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General Mechanism for RecA Protein Binding to Duplex DNA

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RecA protein binding to duplex DNA occurs by a multi-step process. The tau analysis, originally developed to examine the binding of RNA polymerase to promoter DNA, is adapted here to study two kinetically distinguishable reaction segments of RecA–double stranded (ds) DNA complex formation in greater detail. One, which is probably a rapid pre-equilibrium in which RecA protein binds weakly to native dsDNA, is found to have the following properties: (1) a sensitivity to pH, involving a net release of approximately one proton; (2) a sensitivity to salts; (3) little or no dependence on temperature; (4) little or no dependence on DNA length. The second reaction segment, the rate-limiting nucleation of nucleoprotein filament formation accompanied by partial DNA unwinding, is found to have the following properties: (1) a sensitivity to pH, involving a net uptake of approximately three protons; (2) a sensitivity to salts; (3) a relatively large dependence on temperature, with an Arrhenius activation energy of 39 kcal mol^{-1}; (4) a sensitivity to DNA topology; (5) a dependence on DNA length. These results contribute to a general mechanism for RecA protein binding to duplex DNA, which can provide a rationale for the apparent preferential binding to altered DNA structures such as pyrimidine dimers and *Z*-DNA.

1. Introduction

The *Escherichia coli* RecA protein promotes homologous genetic recombination *in vivo* and *in vitro* (for reviews, see Dressler & Potter, 1982; Cox & Lehman, 1987). Its activity depends on DNA binding. *In vitro*, ssDNA‡ binding is necessary to initiate recombination (Shibata *et al.*, 1979*a*,*b*; Cox & Lehman, 1981). RecA protein binds ssDNA co-operatively, forming an extended nucleoprotein filament (Dunn *et al.*, 1982; Flory *et al.*, 1984; Williams & Spengler, 1986). In the presence of ATP, this complex is capable of binding dsDNA, pairing regions of homology, and promoting the exchange of complementary DNA strands (for a review, see Cox & Lehman, 1987). Recombination is not limited to this three-strand reaction. If branch migration continues into a region of two homologous duplexes, a four-strand exchange will result (DasGupta *et al.*, 1981; West *et al.*, 1982). The mechanism of RecA protein-promoted DNA strand exchange is currently not known. Much of homologous recombination *in vivo* is likely to involve four-strand exchanges (Rossignol & Hoedens, 1980). Thus, in these types of reactions, RecA–dsDNA complexes may be the important vehicles of strand exchange.

In addition to homologous genetic recombination, RecA protein plays a central role in regulating the *E. coli* SOS system (for reviews, see Little & Mount, 1982; Walker, 1984). RecA protein stimulates the cleavage of the LexA protein, the repressor of the SOS regulon (Little *et al.*, 1980; Horii *et al.*, 1981). RecA protein also plays a second role in DNA damage repair by facilitating replication across pyrimidine dimers (Ennis *et al.*, 1985). Lu *et al.* (1986) have shown that RecA protein appears to bind preferentially to these dimers. This binding inhibits the 3′→5′ exonuclease activity of DNA polymerase III (Fersht & Knill-Jones, 1983;

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‡ Abbreviations used: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); FI, supercoiled closed circular form of a DNA molecule as isolated from *E. coli* cells; FII, nicked circular form of the same DNA molecule; FIII, linear form of same DNA molecule; FIV, closed circular but relaxed form of the same DNA molecule; FX, closed circular form of the same DNA molecule but underwound by ≥30%; MES, 2(*N*-morpholino) ethanesulfonic acid; bp, base-pairs.

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Lu *et al.*, 1986), allowing replication past the damaged site.

In order further to understand the role of RecA protein in homologous genetic recombination and DNA repair, we have investigated the molecular mechanism of RecA protein binding to dsDNA. We have outlined a multi-step pathway for association (Pugh & Cox, 1987*a*). Identifiable reaction segments† include the following. (1) RecA protein, existing as a monomer or small oligomer, binds ATP and Mg^{2+}. (2) RecA protein interacts weakly with native form dsDNA. This binding is non-specific and the entire DNA is presumed to be available for binding. This low-affinity binding represents a rapid pre-equilibrium with free RecA protein. (3) The low affinity binding is followed by nucleation. At pH values greater than 6, this reaction segment is rate limiting and involves partial DNA unwinding (but not necessarily strand separation) in the vicinity of the bound protein. (4) The nucleated complex is propagated into a nucleoprotein filament by a rapid contiguous addition of RecA protein to its ends. The dsDNA within this complex is underwound by an average of 37% relative to *B*-form DNA (Pugh *et al.*, unpublished results). Under conditions where RecA protein is present in stoichiometric excess, the nucleoprotein filament is stable on FII DNA between pH 5·5 and 7·5, and extends throughout the length of the DNA, hydrolyzing ATP at a steady-state rate ($k_{cat} \approx 22$ to 26 min^{-1}). Reaction segment (1) is based on evidence from the equilibrium dialysis studies of Cotterill *et al.* (1982) and the DNA-independent ATPase activity of RecA protein (Pugh & Cox, 1988). The evidence for reaction segment (2) is largely circumstantial and is based on known properties of the system and precedents established from other DNA-binding proteins. Further justification of this segment is presented below. Segments (3) and (4) and their kinetic properties are based on evidence described in an earlier report (Pugh & Cox, 1987*a*).

For many naturally occurring dsDNAs, free RecA protein requires more than three hours to initiate binding under *in vitro* strand exchange conditions at pH 7·5 (Pugh & Cox, 1987*a*). This kinetic barrier reflects the slow nucleation step. There are many ways to reduce this kinetic barrier. These include reducing the pH, increasing the superhelicity of the DNA, using ATPγS, a non-hydrolyzable ATP analog, and increasing the length of the DNA (Pugh & Cox, 1987*a*). During strand exchange, RecA–ssDNA complexes also promote partial unwinding of a homologous dsDNA, effectively stabilizing the transition state in the strand transfer process (Wu *et al.*, 1983; Schutte & Cox, unpublished results). After strand exchange, RecA protein remains bound throughout the nascent heteroduplex DNA and maintains the DNA in a highly underwound state (Pugh & Cox, 1987*b*). Thus, strand exchange provides another means of circumventing the kinetic barrier in binding. The slow step in binding to dsDNA may also be circumvented by homologous ssDNA fragments. Shibata *et al.* (1982) and Ohtani *et al.* (1982) found that the RecA–ssDNA fragments paired initially with homologous dsDNA, but were eventually released, leaving the duplex "immune" to further pairing. This is consistent with the concept that RecA protein remained bound to the dsDNA. Another entry site onto dsDNA can be provided by ssDNA gaps or tails colinear with the dsDNA (Cassuto & Howard-Flanders, 1986; Shaner & Radding, 1987; Shaner *et al.*, 1987; Lindsley & Cox, unpublished results). RecA protein rapidly and stably binds to ssDNA regions, and may provide a nucleation center for co-operative extension of the RecA–ssDNA filament into the duplex region. Similarly, locally underwound or unwindable regions of dsDNA may provide entry sites for stable dsDNA binding. These include pyrimidine dimers, mismatched base-pairs, *B*/Z-junctions, A+T-rich sequences, cruciforms, and polypurine tracts. Consistent with this is the observation that some of these altered DNA structures are mutagenic and recombinogenic *in vivo* (Rupp *et al.*, 1971; Klysik *et al.*, 1982; Little & Mount, 1982; Walker, 1984; Murphy & Stringer, 1986), and appear preferentially to bind RecA protein *in vitro* (Lu *et al.*, 1986; Blaho & Wells, 1987; Kowalczykowski *et al.*, 1987). This preferential binding can be explained in the context of the general binding mechanism described above.

Binding mechanisms for a number of DNA-binding proteins have been studied in some detail (for reviews, see von Hippel & McGhee, 1972; Kowalczykowski *et al.*, 1981). Many have similar kinetic and thermodynamic properties with respect to DNA binding. These include significant kinetic lags before steady-state or equilibrium is achieved, and sensitivity to small ions, temperature, and DNA composition and topology. The sensitivity to small ions arises from the displacement of anions from the protein and cations from the DNA phosphate backbone upon complex formation. These effects provide information on the details of the association process. The use of salt effects to probe the mechanisms of protein–nucleic acid interaction has been the subject of a number of reviews (Record *et al.*, 1978; Lohman, 1985). For the *E. coli* RNA polymerase, the salt effects on the individual rate constants for promoter binding have been examined in detail (Strauss *et al.*, 1980; Shaner *et al.*, 1983; Roe & Record, 1985; Leirmo *et al.*, 1987). A sensitivity to temperature arises, in many cases, from the enthalpic contribution to DNA helix stability (Record *et al.*, 1981). This has been observed for the phage T4 gene *32* protein, the *E. coli* ssb protein, and the *E. coli* RNA polymerase (von Hippel & McGhee, 1972). At least in the case of RNA polymerase, additional contributions to the temperature dependence arise from a conforma-

† A reaction segment is defined as containing one or more elementary kinetic steps (Radika & Northrop, 1984).

tional change in the protein (Roe *et al.* 1984, 1985). Since partial DNA unwinding is required in the rate-limiting segment of RecA protein binding to dsDNA, the association process might be expected to show a large temperature dependence. Similarly, any condition that reduces the transition temperature for DNA melting would be expected to facilitate the binding of RecA protein to dsDNA.

