Schultz & S. C. Kowalczykowski, unpublished results), possibly implicating the importance of tightly bound recA protein–single-stranded DNA complexes in this reaction.

The first and second observations lead to the conclusion that recA protein has at least two different DNA binding states: a low affinity binding form, which is characterized by the ADP–recA protein complex, and a high affinity form, which is characterized by the ATP–recA protein complex. Because ADP is the product of ATP hydrolysis by the recA protein–single-stranded DNA complex, interconversion between these two states is achieved and controlled by the hydrolytic event. In addition, dissociation of ADP from recA protein results in nucleotide cofactor-free recA protein, which may be considered a third DNA binding form of the protein whose affinity is intermediate between that of the ADP-bound and ATP-bound forms.

The suggestion that recA protein exists in two states has been made based on a correlation of various recA protein enzymatic activities (Cohen *et al.*, 1983). While it is not possible to completely relate our equilibrium states with the two proposed enzymatic states, preliminary binding studies in the presence of ATP and calcium ions under essentially non-hydrolytic conditions (less than 10% of that observed with magnesium ions) have shown that recA protein binds very tightly to εM13 DNA with a salt titration midpoint of ≈450 mM-NaCl (unpublished results). Since the "high protease activity" enzymatic state of recA protein is induced by ATP and calcium ions (Cohen *et al.*, 1983), our results would suggest that the high affinity ATP-bound state is the proteolytically active form of the protein. This conclusion is also in accord with the suggestion that a ternary complex of ATP, recA protein, and single-stranded DNA is the active species in proteolytic cleavage, and with the observation that the non-hydrolyzeable analogue ATP-γ-S is the most effective nucleotide for stimulation of protease activity (Craig & Roberts, 1980).

In addition, these fluorescence studies have shown that the affinity of recA protein is modulated by nucleotide cofactors, and that the structure of the bound DNA within the recA protein–single-stranded DNA complexes is effected by nucleotide binding. The DNA is in a more extended form in the high affinity ATP-bound state than it is in the low affinity ADP-bound (and no cofactor) state. This conclusion complements the results based on electron microscopic studies of complexes of double-stranded DNA and recA protein, which have shown that these complexes exist primarily in two different forms: an extended and unwound structure of duplex DNA formed in the presence of ATP or ATP-γ-S, and a more compact structure, which forms upon ATP hydrolysis (Dunn *et al.*, 1982; Flory & Radding, 1982). Thus, a similar cyclic mechanism appears to exist for the modulation of single-stranded DNA structure by recA protein through ATP hydrolysis, as is proposed to exist for double-stranded DNA complexes (Dunn *et al.*, 1982).

Our equilibrium binding studies also provide a possible molecular explanation for the reported inhibitory effect of ADP on recA protein-catalyzed strand assimilation (Wu *et al.*, 1982; Cox *et al.*, 1983; Kahn & Radding, 1984); namely, that ADP destabilizes the recA protein–single-stranded DNA complex, and that the magnitude of this destabilization increases with increasing ADP concentration (see Fig. 3). Thus, strand assimilation will cease when ADP accumulates to a certain level, due to the inability of recA protein to form stable complexes with single-stranded DNA. Cox *et al.* (1983) have also shown that recA protein will dissociate from single-stranded DNA when the ratio of ADP/ATP reaches values of 0·6 to 1·5, depending on initial conditions. This result is in agreement with the data presented in Figure 4, which shows that complete dissociation occurred when the ADP concentration accumulated to 60% of the input ATP concentration, i.e. an ADP/ATP ratio of 1·5. This interpretation is also consistent with the recent finding by Kahn & Radding (1984) that the pre-synaptic formation of a recA protein–single-stranded DNA complex is sensitive to the ADP concentration. They have reported also that the rate of formation of this complex can be very slow (i.e. up to 15 min). Although under the conditions described here we see little or no lag in the binding of recA protein to εM13 DNA, at higher salt conditions we have observed similar kinetic effects (unpublished results).

