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# Use of ion exchange chromatography for the study of RecA-DNA interaction

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In vitro binding of RecA protein to double-stranded DNA (dsDNA) was studied using ion-exchange liquid chromatography. The method allowed quantification of both free DNA and free protein. The results unambiguously showed a binding stoichiometry of 3 base pairs per RecA monomer. The binding exhibited cooperativity, and the stoichiometry suggested that RecA does not form complexes with two molecules of dsDNA. More than 90% of RecA molecules in the sample were active for DNA binding.

Liquid chromatography; Ion exchange; Protein-DNA interaction; RecA protein; Recombination

## 1. INTRODUCTION

RecA protein is required for general genetic recombination in *Escherichia coli*. In vitro, the purified protein mimics this reaction by promoting strand exchange between homologous DNA sequences in the presence of ATP co-factor [1-5]. Electron microscopy studies show that RecA forms a fiber structure by cooperative binding around DNA in a helical manner [6-8]. On the basis of this observation it was postulated that such a RecA fiber could accomodate two molecules of DNA and bring them close enough to each other to promote exchange of DNA strands [1]. The binding stoichiometry, determined by DNase protection studies [9], linear dichroism spectroscopy [10-12] and gel electrophoresis [13] supports this hypothesis. RecA fibers may in fact bind up to 3 molecules of single-stranded DNA as indicated from a study of the capability of RecA-DNA complexes to stimulate the auto-cleavage of LexA repressor [14]. However, binding of two molecules of double-stranded (ds) DNA has never been observed [12,13,15].

In this work we report direct determination of binding stoichiometry of a dsDNA-RecA complex by ion-exchange high-performance liquid chromatography. Ion-exchange chromatography separates molecules with respect to their electric charges. The dissociation of the DNA-RecA complex in the presence of non-hydrolyzable co-factor analog ATPγS is very slow [15,16]. Since the electric charges of protein, DNA and their complex differ, we may utilize this kinetic stability to separate the compounds by chromatography.

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## 2. EXPERIMENTAL

RecA was purified as described elsewhere [12] using DEAE 5PW (Tosoh) high-performance liquid chromatography for the final step. RecA concentrations were determined spectrophotometrically using the molar absorptivity $E_{280} = 2.17 \times 10^4\ M^{-1} \cdot cm^{-1}$ [17]. Freshly dissolved DNA from salmon testes (Sigma, type III, lot 96F 7125) was used without further purification. The size of the DNA was about 20 kilo base pairs as determined by agarose gel electrophoresis. DNA concentrations were determined spectroscopically using the molar absorptivity $E_{260} = 6.6 \times 10^3\ M^{-1} \cdot cm^{-1}$ (in bases). ATPγS was purchased from Boehringer Mannheim (lot 11747720-81) and used without further purification. DNA-RecA complex was formed by incubating varying amounts of DNA with 10 μM RecA and 50 μM ATPγS for 30 min at 25°C in a buffer containing 20 mM potassium phosphate, pH 6.6, 50 mM NaCl, 1 mM $MgCl_2$ and 1 mM 2-mercaptoethanol.

High-performance liquid chromatography was performed at 4°C using a Waters 650 Advanced Protein Purification System equipped with a DEAE 5 PW packed glass column (Tosoh, anionic exchanger, 8 mm diameter, 75 mm height). The column was equilibrated with buffer containing 40 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. 500 μl of sample solution was diluted to 2.5 ml with the cold equilibration buffer prior to injection into the column. The column was washed with equilibration buffer for 7 min after injection. The elution was subsequently done by increasing the NaCl concentration from 100 mM to 600 mM at a rate of 10 mM/min. The flow rate was 0.8 ml/min at a backpressure of about 280 psi. The elution profile was monitored by absorbance at 280 nm. The base line of the elution profile was recorded by charging only the reaction buffer, and checked after each 2-3 experiments. The experiments showed high reproducibility; differences in peak area between two duplicated experiments were less than 15%.