In this paper, we examine the kinetic properties of the initiation of RecA protein binding to dsDNA in greater detail, to determine the effects of pH, salt, temperature, DNA topology and length on two reaction segments. This association process shares some important features with an analogous system, RNA chain initiation by the *E. coli* RNA polymerase (for a review, see McClure, 1985). Kinetic equations (the tau analysis) have been derived for RNA polymerase–promoter interactions that allow quantitative separation of weak promoter binding and isomerization into a stable, transcriptionally active complex (McClure, 1980). The first of these steps exhibits a dependence on RNA polymerase concentration. The second does not. The activity of the final complex (abortive initiation) is used as an assay to measure the rates of these binding steps. The RecA-dsDNA binding reaction also has a kinetically accessible step (probably a weak binding interaction with dsDNA; segment 2 above), which exhibits a dependence on RecA protein concentration followed by a rate-limiting step (nucleation; segment 3), which does not. The activity of the final complex (ATP hydrolysis) can be used to measure the rates of these steps. The tau analysis is adapted and presented here to examine quantitatively these steps in RecA protein binding to unmodified plasmid DNA. This simplified system provides a useful starting point at which to examine the kinetics of RecA protein binding to DNA containing structural variations that may affect recombination.

2. Materials and Methods

(a) *Reagents*

RecA protein was purified to homogeneity as described (Cox *et al.*, 1981). The concentration of RecA protein in stock solutions was determined by absorbance at 280 nm, using an extinction coefficient of $\varepsilon_{280} = 0{\cdot}59\ A_{280}$ mg^{-1} ml (Craig & Roberts, 1981). The turnover number or k_{cat} for ATP hydrolysis in the presence of excess ϕX174 ssDNA at pH 6·8 was 28 min^{-1}. All preparations used were proficient in DNA strand exchange *in vitro*, and contained no detectable nuclease activity. The $8{\cdot}7 \times 10^3$ bp plasmid pBR322-2 (a dimer of pBR322) and its purification have been described (Pugh & Cox, 1987*a*). The concentrations of dsDNA stock solutions were determined by absorbance at 260 nm, using 50 μg ml^{-1} $A_{260}{}^{-1}$ as a conversion factor. FII DNA was prepared from FI DNA using DNase I, according to the method of Shibata *et al.* (1981). More than 99% of the DNA was present as nicked circles, as determined from scanning densitometry of photographic negatives of DNA samples electrophoresed onto agarose gels and stained with ethidium bromide. This method generates FII DNA containing a single nick. Pyruvate kinase, lactic dehydrogenase, DNase I, NADH, phosphoenolpyruvate, MES, glutamic acid, and ATP were purchased from Sigma. Restriction endonucleases were purchased from New England Biolabs.

(b) *Reaction conditions*

Except where noted, all reactions were performed in MES-NaOH buffer in which the ionized form of the buffer was always 50 mM. Different pHs were obtained by varying the concentration of the neutral form. The pH values reported here were determined at 25 °C. Except where noted, reactions contained, in addition to MES-NaOH buffer, 10 mM-$Mg(CH_3COO)_2$, 1 mM-ATP, 10 μM-pBR322-2 FII dsDNA (measured in nucleotide bases), 1 mM-dithiothreitol, 0·1 mM-EDTA, 2% (v/v) glycerol, ATP-regenerating system (4·5 units pyruvate kinase ml^{-1}, 4·5 units lactic dehydrogenase ml^{-1}, 3 mM-NADH, 5 mM-phosphoenolpyruvate, 1 mM-KCl), and the indicated RecA protein concentrations. All reactions were performed in a volume of 0·5 ml in 0·5 cm path-length, self-masking, quartz cuvettes at 37 °C. Reactions were initiated by the addition of RecA protein after all other components were equilibrated to the final temperature. The integrity of the DNAs used in these experiments were checked before and after each experiment. No detectable degradation of the DNA occurred over the course of these experiments.

(c) *ATPase assay*

Absorbance measurements were obtained on a Perkin–Elmer Lambda 7 double-beam recording spectrophotometer equipped with 2 6-position, thermojacketed cuvette holders attached to a constant temperature water circulator. Cell path length and bandpass were 0·5 cm and 2 nm, respectively. The spectrophotometer and the coupled-enzyme assay for ATP hydrolysis are described in detail elsewhere (Morrical *et al.*, 1986; Pugh & Cox, 1987*a*, 1988). In brief, regeneration of ATP from ADP and phosphoenolpyruvate is coupled to the conversion of NADH to NAD^+, which can be monitored spectrophotometrically by a decrease in absorbance at 380 nm. Due to the high concentration of NADH used in this study, absorbances were measured at 380 nm, instead of 340 nm (its absorbance maximum), so as to remain in the linear region of the spectrophotometer. High concentrations of NADH were necessary to ensure that a steady-state (endpoint) was reached under all conditions. No component of the coupling system limited the observed rate of ATP hydrolysis. ATP is constantly regenerated in this system so that no product accumulation occurs. An extinction coefficient of $\varepsilon_{380} = 1210$ M^{-1} cm^{-1} for NADH was used to obtain steady-state velocities of ATP hydrolysis. All data were fit by linear regression and the standard deviations reported in the text.

3. Model

(a) *A two segment equation for initiation of RecA protein binding to dsDNA*

To put the initial events of this DNA binding reaction in perspective, we first consider the final product. This is a stable nucleoprotein filament within which the dsDNA is extensively underwound. This complex hydrolyzes ATP and is stabilized by co-operative interactions between

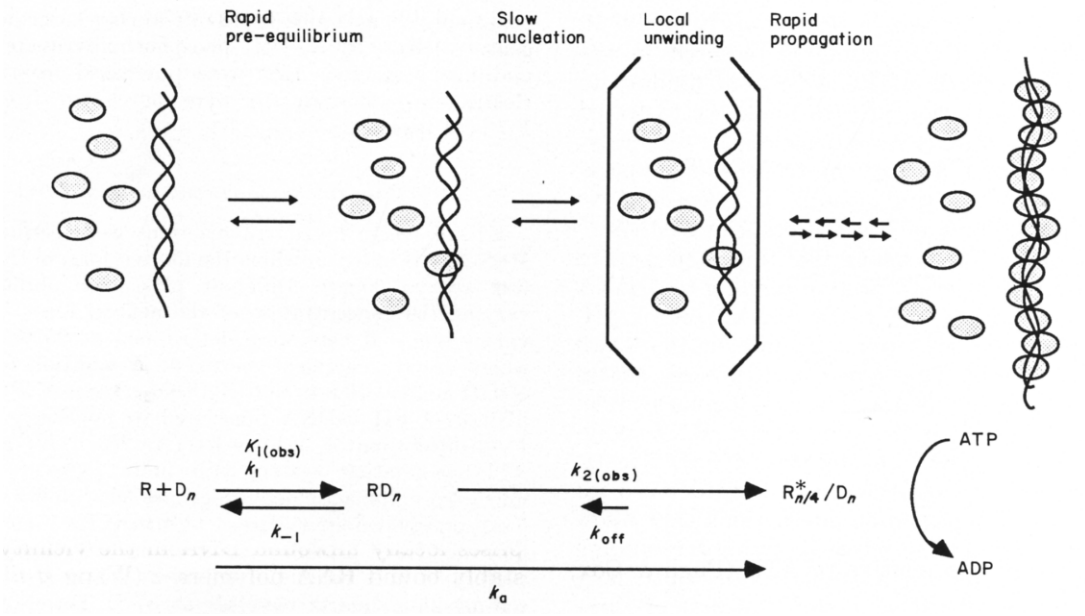


Figure 1. A 2-segment reaction mechanism to RecA protein binding to dsDNA. For details, see the text.

RecA monomers. The addition of each RecA binding unit to a growing complex involves both DNA binding and partial unwinding of the DNA bound. The co-operativity within the complex establishes that the kinetic parameters describing the first binding event will be different from those describing the subsequent binding events. Since subsequent binding (propagation) is fast relative to this first binding event, only the initial binding event is kinetically observed in this study. Under the conditions used in this study, the formation of a complete nucleoprotein filament cannot be separated from the nucleation event, so that each nucleation event is directly manifested by the activity of the final complex.