Since the exact relationship between DNA binding and recA protein enzymatic function has not been established, it is not possible to predict the quantitative effects that changes in ATP/ADP ratio, salt concentration, etc., will have on enzymatic activity. However, if recA protein activity is directly related to the absolute magnitude of the binding affinity that recA protein has for single-stranded DNA, then enzymatic activity should parallel the effects that nucleotide cofactor concentration, salt concentration, and DNA nucleotide composition have on DNA binding affinity described here. Alternatively, if recA protein function depends on a specific configuration of bound proteins such as, for example, a critically sized cluster of contiguously bound ATP–recA protein molecules, then a more complex non-linear correlation should be observed. The exact relationship between DNA binding and enzymatic function will require much more detailed correlation of these properties.

Another consequence of the work presented here is the importance of making a distinction between the effects of ATP binding and ATP hydrolysis, particularly when conditions are such that recA protein is hydrolyzing ATP. Both ATP and ADP have been reported to stimulate the dissociation of recA protein from single-stranded DNA at an identical rate (McEntee *et al.*, 1981*a*) and, under certain conditions, we have observed similar effects (unpublished results). Because ADP causes an equilibrium destabilization of the recA protein–single-stranded DNA complex, it might be expected that this effect would also be manifested in the kinetic dissociation rate (Kowalczykowski *et al.*, 1980), and indeed this has been observed (McEntee *et al.*, 1981*a*; J. P. Menetski and S. C. Kowalczykowski, unpublished results). In the case of dissociation stimulated by ATP (under hydrolytic conditions), it is important to recognize that the product of ATP hydrolysis is the unstable ADP–recA protein–DNA complex which, if the ADP remains bound, will dissociate at a rate identical to that of the ADP–recA protein–DNA complex formed under equilibrium conditions. Thus, we propose that the dissociation of the recA protein–single-stranded DNA complex that occurs in the presence of ATP hydrolysis is not due to destabilization of the complex by ATP binding, but rather is a result of the hydrolysis of ATP to produce ADP; the ADP, in turn, causes a destabilization of the recA protein–DNA complex†. Thus, in our interpretation, the effects of ATP on the affinity of recA protein for single-stranded DNA are twofold: ATP *binding* induces a tight binding form of the protein, whereas ATP *hydrolysis* results in a weak binding, rapidly dissociating form of the protein through the formation of ADP. In this way, ADP can be thought of as a release factor that allows recA protein to cycle on and off single-stranded DNA. Further understanding of these effects will require the results of more detailed kinetic studies, which are in progress.

Quantitative analysis of the DNA binding curves led to the conclusion that the co-operativity parameter, ω, is equal to 50, and is unaffected by NaCl or ADP concentration. This co-operativity value would predict that the average sizes of co-operatively bound recA protein clusters would range from 4 to 11 protein molecules (i.e. 20 to 55 nucleotide residues) at values of DNA saturation varying from 50% to 90%. These values are much lower than the cluster sizes that are observed by electron microscopy (Dunn *et al.*, 1982; Flory & Radding, 1982); however, most of the electron microscopy work is performed using an excess molar ratio of recA protein to DNA in order to form the complexes. Dunn *et al.* (1982) present a micrograph (their Figure 1D) in which the weight ratio of recA protein to single-stranded DNA is 4 to 1, corresponding to a molar ratio of approximately one recA protein monomer per 12 nucleotides, or slightly less than 50% saturation of the single-stranded DNA. The cluster sizes visible in this micrograph are clearly much smaller than full-length complexes of recA protein–fd single-stranded DNA, and we estimate them to be 10% or less than full-length DNA. This corresponds to clusters of approximately 600 nucleotides in length or less which, although still much larger than our estimate based on the εM13 DNA titrations, is not unreasonably different considering the differences inherent in the electron microscopy and fluorescence methods. In addition, these two experimental techniques differ significantly in the salt concentration ranges employed. In the fluorescence work, the co-operativity parameter is determined for the salt range of 130 to 295 mM-NaCl, whereas all of the electron microscopic work is done in the absence of NaCl in low salt buffers of 10 to 30 mM-Tris·HCl. Although we have shown that ω is independent of NaCl in this higher NaCl range, it is possible that differences might be observed at lower salt concentrations because, for example, the DNA binding properties of T4-coded gene 32 protein at NaCl concentrations less than 50 mM have been shown to be different from those observed at higher salt concentrations (Kowalczykowski *et al.*, 1981*b*). Thus, it is likely that an ω value of 50 is representative of recA protein–DNA binding co-operativity in the range of physiological NaCl concentration.

