Equilibrium Binding of Single-stranded DNA to the Secondary DNA Binding Site of the Bacterial Recombinase RecA

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The bacterial recombinase RecA forms a nucleoprotein filament in vitro with single-stranded DNA (ssDNA) at its primary DNA binding site, site I. This filament has a second site, site II, which binds ssDNA and double-stranded DNA. We have investigated the binding of ssDNA to the RecA protein in the presence of adenosine 5’-O-(thiotriphosphate) cofactor using fluorescence anisotropy. The RecA protein carried out DNA strand exchange with a 5’-fluorescein-labeled 32-mer oligonucleotide. The anisotropy signal was shown to measure oligonucleotide binding to RecA, and the relationship between signal and binding density was determined. Binding of ssDNA to site I of RecA was stable at high NaCl concentrations. Binding to site II could be described by a simple two-state equilibrium, \( K = 4.5 \pm 1.5 \times 10^9 \text{ M}^{-1} \) (37 °C, 150 mM NaCl, pH 7.4). The reaction was enthalpy-driven and entropy-opposed. It depended on salt concentration and was sensitive to the type of monovalent anion, suggesting that anion-dependent protein conformations contribute to ssDNA binding at site II.

Homologous recombination plays an essential role in chromosomal segregation and DNA repair. Recombinases act in a filamentous nucleoprotein structure that stretches the DNA and hydrolyzes ATP. Essential features of the structure and function of this recombinase-DNA filament have been conserved from phage to man (1, 2). The most widely studied recombinase is the RecA protein of *Escherichia coli*. Major steps of the strand exchange reaction can be reproduced by RecA protein in vitro in the presence of ATP or a stable analog such as ATPγS (3–5).

Early characterization of this reaction led to the functional definition of two DNA binding sites on the RecA protein (6). ssDNA forms a complex with RecA at site I known as a presynaptic filament. dsDNA then binds at a second site, site II. If the DNA sequences at site I and II are complementary, synapsis can occur, leading to strand exchange. ssDNA can also bind at site II (1, 5, 7–9). The location of sites I and II within the RecA molecule have been investigated by x-ray crystallography (10), cross-linking experiments (11–14), limited protease digestion (15), and properties of mutant RecA proteins (16–21).

Although an equilibrium constant for binding of dsDNA at site II has been reported (22, 23), binding constants for the reaction of ssDNA with site II of RecA protein are unknown. We have recently (24) measured the equilibrium binding parameters for the reaction of ICP8, the ssDNA-binding protein of herpes simplex virus type I, with ssDNA using an approach that combines fluorescence anisotropy and macromolecular binding density function analysis (25). Here we employ this method to study the reaction between site II of the RecA nucleoprotein filament and a 5’-fluorescein-labeled ss 32-mer oligonucleotide.

One of the reasons that equilibrium constants for this reaction have not previously been measured is that ss polynucleotides bind tightly to RecA in the presence of cofactor (26, 27). Use of an oligonucleotide, which may bind less to RecA than do polynucleotides, was expected to overcome this difficulty. We chose an oligonucleotide known to be a substrate for RecA-catalyzed DNA strand exchange (8, 9) and labeled the 5’-extremity with fluorescein. It was necessary to 1) demonstrate that the protein formed a functional complex with the fluorescent oligonucleotide, 2) show that the anisotropy reflected protein binding rather than local effects near the fluorescein label, and 3) experimentally determine the relationship between the anisotropy signal and fractional saturation for the reaction. These conditions were met, and we have characterized the equilibrium binding of ssDNA with site II of the RecA nucleoprotein filament in the presence of ATPγS.

**EXPERIMENTAL PROCEDURES**

RecA protein was purified from *E. coli* as previously described (19) and was free of nuclease activity. Oligonucleotides 1 (5’-F CCA TCC GCA AAA ATG ACC TCT TAT CAA AAG GA-3’), 2 (5’-CCA TCC GCA AAA ATG ACC TCT TAT CAA AAG GA-3’), and 3 (5’-TCC TTT TGA TAA GAG GTC ATT TTT GCG GAT G-3’) were synthesized by Genosys. To prepare double-stranded oligonucleotides 1/3 and 2/3, an equimolar mixture of complementary oligonucleotides in 10 mM Tris acetate, pH 7.4, was heated to 90 °C and slowly cooled to ensure hybridization. Protein and oligonucleotide concentrations were determined by UV spectroscopy with \( e_{260} = 2.17 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) and \( e_{245} = 8.64 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \), respectively. Concentrations of ss oligonucleotides were \( n_\text{nt} \), and ds oligonucleotides were \( n_\text{base pairs} \).