The features of the initial binding event that are important to this study are summarized in Figure 1. In this model, the initial binding event is reduced to two kinetically accessible reaction segments: a rapid pre-equilibrium and nucleation. Here, RecA protein (R) refers to a single nucleating binding unit, which may be a single polypeptide or a small aggregate (relative to the final nucleoprotein filament) of RecA protein. RecA protein binds MgATP in the absence of DNA (Cotterill *et al.*, 1982; Pugh & Cox, 1988). MgATP is required for stable dsDNA binding and so R represents RecA protein with MgATP bound. D_n refers to dsDNA n base-pairs in length. The rapid pre-equilibrium is proposed between $R+D_n$ and RD_n, and is described by $K_{1(obs)}$, where $K_{1(obs)} = k_1/k_{-1}$. RD_n represents RecA protein bound with low affinity and non-specifically along the DNA lattice; the DNA is not underwound, and no ATP hydrolysis occurs. Nucleation and propagation follow, and together are described by the net rate constant $k_{2(obs)}$. On a homogeneous lattice, nucleation can occur potentially at any of n overlapping sites. Nucleation is rate limiting and involves partial unwinding of the DNA in the vicinity of the bound RecA protein to form R^*D_n. This is followed by a rapid addition of RecA protein to the end of the nucleation complex, propagating along the DNA lattice to form a nucleoprotein filament $R^*_{n/4}D_n$, containing $n/4$ RecA monomers. Each segment of the reaction may have one or more elementary kinetic steps, but this simple pathway explains adequately the fundamental kinetic behavior of the system as described below. Since nucleation is rate limiting overall, only the association of the first RecA binding unit can be examined kinetically. Under pseudo-first-order conditions, where the total RecA protein concentration is much greater than the total concentration of steady state binding sites, the reaction is maintained far to the right at steady state. On FII DNA, the nucleoprotein filament represents a stable complex in which the observed net rate constant for depolymerization of the entire filament, k_{off}, is negligible (Pugh & Cox, 1987*a*).

(b) *Justification for the order of events*

Evidence for many features of the model illustrated in Figure 1 has been presented elsewhere (Pugh & Cox, 1987*a*). This and additional available evidence for the order of binding, partial DNA unwinding, propagation, and other events is summarized here.

(1) DNA binding precedes filament formation. Under optimal conditions for RecA protein association with dsDNA (ATP, Mg^{2+}, pH 6), RecA protein does not exist as long protein filaments in the absence of DNA, although relatively small oligomers might escape detection by the method used and may be present (McEntee *et al.*, 1981; Cotterill & Fersht, 1983; Pugh & Cox, 1987*a*; Takahashi *et al.*, 1986). Filamentation in the

presence of DNA occurs with kinetics indistinguishable from DNA binding (Pugh & Cox, 1987*a*). From these observations it is concluded that formation of long filaments occurs subsequent to, or concurrently with, the initial binding event.

(2) The initiation of DNA unwinding precedes filament formation. No DNA-dependent filaments (measured by light-scattering) are observed in the absence of partial DNA unwinding (Pugh & Cox, 1987*a*). The two processes appear to be tightly coupled. Evidence presented in this paper and previously (Pugh & Cox, 1987*a*) shows that a reaction step involving partial DNA unwinding (nucleation) is distinctly rate limiting.

(3) The initiation of stable binding and DNA unwinding is preceded by weak DNA binding of RecA protein. This is based on the observation that, at pH 7·5, ATPγS stimulates the coupled DNA binding and unwinding reaction by almost an order of magnitude relative to ATP (Pugh & Cox, 1987*a*). At pH 7·5, in the presence of ATP instead of ATPγS, association is relatively independent of RecA protein concentration (above saturating levels). Thus, ATPγS appears to act by increasing the rate of the reaction step that is independent of RecA protein concentration. Since there is no reason to believe that ATPγS affects DNA structure, it is inferred that RecA protein binds to DNA with low affinity prior to nucleation and induces the partial unwinding of the DNA. On this basis, it would appear that RecA protein binding is not limited to the trapping of spontaneously unwound DNA, as is found with many helix destabilizing proteins. The binding of RecA protein to dsDNA without concomitant DNA unwinding has been detected by Dunn *et al.* (1982) using electron microscopy. Cotterill *et al.* (1982) have observed that dsDNA affects the intrinsic affinity of RecA protein for various nucleotide cofactors under conditions (pH 8·1) where the initiation of stable DNA binding and unwinding does not readily occur with RecA protein. From this we postulate that binding to native form DNA occurs with very low affinity, forming a rapid pre-equilibrium before nucleation. Such a reaction segment has been proposed in a number of previous reports (Dunn *et al.*, 1982; Pugh & Cox, 1987*a*; Kowalczykowski *et al.*, 1987).

(4) A complex of RecA protein, dsDNA, ATP and Mg^{2+} is required at the rate-limiting step. Pre-incubation of any three of these components for an extended period does not reduce tau, which is defined in this system as the observed lag in ATP hydrolysis at a given RecA protein concentration, when the fourth component initiates the reaction (Pugh & Cox, 1987*a*)†. The observation that tau is affected by DNA properties such as superhelicity and length argues strongly against a slow conformational change in RecA protein prior to binding DNA (Pugh & Cox, 1987*a*).

Since tau decreases with increasing DNA length (Pugh & Cox, 1987*a*; this work), it is assumed that nucleation can occur, to a first approximation, anywhere along the DNA lattice. However, the real probability of nucleation at a given site is likely to be influenced by the local helix structure, composition, and thermal stability.

(c) *The tau analysis*

The abortive initiation assay, developed by McClure (1980), has been used to examine promoter site selection and open complex formation for *E. coli* RNA polymerase. The open complex comprises locally unwound DNA in the vicinity of the stably bound RNA polymerase (Wang *et al.*, 1977; Hsieh & Wang, 1978; Gamper & Hearst, 1982). When RNA chain synthesis is limited to the first few nucleotides of the template, the complex aborts synthesis, releases the oligonucleotide, and reinitiates chain synthesis (McClure, 1980; Cech & McClure, 1980; Hawley & McClure, 1980, 1982). Under the abortive initiation conditions, RNA polymerase does not dissociate out of the open complex to reinitiate chain synthesis, but remains as an open initiation complex that synthesizes oligonucleotides at a steady-state rate. The lag observed in achieving steady-state oligonucleotide synthesis is a measure of the rate of open complex formation.

Many individual kinetic steps are presumably involved in the weak binding of RNA polymerase at the promoter, and subsequent isomerization into a transcriptionally active open complex (Hawley & McClure, 1982; Roe *et al.*, 1984, 1985). Roe *et al.* (1985) have established a three-step mechanism for open-complex formation, with an intermediate detected between formation of the closed and open complexes. However, the fundamental kinetics can be explained satisfactorily by grouping the elementary steps into two reaction segments according to the scheme of McClure (1980):

$$R + P \underset{k_{-1}}{\overset{K_1}{\underset{k_1}{\rightleftarrows}}} RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} RP_o \overset{NTPs}{\searrow}_{RNA}, \qquad (1)$$

where R (for eqns (1) and (2) only) is unbound RNA polymerase, P is the uncomplexed promoter, RP_c is the intermediate closed promoter complex, and RP_o is the transcriptionally active open complex. K_1 ($=k_1/k_{-1}$) is the apparent association equilibrium constant for formation of RP_c; k_2 and k_{-2} are the isomerization rate constants for the forward and back reaction, respectively. For many promoters R + P and RP_c are in rapid pre-equilibrium, while k_2 is the rate-limiting step and k_{-2} is small relative to k_2 (McClure, 1980; Hawley *et al.*, 1982). McClure has derived an equation that relates the time lags (tau) in the approach to steady-state oligonucleo-

† When ATP is used to initiate the reaction, the lag is increased somewhat and the steady-state velocity is decreased. This is due to a slow, pH-dependent, irreversible precipitation of the protein in the absence of ATP at low pH.

tide production in the abortive initiation assay to the kinetic parameters described above:

$$\text{tau} = \frac{1}{k_2} + \frac{1}{k_a\,[\text{R}]}, \qquad (2)$$

where k_a ($=K_1k_2$) is the apparent second-order association rate constant. The validity of this analysis is based on four assumptions (McClure, 1980); (1) RP_c is at steady state, (2) reactions are performed under pseudo-first order conditions of excess RNA polymerase (total [R]≫total [P]), (3) k_{-2} is very small, and (4) $k_{-1} \gg k_2$. These requirements are met, to a first approximation, for RNA polymerase with certain promoters.

The same analysis is adapted here to examine the association of RecA protein with dsDNA. We have shown that ATP hydrolysis is a valid measurement of stable DNA binding and unwinding by RecA protein (Pugh & Cox, 1987*a*). As described below, a significant lag exists before steady-state ATP hydrolysis is observed (Pugh & Cox, 1987*a*; Kowalczykowski *et al.*, 1987). This lag can be related to the kinetic parameters of Figure 1 by equation (2), where [R] denotes RecA protein concentration. In the kinetic scheme above, the final RecA–dsDNA complex is analogous to the RP_o state for RNA polymerase. The steady-state rate of ATP hydrolysis promoted by the complex provides a simple method for quantifying complex formation, just as abortive initiation is used to quantify RP_o.

(d) *Criteria for tau analysis*

To adapt McClure's tau analysis in this system, the following assumptions are made and justified.

(1) RD_n is at steady state.

(2) The total RecA protein concentration approximates the free RecA protein concentration. This condition is achieved by using high concentrations of RecA protein such that pseudo-first-order kinetics are observed. Since RecA protein binds the entire duplex at a density of one polypeptide per 4 bp (Pugh & Cox, 1987*a*), the total concentration of steady-state DNA binding sites is 1/4 of the total concentration of nucleotide base-pairs. For the experiments presented in this paper, the concentration of steady-state DNA binding sites is 1·25 μM (5 μM bp or 10 μM bases). Tau plots are constructed with RecA protein concentrations ranging from 4 to 40 μM (3.2- to 32-fold excess).