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## 3. RESULTS AND DISUSSION

RecA, DNA and ATPγS were charged separately on the DEAE 5PW column following incubation under conditions identical to those used for complex formation. ATPγS was weakly retained and eluted at about 120 mM NaCl (Fig. 1a). Side peaks, in addition to the main peak, were observed indicating the possible presence of hydrolyzed ATP (ADP and AMP) and/or contaminants. RecA was well retained by this anionic exchange column and eluted at 360 mM NaCl as a single peak (Fig. 1a). The elution profile was similar to that observed during RecA purification indicating the absence of degradation during the incubation. Double-stranded DNA was strongly retained and eluted at 440 mM NaCl (Fig. 1a). Thus the 3 elements of DNA-RecA-ATPγS complex can readily be separated by this chromatography. In all cases the recovery of material was better than 90%, and the eluted peak size was proportional to the amount of charged material for both DNA (cf. Fig. 2) and RecA (not shown) in the range of concentrations used for this work.

DNA-RecA-ATPγS complex was formed by incubation of 10 μM RecA, 30 μM DNA and 50 μM ATPγS for 30 min at 25°C. When the mixture was charged the peak areas of RecA and DNA were reduced to 7% and 2%, respectively, of their value when charged individually (Fig. 1b). A significant amount of material passed through the column prior to application of the NaCl gradient. The UV absorption spectrum of this material was characterized by: (i) large absorption at 230 nm indicating the presence of RecA; and (ii) an $A_{260}/A_{280}$ ratio consistent with the presence of DNA and/or ATPγS. This result suggests that the DNA-RecA complex is not retained in the column, probably due to size exclusion. The limiting exclusion size of the column is about 10 000 kDa, and the expected molecular mass of the complex is larger than 250 000 kDa if the stoichiometry is 1 RecA per 3 base pairs of DNA (see below).

Due to the slow association kinetics of DNA-RecA-ATPγS [15,16], a 30 min incubation time was used for complex formation. The elution profile was not significantly altered by increasing the incubation of the mixture for up to 2 h. Fig. 1b shows the elution profile of a RecA, DNA and ATPγS mixture charged without incubation; the resulting elution profile is a superimposition of the 3 separated elements (Fig. 1b). When the complex was dissociated by inactivating RecA with the oxidized form of dithiothreitol, the DNA was quantitatively recovered (results not shown), showing that the disappearance of the DNA peak was not due to degradation of DNA. A mixture of RecA and DNA which had been incubated without ATPγS eluted as the individual two elements. This confirms the lack of complex formation in the absence of co-factor [18]. A mixture of ATPγS and RecA also eluted as separate elements, although the peak of RecA became slightly larger. These observations are all consistent with the notion that the disappearance of the free DNA and RecA peaks are related to complex formation.

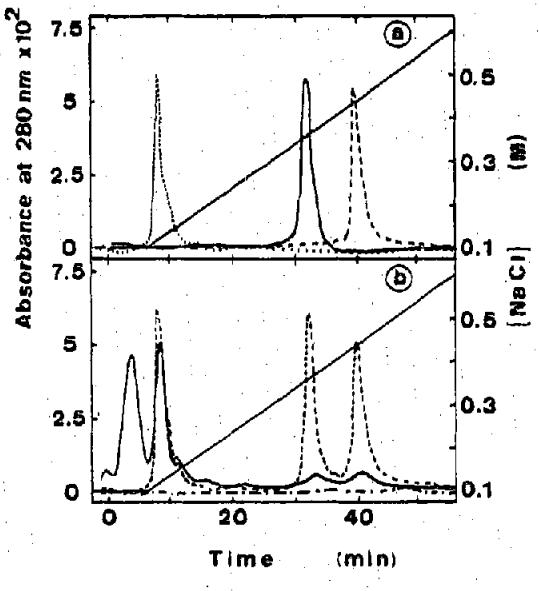


Fig. 1. (a) Elution profiles of 30 μM (in base pairs) dsDNA (----), 10 μM RecA (—) and 50 μM ATPγS (------), separately charged on a DEAE 5PW column and eluted with a linear NaCl gradient (—) as described in section 2. The elution profiles were monitored by the absorption at 280 nm. (b) Elution profiles of a mixture of 30 μM DNA, 10 μM RecA and 50 μM ATPγS charged immediately afer mixing (---), or after 30 min incubation at 25°C (—). The samples were eluted and monitored as in (a). NaCl gradient (—) and a typical absorption baseline (---) are also shown.