Another possibility is that the co-operativity parameter for binding to εM13 DNA is different from that of unmodified DNA. Although this possibility cannot be eliminated completely until analogous quantitative studies are performed using unmodified DNA, previous binding studies with gene 32 protein using etheno-modified polynucleotides have shown that, for this protein, the co-operativity parameter is unaffected by the chemical modification (Newport *et al.*, 1981). This is true even though the affinity of gene 32 protein for the etheno-modified polynucleotide is higher than it is for the unmodified polynucleotide, just as is the case for the binding of the recA protein to the two forms of M13 single-stranded DNA. Thus, based on this precedent, it is likely that the properties of the recA protein–εM13 DNA complexes are representative of those formed with unmodified DNA, except that the affinity is higher for the modified DNA.

One of the questions that can be asked regarding the various recA protein-catalyzed reactions is, what is the role of ATP hydrolysis? The results presented here have shown that nucleotide cofactors modulate the affinity of recA protein for single-stranded DNA, with ATP increasing the affinity and ADP decreasing the affinity. In order to place the significance of these qualitative statements into a semi-quantitative physiological context, the values of $K\omega$ for recA protein–εM13 DNA binding, in the presence and absence of ADP, have been extrapolated to 220 mM-NaCl and are presented in Table 4. This salt concentration has been estimated to approximate the effective physiological salt concentration within *E. coli* (Kao-Huang *et al.*, 1977; von Hippel *et al.*, 1982). The reciprocal of $K\omega$ is the free recA protein concentration required for half-saturation of the single-stranded DNA but, due to the co-operative nature of recA protein–single-stranded DNA binding, it can be considered to be approximately equal to the concentration necessary for single-stranded DNA binding to occur. The numbers in Table 4 show that in the absence of any nucleotide cofactor, the recA

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† The effect of dTTP is also to stimulate dissociation of the protein even though it is not hydrolyzed (Weinstock *et al.*, 1981*a*); however, dTTP binding results in an equilibrium destabilization of the recA protein–εM13 DNA complex that is even greater than that induced by ADP (unpublished results).

Table 4

Values of the apparent affinity, Kω, *and its reciprocal, estimated at physiological salt concentration*

	$K\omega$(M^{-1})	$1/K\omega$(μM)
With ADP (100 μM)	$1{\cdot}4 \times 10^4$	71
No cofactor	$3{\cdot}4 \times 10^5$	2·9

Assumed to be 220 mM-NaCl based on Kao-Huang *et al.* (1977) and von Hippel *et al.* (1982).

protein–εM13 DNA complex will form at a free recA protein concentration greater than 3 μM but, if 100 μM-ADP is present, the protein–DNA complexes are unable to form unless the recA protein concentration exceeds ≈70 μM. The basal level of recA protein within *E. coli* has been estimated to be ≈1300 molecules per cell (Sales & Paoletti, 1983), which corresponds to a total concentration of approximately 2 to 3 μM. Thus, if all of the recA protein were "free", the no cofactor recA protein–εM13 DNA complex would be marginally stable, whereas the complex in the presence of ADP would be unable to form under these approximate physiological conditions. Note also that the affinity of the natural single-stranded M13 DNA substrate is less than that of the εM13 modified substrate by at least a factor of ten and probably more. This, in turn, would require that the free recA protein concentrations needed for natural single-stranded DNA binding would be at least tenfold higher than those suggested by Table 4. Therefore, in order to insure that complexes of recA protein and single-stranded DNA will form under these physiological conditions, it is somehow necessary to increase the affinity of recA protein for single-stranded DNA. We would propose that this increase in affinity is accomplished by ATP binding. However, in order to act enzymatically, the bound recA protein must also be able to dissociate from the DNA at a reasonable rate. If the stability of the protein–DNA complex in the presence of ATP (and in the absence of hydrolysis) is comparable to that of the ATP-γ-S complex, then the dissociation half-time would be prohibitively too long. One way to circumvent this "tight binding" dilemma is to hydrolyze the ATP to form ADP, which serves both to destroy the ATP and to produce a release factor that allows dissociation of the bound recA protein. Thus, as mentioned earlier, the ATP hydrolytic event serves to produce ADP, which permits rapid dissociation of the recA protein–DNA complex. This dissociated recA protein is now free to bind at a new DNA site and then to "act" in some, as yet undetermined, way to catalyze the strand assimilation reaction.