(3) k_{off}, the observed net rate constant for depolymerization of the entire filament through k_{-2}, is very small. k_{-2} is defined as the rate constant for dissociation of the last RecA protein binding unit present in a decaying complex, $R^*D_n \rightarrow RD_n$. The validity of this assumption is based on experiments (Pugh & Cox, 1987*a*) in which RecA–dsDNA complexes were shifted from near pH 6·0 to pH 7·5, where initiation of binding is extremely slow. No changes in the steady-state level of binding was observed, indicating that complexes formed on FII DNA are stable and that complete dissociation occurs at a negligible rate. The value of k_{off} may be determined by the relative rates of polymerization and depolymerization of the filament and so is likely to be defined by the concentration of RecA protein. Maintaining high levels of the protein helps to ensure that k_{off} is small. It is important to note that elementary rate constants for dissociation of RecA monomers or small units of RecA protein may be significant. Rapid association and dissociation of individual RecA protein binding units from the final complex would not be detected here. If this occurs, it reflects a property of the final complex at steady state and does not contribute to the observed kinetics of complex formation.

(4) RD_n is in a rapid pre-equilibrium with $R + D_n$, where $k_1[R]$, $k_{-1} \gg k_{2(obs)}$. This assumption is justified above and is a reasonable expectation, on the basis of the observation that RecA protein associates rapidly (on the time scale of $k_{2(obs)}$) with ssDNA (Pugh & Cox, 1987*a*), underwound dsDNA (Pugh & Cox, 1987*a*; this study), functional DNA analogs (Chabbert *et al.*, 1987; Pugh & Cox, 1988), and RecA–dsDNA filaments (Pugh & Cox, 1987*a*). This weak binding has not been measured directly. It is inferred that k_{-1} must be large, resulting in a small $K_{1(obs)}$.

(5) Nucleation is slow relative to propagation. This assumption is strongly supported by two observations (Pugh & Cox, 1987*a*). (1) In the presence of saturating concentrations of RecA protein, intermediates in the DNA unwinding process cannot be detected during the lag period; the DNA appears to be either completely bound or completely unbound. (2) The time lag decreases with increasing DNA length (n). This is expected if nucleation is rate limiting. With longer DNAs there are more potential nucleation sites (n). This is also consistent with the observation by Kowalczykowski *et al.* (1987) that the lag in binding is reduced by increasing the temperature of the reaction or by increasing the A + T content of the DNA.

This is perhaps the key factor that permits the use of the McClure analysis in this RecA–dsDNA binding reaction. The analysis is not complicated by the size of the final RecA nucleoprotein filament or by co-operativity within it. This is because the formation of this final complex is kinetically indistinguishable from the first binding event in the association pathway. Co-operativity plays little or no role in this nucleation event (this study), which governs the overall process.

(6) The observed velocity of ATP hydrolysis is proportional to the final state of DNA binding. The validity of this statement has been established (Pugh & Cox, 1987*a*).

(e) *ATP hydrolysis as a means for measuring tau*

Under solution conditions that are optimal for DNA strand exchange, RecA protein is, to a first

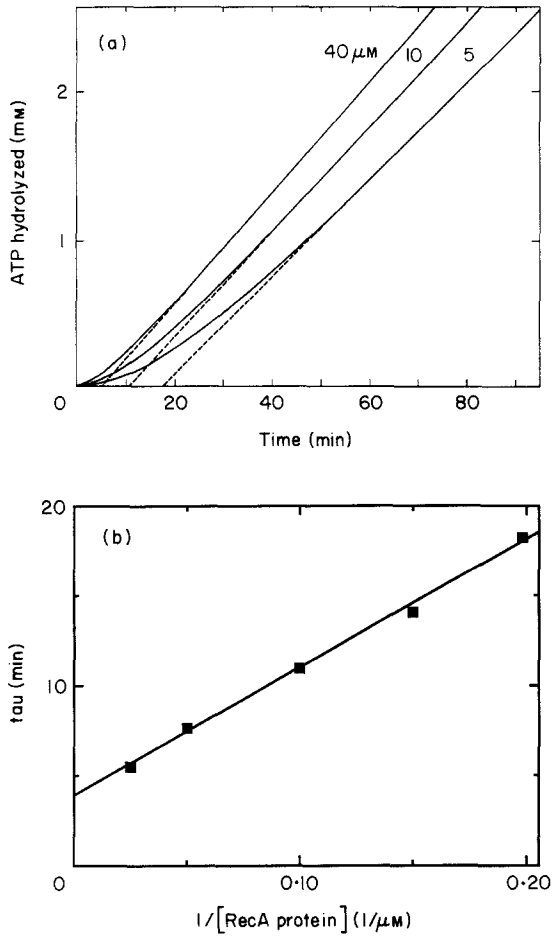


Figure 2. Time lags in ATP hydrolysis by RecA protein. (a) Reactions were performed at pH 6·26 (52% anion) and contained 10 μM-nicked circular FII dsDNA (measured in nucleotide bases), 1 mM-ATP, 10 mM-$Mg(CH_3COO)$. Reactions were initiated by the addition of the indicated concentration of RecA protein. For a more detailed description of conditions, see Materials and Methods. (b) The time lag (tau) in the approach to steady-state ATP hydrolysis is plotted as a function of the reciprocal of the total RecA protein concentration.

approximation, a DNA-dependent ATPase. A small but detectable amount of DNA-independent ATP hydrolysis is present (Weinstock *et al.*, 1981*a*,*b*; Pugh & Cox, 1988). The DNA-independent reaction proceeds at a steady-state rate and is proportional to the RecA protein concentration under these conditions. Under the standard reaction conditions employed in this study, the turnover number or k_{cat} for DNA-independent ATP hydrolysis at pH 6·5 was 0·12(±0·02) min^{-1}. This activity represents less than 1% of that measured in the presence of DNA. Under the higher salt conditions used in this study, the DNA-independent k_{cat} ranged from 0·2 to 0·5 min^{-1}, depending upon the salt type and concentration. For a more detailed description of this phenomenon, (see Pugh & Cox, 1988). Steady-state velocities measured in the presence of DNA were corrected for DNA-independent ATP hydrolysis. DNA-independent ATP hydrolysis reaches steady-state without a detectable lag. Thus, tau values obtained in the presence of DNA required no correction.

Once RecA protein has saturated the DNA lattice, the amount of ATP hydrolyzed increases linearly with time. Figure 2(a) shows a typical time-course of ATP hydrolysis in the presence of dsDNA and excess RecA protein at pH 6·26. When corrected for the small amount of DNA-independent ATP hydrolysis, the steady-state velocity of the DNA-dependent ATP hydrolysis remained constant over a wide range of excess (3 to 32-fold) RecA protein. The observed steady-state velocity is limited only by the DNA concentration. All other components are present in excess, representing pseudo-first-order conditions. The k_{cat} for ATP hydrolysis in the experiment of Figure 2(a) was 26·4(±0·5), min^{-1}, assuming a binding density of one RecA monomer per 4 bp.

As shown in Figure 2(a), significant lags are observed before steady-state ATP hydrolysis is achieved, even in the presence of a vast excess of RecA protein. With increasing concentrations of RecA protein the lag to steady-state decreased. The time lag (tau) in this system is determined by extrapolating the linear steady-state rate back to the x-axis where zero ATP is hydrolyzed. The time value at the intersection with the x-axis is tau, and it represents the average time required to form a complete RecA–dsDNA nucleoprotein complex. This statement is corroborated by kinetic studies using DNA unwinding, DNase I protection, and 90° light-scattering as assays for DNA binding (Pugh & Cox, 1987*a*). When tau was plotted as a function of the reciprocal RecA protein concentration according to equation (2), a straight line was obtained with a finite y-intercept (tau intercept) (Fig. 2(b)). The equation for this line, in units of μM and minutes, is:

$$\text{tau} = (3{\cdot}8 \pm 0{\cdot}1) + (71 \pm 3)\,[\text{RecA protein}]^{-1}.$$

The reciprocal of the tau intercept is $k_{2(obs)}$, the reciprocal of the slope is k_a, and $K_{1(obs)} = k_a/k_{2(obs)}$. The tau plot shown in Figure 2(b) is for the association of RecA protein with dsDNA at pH 6·26. Under these conditions, $k_a = (1{\cdot}4 \pm 0{\cdot}1) \times 10^4\ M^{-1}\ min^{-1}$, $k_{2(obs)} = 0{\cdot}25(\pm 0{\cdot}01)\ min^{-1}$, and $K_{1(obs)} = (5{\cdot}6 \pm 0{\cdot}4) \times 10^4\ M^{-1}$. The experiments in this study were performed in the pH range 6·0 to 6·7 so as to provide a convenient measure of tau. At pH values near 7·5, tau was too large (hours) to measure accurately. Also, the dependence on RecA protein concentration that is reflected by $K_{1(obs)}$ is evident only at pH values near 6. The linearity of the tau plot as a function of the reciprocal RecA protein concentration indicates that binding is first order with respect to RecA protein concentration, suggesting that little or no co-operativity exists in the initial binding event.