Strand exchange in vitro was carried out following published methods (8). Presynaptic complexes were formed by reacting 15 μM RecA, 1 mM ATPγS, and 45 μM nt ss oligonucleotide 15 min at 37 °C in TMDG buffer (20 mM Tris acetate, 2 mM MgCl₂, 1 mM dithiothreitol, and 5% glycerol, pH 7.5). The (MgCl₂,COO⁻), concentration was then increased to 12 mM, and after 5 min of incubation, strand exchange was initiated by the addition of ds oligonucleotide (45 μM base pairs). 10-μl aliquots were removed from the reaction at various times and deproteinized at room temperature by adding 50 mM EDTA, 1% SDS. After 5 min, 5 μl of glycerol/bromphenol blue were added, and samples were stored on ice prior to 20% polyacrylamide gel electrophoresis (0.1% Tris, 90 mM boric acid, 1 mM EDTA, pH 8.4) for 75 min at 150 V. Strand exchange was quantified from fluorescence intensities on wet gels using a PhosphorImager Storm 840 apparatus (Molecular Dynamics) in the Fluorescence Blue operating mode. The intensity of the signal from ds...
Fig. 1. DNA strand exchange of fluorescent labeled ss oligonucleotide with unlabeled ds oligonucleotide. a, the presynaptic complex formed between RecA (15 μM) and oligonucleotide 1 (45 μM nt) was subsequently reacted with unlabeled double-stranded oligonucleotide 1/2 (45 μM base pairs) for the indicated times at 37 °C in the presence or absence of RecA protein and ATPγS. Migration of ss and ds oligonucleotide during SDS-polyacrylamide gel electrophoresis is indicated to the left of the gel. Circles are RecA protein, lines are DNA, and F is fluorescein. b, kinetics of strand exchange at 25 °C (●) and 37 °C (x). ds DNA formed in the absence of protein was subtracted.

oligonucleotide was corrected for quenching (see “Results”).

Fluorescence experiments were performed by a model MOS-400 spectrofluorometer from Bio-Logic (Claix, France). Samples were excited at 490 nm and stirred continuously at various temperatures ± 1 °C in a 1.0 × 0.4-cm temperature-controlled cuvette with the 4-mm path oriented in the direction of the exciting light. Inner filter effects were negligible. The pH was measured at the beginning and end of control experiments to assure that it remained constant during titrations. Addition of RecA protein led to a 3-nm red shift, with no change in the shape of the fluorescence emission spectrum of oligonucleotide 1. Therefore, to optimize the sensitivity of the experiments, total spectral intensity was subsequently measured using a 515-nm cutoff filter. Fluorescence and fluorescence anisotropy were measured using a photoelectric modulator (Hinds) in 1/2 mode. Results are reported as either A\textsubscript{obs} or A = A\textsubscript{max} - A\textsubscript{obsm} / (1 - φ); A\textsubscript{obsm} is the observed anisotropy, A\textsubscript{m} is the signal of the oligonucleotide alone, and φ is the estimated fraction of bound oligonucleotide, (A\textsubscript{max} - A\textsubscript{m}) / A\textsubscript{m}, where A\textsubscript{max} is the anisotropy of oligonucleotide complexed to RecA.

RESULTS

Functional Characterization of RecA Filament with Fluorescent Oligonucleotide—We first determined whether RecA protein catalyzed the strand exchange reaction with the fluorescent-labeled oligonucleotide. When the spontaneous background reaction was subtracted from the data, 47 ± 7% strand transfer was observed (Fig. 1), which required protein, ATPγS, and sequence homology (data not shown). The extent of this reaction was independent of the placement of the fluorescein label in the ss or ds substrate and identical for reactions carried out at 37 °C and 25 °C within experimental uncertainty (Fig. 1b). These results are consistent with the extent of strand exchange previously reported for the oligonucleotide without fluorescein label (8, 9). Hence, the fluorescein label did not appear to perturb the strand exchange reaction, and the subsequent fluorescence spectroscopy studies were carried out in reaction conditions where strand exchange takes place.

The stoichiometry of the reaction was determined from the spectral changes observed during the titration of fluorescent ss oligonucleotide by RecA protein (Fig. 2). RecA quenched fluorescent oligonucleotide 1 by 30 ± 4%. The initial anisotropy of oligonucleotide, 0.027 ± 0.005, increased to 0.19 ± 0.01, indicating a decreased mobility of the protein-DNA complex. The extent of spectral changes was independent of ATPγS. Stoichiometry, determined from the ratio of oligonucleotide to RecA protein necessary to saturate the signal, was 5.8 ± 0.3 nt/RecA. This value is in agreement with the results of previous studies showing that RecA protein has two sites with binding site sizes of 3 nt/RecA molecule (1, 7).