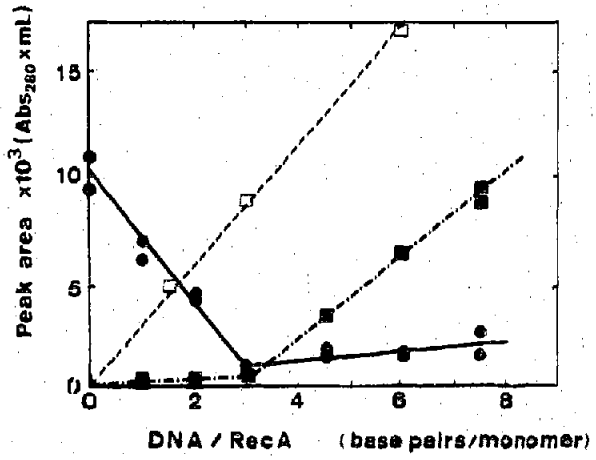


Fig. 2. Amounts of free DNA and free RecA as a function of DNA/RecA ratio. The complexes were formed by incubating 10 μM RecA and 50 μM ATPγS with various amounts of DNA. The mixtures were chromatographed on a DEAE 5PW column as described in section 2. The amounts of free DNA (■) and of free RecA (•) were estimated from the areas of corresponding peaks. The DNA peak area when only a corresponding amount of DNA was charged is also presented (□).

In order to determine the binding stoichiometry, we varied the DNA/RecA ratio in the incubation mixture by systematically altering the DNA concentration. The amounts of free DNA and RecA were determined from the elution profile by measuring the area under the corresponding peaks (Fig. 2). When the DNA/RecA ratio was below 3 base pairs per RecA monomer subunit only very small amounts of free DNA were eluted (1–2% of charged amounts). At higher DNA/RecA ratios, the amount of free DNA eluted was proportional to the amount of DNA in excess of a 3:1 ratio (Fig. 2). The peak corresponding to free RecA decreased with increasing concentration of DNA, reaching a minimum at a DNA/RecA ratio of 3 base pairs of DNA per RecA monomer. At higher DNA/RecA ratios, the RecA peak remained small. The minimum value was about 10% of the total amount of RecA charged, indicating that at least 90% of RecA molecules are active for double-stranded DNA binding.

Our results clearly show that the DNA/RecA binding stoichiometry is 3 base pairs per RecA monomer as previously indicated by transmission electron microscopy [8] and linear dichroism [12,15] studies. There is only one binding stoichiometry suggesting the absence of a stable complex of RecA with two molecules of dsDNA. When the DNA/RecA ratio was higher than 3, the amount of free DNA eluted was only slighly less than the amount of excess DNA charged (Fig. 2). Thus, instead of a simple statistical distribution, almost all RecA molecules were bound to a limited number of DNA molecules. This result suggests a high binding cooperativity, in agreement with electron microscopy observations [7,8].

The liquid chromatography technique described in this paper allows the simultaneous determination of free DNA and free protein, and therefore permits an unambiguous determination of binding stoichiometry. Most common biochemical techniques, such as gel shift and filter binding assays, measure only the concentration of free DNA. Binding stoichiometries are estimated by assuming that all protein molecules are homogeneous and active. Our method has the advantage of providing an experimental examination of these assumptions. Knowing the free protein concentration also allows determination of other binding parameters such as association constants. The measurements are very reproducible. The method described here could easily be used for the study of other kinetically stable protein-DNA complexes.

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