ATP hydrolysis was measured over a range of DNA concentrations from 4 μM to 10 μM in the presence of excess RecA protein (not shown). The steady-state velocities were proportional to DNA concentration. However, the tau values showed no

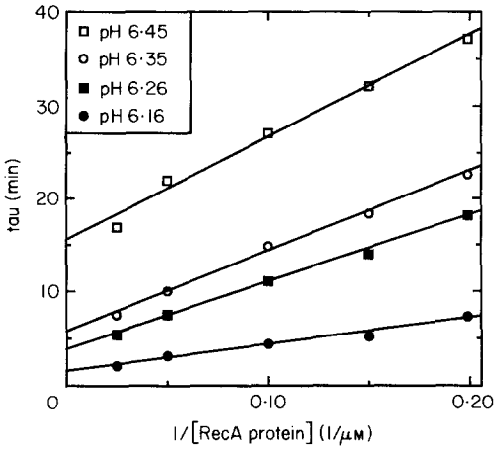


Figure 3. Tau plots as a function of pH. Reactions were performed as described in Fig. 2 and in Materials and Methods, except that the pH was varied as indicated. In this experiment, each pH value is precise to within 0·005 unit. Each pH value is accurate to within 0·05 unit of the indicated values, when measured on different days or with fresh buffer.

significant dependence on DNA concentration. This is expected, since tau measures the average length of time to form a single RecA–dsDNA complex.

The average error in the data throughout this study was less than 10% when reproduced using the same reagents on the same day. In experiments performed on different days or using different preparations of RecA protein and other reagents, the error was 10 to 20%. This error is most likely due to the high sensitivity of tau to pH, temperature, ion concentration, etc. (this study), all parameters that might vary slightly in these complicated reaction mixtures from one set of experiments to the next. Therefore, the accuracy of the kinetic constants determined here are considered to be approximate. However, each set of experiments shown were performed with the same set of reagents on the same day so that trends within an experiment could be quantified. These trends were highly reproducible under a variety of solution conditions. The standard deviations within a set of experiments are reported.

4. Results

(a) *pH effect*

The stable association of the first RecA protein binding unit with dsDNA is pH dependent, with the process requiring the net uptake of approximately two protons (Pugh & Cox, 1987*a*). To determine the effect of pH on individual reaction segments, lags in achieving steady-state ATP hydrolysis were measured over a convenient pH range (pH 6·0 to 6·7), and tau plots were constructed. As shown in Figure 3, the tau plots were linear and showed a sensitivity to pH. The tau-intercepts and slopes were significantly increased by small increases in pH. Thus, $k_{2(obs)}$ and

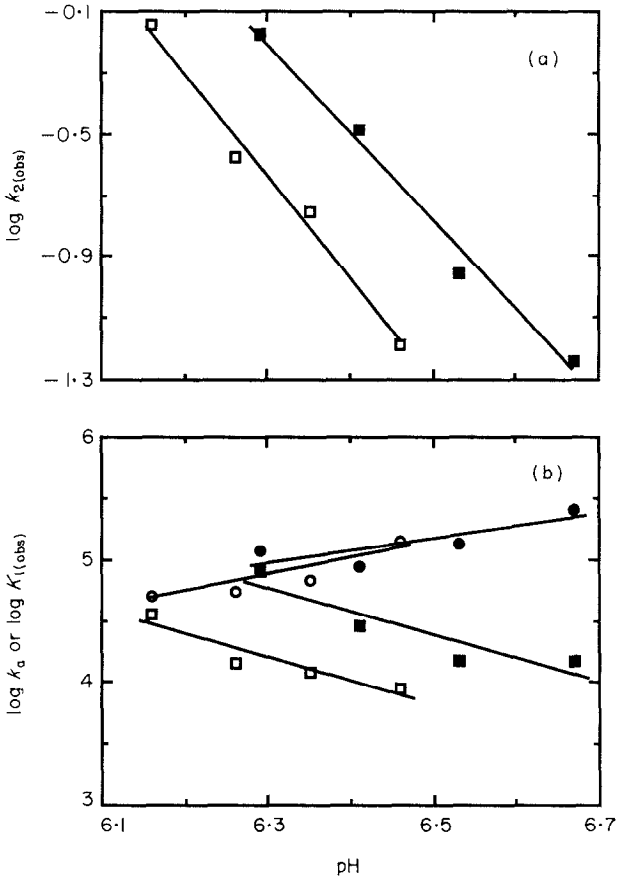


Figure 4. Dependence of $k_{2(obs)}$, k_a, and $K_{1(obs)}$, on pH. Eqn (2) and the data from Fig. 3 were used to obtain $k_{2(obs)}$, k_a, and $K_{1(obs)}$ in MES-NaOH buffer (open symbols). The experiment of Fig. 3 was repeated using MES-KOH buffer and the kinetic parameters obtained from it are plotted here (filled symbols). The log of each is plotted as a function of pH. In (b) the symbols are □, ■, for log k_a, and ○, ● for log $K_{1(obs)}$.

k_a both contain at least one pH-dependent step. The k_{cat} for ATP hydrolysis showed no significant change over this pH range. In these experiments, the measured k_{cat} was 26(±1) min^{-1}.

The net change in the number of protons involved in each segment of the reaction can be determined by plotting the logarithms of the various kinetic rate constants or equilibrium constants as a function of the pH (Record *et al.*, 1978). In Figure 4(a), log $k_{2(obs)}$ is plotted as a function of pH under two ionic conditions. One set of observations was made in the presence of 50 mM-Na^+ and the other in the presence of 50 mM-K^+. Over the pH range tested, the slope appeared to be the same for each set of experiments. The equation for the average of the two lines is:

$$\log k_{2(obs)} = 19(\pm 2) + 3{\cdot}1(\pm 0{\cdot}3) \log [H^+].$$

The slope of the line 3·1(±0·3) indicates that approximately three protons are taken up by RecA protein and/or dsDNA during the nucleation segment under these conditions. The linearity of the plot asserts that the pK_a for each of the functional groups protonated lies below 6.

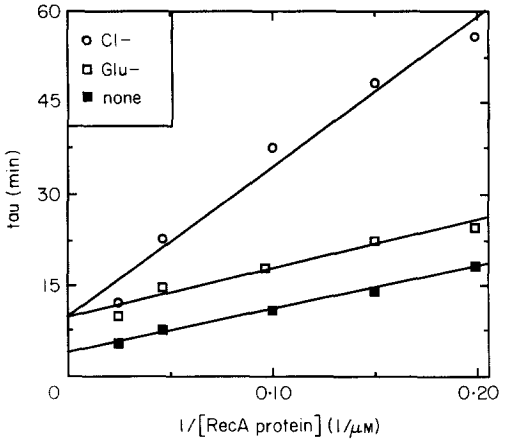


Figure 5. Tau plots as a function of salt concentration and type. Reactions were performed at pH 6·26 as described in Fig. 2 and Materials and Methods, except that 150 mM-NaCl or NaGlu was included in the reactions as indicated.

To determine the net change in protons bound through the two segments of the association reaction, log k_a was plotted as a function of pH as shown in Figure 4(b). The equation of the average of the two lines is:

$$\log k_a = 16(\pm 1) + 1{\cdot}9(\pm 0{\cdot}1) \log [H^+].$$

The same relationship was obtained when 90° light-scattering was used to measure DNA binding (data not shown). Thus, it appears that a net uptake of approximately two H^+ is required for the overall association of a single RecA protein binding unit (one or more monomers) to an isolated DNA site under these conditions.

From the relationship $k_a/k_{2(obs)} = K_{1(obs)}$, $K_{1(obs)}$ was determined and plotted as a function of pH (Fig. 4(b)). The equation for the line is:

$$\log K_{1(obs)} = -3(\pm 2) - 1{\cdot}2(\pm 0{\cdot}4) \log [H^+].$$

Over this pH range, the data indicate that approximately one H^+ is released in forming the weak pre-nucleation complex.

(b) *Salt effect*

As with most DNA-binding proteins, DNA binding by RecA protein is sensitive to the ionic environment. This sensitivity can be used to provide molecular details on this binding interaction. Protein–nucleic acid interactions studied *in vitro* are performed typically in solutions containing NaCl. It has been demonstrated recently that glutamate is a more physiologically relevant anion than choride (Richey *et al.*, 1987). Glutamate and acetate dramatically enhance protein–nucleic acid interactions *in vitro* relative to chloride (Leirmo *et al.*, 1987). This enhancing effect is observed with RecA protein binding to ssDNA (Menetski & Kowalczykowski, 1985; Roman & Kowalczykowski, 1986; Kim & Cox, unpublished results). To assess the effects of salt on the association of RecA protein with dsDNA, ATP hydrolysis was examined at various RecA protein concentrations under pseudo-first-order conditions, as described above, in the presence and absence of 150 mM-NaCl or NaGlu. Tau plots for these reactions are shown in Figure 5.