Addition of NaCl to the complex formed in the absence of cofactor increased the fluorescence and decreased the anisotropy (Fig. 2b). In both cases the final signal was equal to that of free oligonucleotide at these concentrations of NaCl, and the salt titration midpoint (STMP) was 115 ± 10 mM NaCl. In the presence of ATPγS (Fig. 2d), addition of NaCl increased the fluorescence signal by 16 ± 5% (which can be accounted for by increased oligonucleotide fluorescence in the presence of salt (24)) and decreased the anisotropy by 11 ± 6%. These results show that in the absence, but not in the presence, of ATPγS, NaCl can dissociate oligonucleotide from the complex formed with excess RecA. A similar effect of cofactor on the stability of complexes with polynucleotides has been reported, although STMP values are greater for polynucleotides than for oligonucleotides (26, 27).
Determination of Reaction Conditions for Equilibrium Binding—The RecA protein has two DNA binding sites. In the previous experiments, oligonucleotide bound stoichiometrically to protein, and the complex formed in the presence of excess RecA, presumably binding at site I, was stable in the presence of high concentrations of NaCl. We wished to confirm this result and, if possible, to find equilibrium conditions for binding to the individual sites. To this end we attempted to make complexes with fluorescent oligonucleotide in either site I or site II and titrated them with various salts to find conditions where oligonucleotide bound incompletely to protein (Fig. 3). The anisotropy of the oligonucleotide alone increased with salt concentration, and this phenomenon was taken into account in the determination of the STMP.

Reacting 1 μM RecA with 3 μM fluorescent oligonucleotide 1 formed a complex between oligonucleotide 1 and site I (1, 7). Anisotropy of this complex was 0.18 and decreased only slightly on addition of 800 mM NaCl (Fig. 3a). This result shows that ssDNA binding at site I was insensitive to NaCl, as suggested by the results in Fig. 2d. However, when 6 or 9 μM oligonucleotide 2 was equilibrated with RecA prior to adding oligonucleotide 1, the fluorescence anisotropy decreased with addition of NaCl; the final anisotropy was the same as for oligonucleotide alone at these salt concentrations, indicating that all fluorescent oligonucleotide was displaced (STMP = 170 mM). Equilibration of 1 μM RecA with 1.5 or 3 μM nonfluorescent oligonucleotide 2 prior to addition of oligonucleotide 1 gave comparable results, although fluorescent oligonucleotide was not completely displaced. The STMP was the same in all cases, indicating a second mode of binding to RecA, with different salt sensitivity than the complex with site I. In the case where 3 μM oligonucleotide 2 was equilibrated prior to addition of equimolar oligonucleotide 1, the fluorescent oligonucleotide could not be entirely removed by NaCl, showing that some oligonucleotide 1 was bound in site I; these results indicate that although stoichiometric binding at site I is insensitive to salt, it may be kinetically labile, at least at higher salt concentrations.

Similar experiments were carried out using other salts (Table I). The STMP value of KCl, 170 mM, was the same as NaCl. Na(CH$_3$COO) was less efficient (STMP = 240 mM). MgCl$_2$ readily dissociated these complexes (STMP = 55 mM). High concentrations of MgCl$_2$ also displaced oligonucleotide 1 from site I (STMP = 450 mM) (Fig. 3b). To confirm this latter observation, 5 μM fluorescent oligonucleotide 1 was titrated with RecA as in Fig. 2; at the end of the titration, oligonucleotide was less efficient (STMP cleotide 1 in site I and site II.

\[ \text{STMP}_{\text{cleotide 1 in site I and site II.}} \]

\[ \text{anisotropy of fluorescent oligonucleotide 1, MgCl}_2 \]

\[ \text{prior to addition of 3 μM oligonucleotide 1, a fluorescent oligonucleotide that has the same sequence. The resulting complex was titrated with (a) NaCl or (b) MgCl}_2. \]

\[ \text{RecA and 100 μM ATPγS, TMDG buffer; 25 °C) }} \]

\[ \text{TABLE I} \]

| Salt     | Site I | Site II |
|----------|--------|---------|
| NaCl     | n.o.$^a$ | 170 ± 8 |
| KCl      | n.o.$^a$ | 170 ± 8 |
| Na(CH$_3$COO) | n.o.$^a$ | 240 ± 10 |
| MgCl$_2$ | 450 ± 40 | 55 ± 8  |

$^a$ Not observed for salt concentrations less than 1 M.

was in the presence of excess RecA and therefore bound primarily at site I. This complex, which was insensitive to NaCl (Figs. 2d and 3a), was nevertheless dissociated by MgCl$_2$ (STMP = 485 mM) (data not shown). Finally, MgCl$_2$ disrupted a complex of 1 μM RecA, 1.5 μM oligonucleotide 2, and 3 μM oligonucleotide 1 by a two-step process (Fig. 3b).