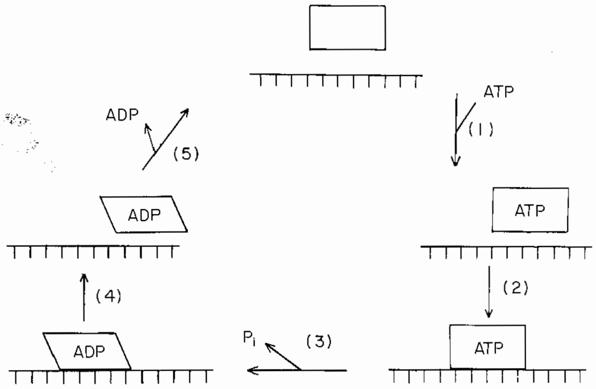

Figure 9. recA protein–single-stranded DNA binding cycle: (1) ATP binding to free recA protein. (2) ATP–recA protein complex binding to DNA. (3) ATP hydrolysis. (4) ADP–recA protein complex dissociation from DNA. (5) ADP dissociation to form free recA protein.

The steps described above constitute a recA protein–single-stranded DNA binding cycle, which is illustrated by Figure 9. Since the no cofactor–recA protein–single-stranded DNA complex is likely to be only marginally stable under physiological conditions, the first step required in the cycle would be the formation of a stable recA protein–ATP–single-stranded DNA complex. This complex could form either by the binding of a recA protein–ATP complex to DNA or by the binding of ATP to the few DNA bound recA protein molecules that would necessarily be present. Although the first possibility is shown in Figure 9 (steps 1 and 2), there is no information to favor either path. The third step involves the hydrolysis of ATP to produce ADP (and inorganic phosphate), which would be necessary in order to release the bound recA protein molecule. In step 4, we show the unstable recA protein–ADP complex dissociating from the DNA, followed by step 5, which is the dissociation of ADP from the recA protein (it is possible that steps 4 and 5 may occur in reverse order, but so far we favor this scheme based on preliminary kinetic work). Thus, one minimal role of ATP hydrolysis is to allow the bound recA protein to recycle on and off single-stranded DNA although, clearly, ATP hydrolysis is likely to play a role in some additional aspects of the strand assimilation process such as unwinding of duplex DNA, searching for homology, or branch migration.

As mentioned above, Figure 9 is a schematic representation of the recA protein–single-stranded DNA binding cycle and complicating (but ultimately essential) features, such as potential parallel association and dissociation pathways as well as the placement and function of other recA protein molecules and duplex DNA, have not been shown. For simplicity, only one recA protein molecule is shown in Figure 9; however, since recA protein binds co-operatively, it would be more appropriate to view the bound recA protein molecule as part of a co-operative cluster of recA protein molecules rather than just an isolated single protein molecule. Based on theoretical considerations, it is more likely, by a factor of ω, that the dissociation of a protein from a co-operatively bound cluster of proteins will occur at the end of a cluster (Kowalczykowski *et al.*, 1980). Thus, the dissociation of a recA protein molecule from the end of a co-operatively bound cluster is 50-fold more likely to occur than dissociation from the middle of the cluster. If the recA protein association–dissociation cycle is polar, with dissociation at one end, and association at the other end of a co-operatively bound cluster, then such a mechanism could account for the observed polarity of branch migration catalyzed by recA protein.

Finally, in addition to being bound to single-stranded DNA, recA protein must also be bound to double-stranded DNA. Thus, rather than viewing the recA protein as dissociating from the single-stranded DNA, it may be more appropriate to think of the single-stranded DNA as dissociating from and reassociating with the recA protein–duplex DNA complex upon ATP hydrolysis, thereby perhaps providing a means of translocating the DNA molecules relative to one another during a homology search process. Clearly, further equilibrium and kinetic work is necessary, and studies in progress will hopefully provide information on these mechanistic questions.

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