Table 1

Kinetic parameters for RecA protein binding to dsDNA under various conditions

Condition	k_a ($\times 10^{-4}$) (M^{-1} min^{-1})	$k_{2(obs)}$ (min^{-1})	$K_{1(obs)}$ ($\times 10^{-5}$) (M^{-1})	k_{cat} (min^{-1})
[Ion] and type†				
50 mM-Na^+	1·4±0·1	0·25±0·02	0·6±0·1	26±1
200 mM-Na^+, 150 mM-Cl^-	0·4±0·0	0·10±0·05	0·4±0·1	12±0
200 mM-Na^+, 150 mM-Glu^-	1·2±0·2	0·10±0·02	1·2±0·3	20±1
Temperature‡				
30·7 °C	1·0±0·1	0·04±0·00	2·2±0·3	14±0
33·7 °C	1·2±0·1	0·09±0·01	1·4±0·2	19±0
36·9 °C	2·9±0·2	0·16±0·01	1·8±0·3	24±2
Topology‡				
FI	6·6±0·6	0·26±0·01	2·6±0·3	15±2
FII	2·9±0·2	0·16±0·01	1·8±0·2	24±2
DNA length (average)†				
436 bp	1·1±0·1	0·05±0·00	2·0±0·3	23±2
1454 bp	1·6±0·2	0·08±0·01	2·0±0·3	21±2
4360 bp	1·7±0·2	0·14±0·01	1·2±0·2	23±2
8720 bp	2·9±0·2	0·16±0·01	1·8±0·2	24±2

The kinetic parameters are derived from the data shown in the Figures for a particular condition. See Materials and Methods for reaction conditions.

† Each reaction also contained 50 mM-MES^-, 20 mM-CH_3COO^-, and 10 mM-Mg^{2+}. Except for the phosphenol pyruvate, all ionic species were present at concentrations less than 2 mM. Reactions were performed at pH 6·26±0·00 (measured at 25 °C, 52% anion).

‡ Reactions were performed at pH 6·50±0·04 (measured at 25 °C, 66% anion).

Three salt effects are apparent. (1) The tau intercept is increased with the addition of 150 mM of either salt. (2) The tau intercept in the presence of 150 mM-NaCl does not appear to be significantly different from that in the presence of 150 mM-NaGlu. The error in the data does not allow small effects to be detected. (3) The slope of the plot appears to be significantly increased in the presence of added NaCl but not that of NaGlu.

Salt concentrations and evaluation of the kinetic parameters are presented in Table 1. NaCl appeared to decrease k_a more than NaGlu. Both salts appeared to decrease $k_{2(obs)}$ similarly, relative to their absence. However, NaCl did not appear to affect $k_{1(obs)}$ while NaGlu appeared to increase it. When other salts were tested at a single RecA protein concentration, all produced an increase in tau (Table 2). Tau increased more with certain salts than with others in the following pattern: NaCl, $KCl > NH_4Cl$, $K(CH_3COO) >$ NaGlu > no salt. This sequence generally follows the Hofmeister series (von Hippel & Schleich, 1969; Jencks, 1969; Arakawa & Timasheff, 1982; Leirmo *et al.*, 1987).

The apparent k_{cat} for DNA-dependent ATP hydrolysis was also salt sensitive. NaCl reduced the k_{cat} dramatically, while NaGlu caused only a small reduction relative to its absence (Tables 1 and 2). With other salts, the reduction in k_{cat} (relative to its absence) followed the pattern: NaCl, $KCl > NH_4Cl$, $K(CH_3COO) >$ NaGlu > no salt (Table 2). This apparent reduction in k_{cat} could be a consequence of a direct effect of the salt on the ATP turnover on the enzyme. Alternatively, the salts could be shifting the DNA-binding equilibrium of RecA protein, causing a decrease in binding density and thereby indirectly reducing the k_{cat} . If the binding density is adversely affected, then increasing the RecA protein concentration should cause an increase in RecA protein binding to the DNA and an increase in the measured k_{cat}. When tested with up to a 32-fold stoichiometric excess of RecA protein, no change in the apparent k_{cat} was observed with any of the salts tested. This suggested that the various salts (up to a 150 mM) affected the ATPase activity directly.

Table 2

Tau and k_{cat} *in the presence of various types of salts*

Salt	tau (min)	k_{cat} (min^{-1})
−†	9	29
NaGlu	22	23
NH_4Cl	31	20
$K(CH_3COO)$	32	20
NaCl	40	16
KCl	41	16

Reactions were performed at pH 6·5, in the presence of 10 μM-FII dsDNA, 20 μM-RecA protein and 100 mM of the indicated salt, under the ionic conditions described in Table 1.

† No additional salt added above the standard solution conditions.

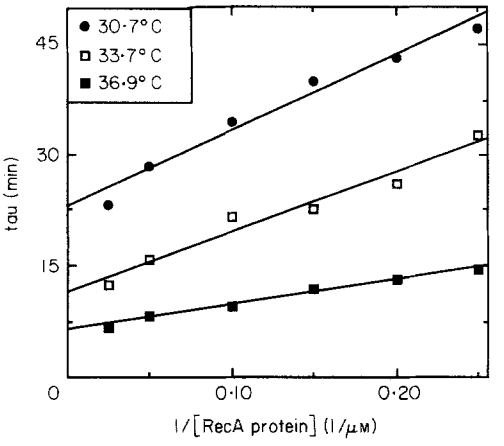


Figure 6. Tau plots as a function of temperature. Reactions were performed at pH 6·50 (66% anion) as described in Fig. 2 and Materials and Methods, except that the temperature was varied as indicated.

(c) *Temperature effect*

Time lags were measured at three temperatures and tau plots were constructed (Fig. 6). Tau increased with decreasing temperature. The values of the kinetic parameters are presented in Table 1. An Arrhenius plot of the temperature (t) dependence of $k_{2(obs)}$ was linear in this temperature range (not shown). The equation for the line is:

$$\ln k_{2(obs)} = (62 \pm 1) = (2{\cdot}0 \pm 0{\cdot}0) \times 10^4 \, t^{-1}.$$

From the values of $k_{2(obs)}$, an Arrhenius activation energy was estimated to be 39 kcal mol^{-1} (1 kcal = 4·184 kJ). $K_{1(obs)}$ showed no significant dependence on temperature. The overall association process required approximately 33 kcal mol^{-1}, a value similar to that determined by Kowalczykowski *et al.* (1987) for RecA protein binding to poly(dA-dT). The pK_a of the MES buffer used in our studies has a small temperature dependence. An increase of 0·02 pH unit is expected when the temperature is decreased from 36·9 °C to 30·7 °C. Using the equation for the pH dependence of $k_{2(obs)}$, the pH change is expected to contribute an increase of approximately 15% to $k_{2(obs)}$ at the lower temperature relative to the rate at 36·9 °C.

(d) *Effect of DNA topology*

We have shown that RecA protein binds much more rapidly to supercoiled DNA than to relaxed DNA (Pugh & Cox, 1987*a*). In particular, tau was reduced more than 20-fold at pH 7·5 by DNA that was underwound by >30%. The effect of underwound DNA on the kinetic parameters is examined more closely here using FI DNA, which is covalently closed circular DNA as isolated from *E. coli* cells. FI DNA is underwound approximately 5% relative to its relaxed counterpart (Gellert, 1981; Schutte & Cox, unpublished results). The tau plot for FI DNA is compared to that for FII DNA

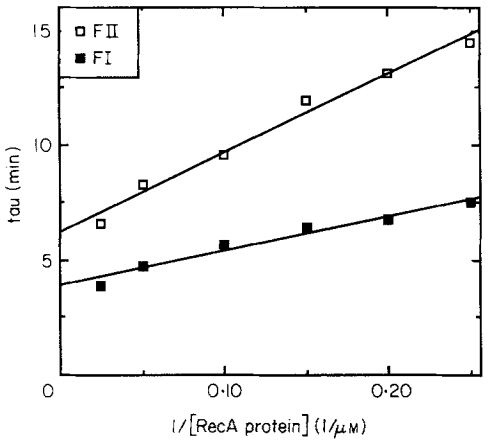


Figure 7. Tau plots as a function of DNA topology. Reactions were performed at pH 6·50 as described in Fig. 2 and Materials and Methods, except that the DNA form was varied as indicated.

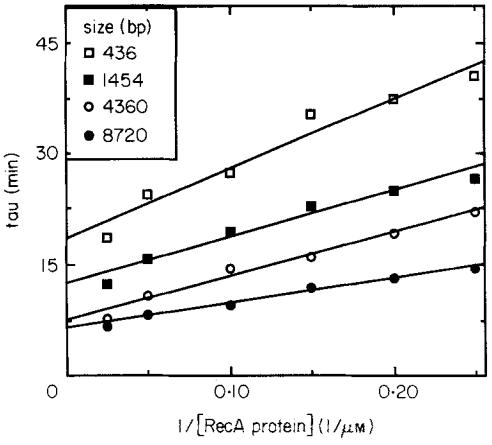


Figure 8. Tau plots as a function of DNA length. Reactions were performed at pH 6·50 as described in Fig. 2 and Materials and Methods, except that the average DNA length was varied as indicated. The DNA sizes were varied by cleaving the plasmid pBR322-2 with the following restriction endonucleases: 436 bp average size, enzymes *Bam*HI, *Dde*I and *Nru*I (cut to >99% completion with the following size distribution (bp): 162, 166, 409, 426, 448, 465, 540, 542, 597, 607); 1454 bp average size, enzymes *Acc*I and *Pst*I (cut to 94% completion with the following size distribution (bp): 1363, 1404, 1595); 4360 bp average size, enzyme *Bam*HI (cut to >99% completion with all molecules 4360 bp). The 8720 bp DNA was in a nicked circle form (>99% FII).

in Figure 7. The tau values were reduced by nearly 50% relative to FII DNA. This corresponded to >60% increase in $k_{2(obs)}$ (Table 1). The value of $K_{1(obs)}$ was increased by approximately twofold. In these experiments, >98% of the DNA was FI, and less than 5% became nicked during the course of the reaction. Since RecA protein will bind stably only to dsDNA that is underwound, the topological limitations of FI DNA allow only partial saturation of the DNA lattice. In these experiments, a k_{cat} of 24(±2) min^{-1} was obtained on FII dsDNA, which represents full saturation of the lattice at a density of one RecA monomer per 4 bp. If the k_{cat} is used as a measure of DNA binding, then the k_{cat} on FI DNA (14(±0) min^{-1}) suggests that approximately 60% of the FI DNA is bound by RecA protein. This correlates well with conclusions made when electron microscopy was used to observe binding (Dunn *et al.*, 1982; Stasiak & Di Capua, 1982).

(e) *DNA length effect*

If the general binding mechanism shown in Figure 1 is an accurate reflection of the observed kinetics, then a linear relationship between $k_{2(obs)}$ and DNA length is expected for a homogeneous DNA lattice (ignoring end effects), under conditions of excess RecA protein. However, with non-homogeneous DNA (M13, ϕX174, pBR322, etc.), this relationship is expected to be modulated somewhat by A+T-rich regions and regions of altered secondary structure, where the energy barrier for nucleation is substantially reduced. None the less, longer DNAs are likely to have more of these preferential nucleation sites. We have noted previously (Pugh & Cox, 1987*a*) that tau, in general, decreases with increasing DNA length, despite the use of different types of DNA. Here, the effect of DNA length on the separate kinetic segments of the binding pathway is examined. The plasmid pBR322-2 (8·7 kb) was cleaved with various restriction endonucleases to generate a population of shorter DNA fragments with a narrow size range. Each reaction contained the same total concentration and composition of nucleotides. The resulting tau plots are shown in Figure 8. In every case tau decreased with increasing DNA length. Table 1 lists the kinetic parameters. Nucleation appeared to be affected the most, with $k_{2(obs)}$ increasing linearly with increasing DNA length. No significant trend was observed for $K_{1(obs)}$ over this size range. Steady-state rates of ATP hydrolysis were independent of DNA length over the range tested. This indicates that the RecA nucleoprotein filament is stable over a wide range of DNA lengths, under these conditions.

5. Discussion

This paper presents an approach to dissecting the kinetic mechanism of RecA protein binding to dsDNA using a method (McClure, 1980) derived for *E. coli* RNA polymerase. This approach was used to make an initial assessment of the involvement of protons, cations, anions, temperature and DNA length and superhelicity in the binding of RecA protein to dsDNA. This survey also delineates productive paths for future efforts. Further studies are necessary to determine more specifically the roles of each of these parameters in binding. The details of the model presented below provide an explanation for the pH sensitivity of the reaction, as well as a framework to help to elucidate the mechanism by which variations in DNA structure (e.g. *Z*-DNA) affect recombination. The primary

conclusions of this study are as follows. (1) There are multiple kinetic steps in the association of RecA protein with dsDNA to form a stable nucleoprotein filament (Pugh & Cox, 1987*a*). These steps can be grouped into two experimentally approachable reaction segments as described in Figure 1. (i) a RecA protein concentration-dependent process that is likely to be a rapid pre-equilibrium to form an unstable RecA–dsDNA complex (pre-nucleation complex) in which the DNA remains in its native wound state; and (ii) nucleation, which involves a slow initiation of DNA unwinding to form a nucleation complex. A rapid propagation of the nucleation complex into a stable nucleoprotein filament, within which the dsDNA is extensively underwound, follows this slow step. The addition of RecA protein to the growing ends of the filament during propagation is too rapid to be observed and does not affect the tau analysis. (2) Both rapid pre-equilibrium and nucleation are pH-sensitive. Approximately one H^+ was released in forming the pre-nucleation complex over the approximate pH range of 6·1 to 6·7. Nucleation is strongly pH dependent, with an apparent net uptake of approximately three protons in this reaction segment. The overall apparent second-order association rate constant (k_a) showed a second-order dependence on pH, as reported by Pugh & Cox, (1987*a*). (3) The association process is salt sensitive with a net release of ions upon complex formation. Both the rapid pre-equilibrium and the nucleation segments of the reaction appeared to have salt-sensitive steps. In particular, the nucleation segment did not appear to be affected by anion type, suggesting that it was cation sensitive. The number of cations and anions involved in the association has not been determined. (4) Nucleation is temperature sensitive, probably reflecting the requirement partially to unwind the DNA. (5) Underwinding the dsDNA increases the rate of nucleation. (6) The rate of nucleation also increases, with DNA length, corresponding to an increase in the number of potential nucleation sites. Each of these conclusions will be discussed in more detail below.

(a) *A general mechanism for the association of RecA protein to duplex DNA*

The two reaction segments shown in Figure 1 satisfactorily explain the fundamental kinetic observations for RecA protein binding to dsDNA. The work described here, however, suggests that multiple reaction steps occur within each of these segments. Association is a multi-step process involving protons, cations, anions, ATP, Mg^{2+}, and protein–protein interactions. DNA length, topology, and composition also affect the rate of binding. We postulate the existence of a rapid binding pre-equilibrium based on several indirect kinetic observations. As yet, we have no direct evidence for this interaction. The small size of $K_{1(obs)}$ suggests that this interaction would be difficult to detect. The nucleation segment of the reaction involves the initiation of stable DNA binding and unwinding, and is rate-limiting overall.

There are presumably many other kinetic steps in the overall association process that are not observable using this technique. For example, two reaction segments can also be postulated for the addition of each RecA protein binding unit to the end of the growing filament during the rapid propagation phase of the reaction: one for binding and the other for partial DNA unwinding. New approaches will be necessary to examine these segments of the reaction.

(b) *pH effects*

Weinstock *et al.* (1981*a*,*b*) originally showed that the dsDNA-dependent ATPase activity of RecA protein was strongly pH dependent. We have shown (Pugh & Cox, 1987*a*) that this dependence reflects a long lag to steady-state DNA binding. The initiation of DNA binding shows an approximately second-order dependence on H^+ concentration overall, with the nucleation segment of the reaction showing a third-order dependence. The propagation steps are kinetically fast and the final complexes are stable on FII DNA between pH 5·5 and pH 7·5. The slow binding of RecA protein to dsDNA at physiological pHs is explained by these pH effects, and is therefore readily accommodated within the model outlined in Figure 1.

The second-order dependence of k_a and third-order dependence of $k_{2(obs)}$ on pH ensures that $k_{2(obs)}$ decreases more with increasing pH than k_a. As a result, at pH 7·5, equation (2) may be dominated primarily by the first term, $1/k_{2(obs)}$. This term is independent of RecA protein concentration, which would explain the apparent insensitivity of the binding kinetics at pH 7·5 to RecA protein concentration (Pugh & Cox, 1987*a*; Kowalczykowski *et al.*, 1987).

It is not known whether this pH effect is caused by protonation of RecA protein, the DNA, or both. The structure of RecA protein appears to be affected by pH. The very low DNA-independent ATPase activity of RecA protein can be stimulated over tenfold by reducing the pH from 7·5 to 6·0 (Weinstock *et al.*, 1981*a*,*b*; Pugh & Cox, 1988). This suggests that RecA protein may become protonated at low pH. This protonation may also be involved in the DNA-binding process.

DNA structure is also affected by pH (Pulleybank *et al.*, 1985). DNA may become protonated at lower pH, causing the DNA to become underwound. Pulleybank *et al.* (1985) proposed that the N-3 position of cytosine may become protonated, leading to Hoogstein base-pairing with guanosine. Polypurine tracts show a pH-dependent sensitivity to the single-strand-specific nuclease S_1 (Pulleybank *et al.*, 1985), indicating that such regions may be underwound or locally single stranded. Such altered secondary structure may produce DNA underwinding and a

conformation favorable for RecA protein nucleation.

(c) *Salt effects*

The salt studies presented in this paper show an involvement of small ions in the binding of RecA protein to dsDNA. The presence of NaGlu appeared to stimulate $K_{1(obs)}$. The nature of this effect is not clear. It appeared that the slow nucleation segment was salt sensitive but unaffected by the nature of the anion. The two anions tested differ significantly in their interaction with proteins according to the Hofmeister series (von Hippel & Schleich, 1969; Jencks, 1969; Arakawa & Timasheff, 1982; Leirmo *et al.*, 1987). If anions played a role in the rate-determining step, different effects on $k_{2(obs)}$ would be expected with Cl^- and Glu^-. Although more work is necessary to address this point, the preliminary conclusion is that there is no net change in the number of bound anions during the rate-limiting nucleation step, and so the observed salt sensitivity may be predominantly a cation effect.

It has recently been demonstrated that Glu^-, rather than Cl^-, is the physiologically relevant anion in *E. coli* (Richey *et al.*, 1987). The Cl^- and Glu^- effects on k_a presented here are qualitatively similar to those observed by Leirmo *et al.* (1987) on site-specific DNA binding by the *E. coli Eco*RI restriction endonuclease and RNA polymerase. Leirmo *et al.* found that k_a for RNA polymerase association with the λP_R promoter exhibited a similar dependence on both KCl and KGlu. However, NaGlu allowed the protein–DNA interactions to be maintained at higher and more physiologically significant concentrations than KCl. The results presented here suggest that a similar situation exists for RecA protein. A number of RecA protein reactions proceed efficiently in the presence of physiologically relevant concentrations of glutamate (or acetate), but are inhibited by low concentrations of chloride (Menetski & Kowalczykowski, 1985; Roman & Kowalczykowski, 1986; Kim & Cox, unpublished results). Glu^- appears to have a lower affinity for, or be preferentially excluded from, a protein relative to Cl^- (Arakawa & Timasheff, 1982, 1984; Leirmo *et al.*, 1987). In the DNA binding site of RecA protein, it would be energetically more favorable to displace Glu^- than Cl^- upon formation of the pre-nucleation complex. Evidence presented by Pugh & Cox (1988) indicates that both Glu^- and Cl^- interact with RecA protein. The evidence also suggests that Cl^- may have a higher affinity for this protein than Glu^-.

(d) *Temperature effects*

DNA melting or unwinding proteins tend to have a large temperature dependence in their DNA association reactions. This arises, in many cases, from an enthalpic contribution to DNA helix stability. Since the nucleation segment of the reaction involves partial DNA unwinding, it is expected that this segment be temperature sensitive. Evaluation of the intercepts of the tau plots as a function of temperature reveals that $k_{2(obs)}$ increased with increasing temperature. $K_{1(obs)}$ did not appear to vary significantly with temperature. The temperature effect on $k_{2(obs)}$ is consistent with the large enthalpic contribution to DNA helix stability. Since the two DNA strands in RecA-dsDNA complexes appear to be hydrogen-bonded (Di Capua *et al.*, 1987), even though the helical twist could be reduced by as much as 30 to 50%, the activation energy for nucleation cannot be equated to that for DNA melting, which ranges from 6 to 10 kcal mol^{-1} bp^{-1} (Record *et al.*, 1981). If RecA protein does melt dsDNA, then, on the basis of the activation energy measured here, 4 to 7 bp of DNA would be melted during nucleation. The relatively temperature-independent $K_{1(obs)}$ suggests that the rapid pre-equilibrium contains relatively little enthalpic contribution. The temperature effect on $K_{1(obs)}$ and $k_{2(obs)}$ in this system is again qualitatively similar to that of the *E. coli* RNA polymerase binding to the λP_R promoter, in which the rapid pre-equilibrium was shown to be temperature insensitive while nucleation isomerization was strongly temperature dependent (Roe *et al.*, 1984, 1985).

(e) *Effect of DNA topology*

RecA protein binds more rapidly to supercoiled DNA than to relaxed DNA (Chabbert *et al.*, 1986; Pugh & Cox, 1987*a*; this study). It appears that the lag to steady-state binding decreases in rough proportion to the superhelical density or degree of underwinding of the DNA cofactor. The length of the lags followed the pattern: FII, FIII, FIV, > FI ≫ FX ≫ ssDNA. Increasing the superhelical density of the DNA caused an increase in $k_{2(obs)}$. Since the slow step in stable binding is the initiation of DNA unwinding, RecA protein will nucleate more rapidly on DNA that is more highly underwound.

The RecA protein binding density on FII, FIII, and FX DNA is one monomer per 4 bp (4 bases on ssDNA) (Pugh & Cox, 1987*a*). Using ATP hydrolysis as a measure of DNA binding we observe that the binding density of RecA protein on FI DNA is reduced to approximately 60%. Similar observations were made by Dunn *et al.* (1982) and Stasiak & Di Capua (1982) using electron microscopy, and by Shibata *et al.* (1979*a*) using ATP hydrolysis. These observations are consistent with the requirement that RecA protein binds stably only to DNA that is locally underwound. FI DNA is topologically precluded from unwinding to the extent necessary for complete RecA protein binding along the length of the DNA.

(f) *DNA length effect*

Over the range 0·4 to 8·7 kb, $k_{2(obs)}$ increased with increasing DNA length. To a first approximation,

$K_{1(obs)}$ appeared to be independent of DNA length over the range tested, 0·4 to 8·7 kb. The k_{cat} for ATP hydrolysis remained constant over this size range, suggesting that the binding density remained constant. These observations are expected for the model shown in Figure 1. Longer DNAs have more potential nucleation sites and so $k_{2(obs)}$ is expected to be proportional to the length of the DNA. This assumes that there is only one nucleation event per DNA molecule. Under the conditions of this study where propagation is fast, this assumption holds true. In the model proposed here the DNA is assumed to be homogeneous, such that nucleation occurs with equal probability anywhere along the length of the DNA. The DNA used in this study is non-homogeneous with respect to sequence. Since RecA protein nucleates faster on A+T-rich DNA (Kowalczykowski *et al.*, 1987), it is likely to nucleate preferentially at A+T-rich regions in plasmid DNA. Strong sites of preferential nucleation would be expected to eliminate any dependence of $k_{2(obs)}$ on DNA length, providing the added DNA length does not contain these DNA sites.

(g) *Implication for preferential binding to altered DNA structures*

The evidence presented here and previously (Pugh & Cox, 1987*a*) establishes a general mechanism for RecA protein binding to dsDNA. The conditions used in this study do not represent the physiological state of the cell. The low pHs used provide conditions for studying the association process over a convenient time. However, work done to date *in vitro* under physiological conditions is readily accommodated by the DNA binding pathway outlined here, although further work is needed. RecA–dsDNA nucleoprotein filaments formed at pH 7·5, a more physiological representation, have many of the same properties as those formed at low pH (Pugh & Cox, 1987*a*). There are some interesting differences in the RecA dsDNA filament at the two pH values. For example, the stability of the filament on linear (FIII) dsDNA is decreased at pH 7·5 (Lindsley & Cox, unpublished results). Nevertheless, the association pathway presented here is applicable generally. This model also provides a framework for studies of RecA protein binding to DNA substrates with damage or structural variations that may be more representative of the physiological substrate for RecA protein. The rate of stable binding to undamaged FII plasmid DNA at pH 7·5 is extremely slow (Pugh & Cox, 1987*a*). Damaged DNA may be locally underwound and exhibit partial or full ssDNA character, providing an entry point for RecA protein. The observation that RecA protein rapidly associates with highly supercoiled DNA at pH 7·5 (Pugh & Cox, 1987*a*) suggests that RecA protein could initiate binding at locally underwound or unwindable regions such as DNA mismatches, pyrimidine dimers, polypurine tracts, cruciforms, *B*/*Z*-DNA junctions, A+T-rich stretches, and single-stranded gaps. Lu *et al.* (1986) have observed that RecA protein binding to dsDNA *in vitro* is enhanced by prior irradiation of the DNA with ultraviolet light (known to cause pyrimidine dimers in DNA). *In vivo*, pyrimidine dimers are highly recombinogenic and mutagenic (Little & Mount, 1982; Walker, 1984). Blaho & Wells (1987) have reported that DNA in the *Z* conformation improves the binding of RecA protein to dsDNA. In *E. coli*, plasmids containing the *Z*-DNA-forming sequence poly(dCA-dGT) are highly recombinogenic in a RecA-dependent manner (Klysik *et al.*, 1982). Nordheim & Rich (1983) have suggested that within the $B \rightleftharpoons Z$ equilibrium lies unwound DNA that may facilitate the recombination process. Kowalczykowski *et al.* (1987) have shown that the lag to steady-state DNA binding is greatly reduced in poly d(A−T) DNA relative to DNA from the phage M13. *In vivo*, polypurine tracts exhibit increased RecA-mediated recombination (Klysik *et al.*, 1982). Evidence has also been presented that single-stranded gaps reduce the lag to steady-state binding (Shaner & Radding, 1987; Lindsley & Cox, unpublished results). All of these effects can be reconciled by the model shown in Figure 1. These DNAs, or local structural variations in the DNA, could simply represent lower kinetic barriers to nucleation. Any local DNA structure that alters DNA twist in the direction of the final bound conformation of the DNA will likely have a faster rate of nucleation relative to most other naturally occurring DNAs. Recent results indicate that the observed improvement in binding to DNA in the *Z*-form can be explained in this way (Kim & Cox, unpublished results).

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