These results demonstrate the existence of two types of oligonucleotide binding to RecA, with different sensitivities to salts. Oligonucleotide binding at site I (3 nt/RecA protein) was stable in the presence of both monovalent cations studied. The second mode of binding, observed when site I was occupied by unlabeled oligonucleotide, was disrupted by salt (STMP = 170 mM NaCl). Stoichiometric analysis of the data is not possible because binding was not quantitative at high salt concentrations. It nevertheless seems reasonable to suppose that the second mode of binding corresponds to the reaction of oligonucleotide with site II.

Equilibrium Constant for the Reaction of Oligonucleotide with Site II of RecA—Based on these results, the equilibrium constant for binding of single-stranded oligonucleotide to site II of the RecA filament was determined at 25 °C in 150 mM NaCl, 100 μM ATPγS, pH 7.5. The formation of the presynaptic complex is quantitative in these reaction conditions (see above), and the concentration of unreacted RecA can be neglected at protein and DNA concentrations where bound and free oligonucleotides are present. Consequently, the titration can be described by the equilibrium (Fig. 4a)

\[ \text{RN1} + \text{N} \rightleftharpoons \text{RN2} \]

\[ \text{REACTION I} \]

where RN1 is the presynaptic complex, RN2 is the complex with ss oligonucleotides in both site I and site II, and N is the...
free nucleotide concentration (m nt). RT is equal to RN1 + RN2, which is the total protein concentration (m protein monomer). Because RN2 has two DNA binding sites, this complex enters once into the equation for mass for the protein and twice in the expression for concentration of nucleotides

$$NT = N + Nb = N + n(RN1 + 2 \cdot RN2)$$  \hspace{1cm} (Eq. 1)

where NT is the total nucleotide concentration, Nb is the bound nucleotide concentration (m nt), and n is the binding site size (nt/protein). The equilibrium constant, \(K = [RN2]/[RN1][NT]\), was determined using the macromolecular binding density function method (25). This approach is based on the principle that all reaction conditions that produce the same fractional saturation, \(\theta = Nb/NT = n(RN1 + 2 \cdot RN2)/NT\), will have the same spectroscopic signal. Application of this approach to the RecA nucleoprotein filament assumes that the anisotropy signal is the same when fluorescent oligonucleotide is bound in either site (see “Discussion”). Combining the expressions for \(\theta\) and RT yields Equation 2.

$$RT = (\theta(2n))NT + (RN1)/2$$  \hspace{1cm} (Eq. 2)

We titrated five concentrations of fluorescent oligonucleotide 1 with RecA protein (Fig. 4b). The observed signal was corrected for the anisotropy of unbound oligonucleotide (see “Experimental Procedures”). Each value of A gave five pairs of concentrations, (RT, NT), which have the same fractional saturation. For anisotropy values in the range of 0.02 to 0.12, plots of RT as a function of NT for the sets (RT, NT) were linear (data not shown), and \(\theta\) and RN1 were determined for each plot using Equation 2.

Each value of \(\theta\) calculated by this method corresponds to a unique anisotropy, and in this way the dependence of A on fractional saturation can be determined (Fig. 5a). Results for A < 0.1 were reproducible in three separate titrations. The variations observed above this value of A were not a consequence of nonlinear plots of Equation 2. The plot of A versus \(\theta\) showed small significant positive curvature, indicating that the mobility of the fluorophore decreased to a greater extent at high levels of binding than at low levels, as was previously observed for ICP8 protein (24). The graph of \(\theta\) as a function of presynaptic filament concentration RN1 (Fig. 5b) is a model-free binding isotherm that is independent of the relationship between A and \(\theta\) (25).

The model-free binding isotherm was fit using the simple two-state equilibrium described by Reaction I. For this model, \(\theta = (C + 1)/(C + 1 + (1/(2n-K\cdot RN1)))\), where C = RN1/2RN2. We first assumed that C could be ignored (C \(\ll\) 1; (1/K) \(\ll\) N) and fit the isotherm using Equation 3.

$$\theta = 1/(1 + (1/(2 \cdot n \cdot K \cdot RN1))) \hspace{1cm} (Eq. 3)$$

Theoretical curves for \(K = 1.5 \times 10^{6} \text{m}^{-1}\), \(K = 1.25 \times 10^{6} \text{m}^{-1}\), and \(K = 1.0 \times 10^{6} \text{m}^{-1}\) are shown in Fig. 5b. Using these values, C \(\ll\) 1 as long as the free nucleotide concentration N \(\gg\) 1 \(\mu\text{m}\), which appears to be the case at least for the titrations of 6, 9, 12, and 15 \(\mu\text{m}\) oligonucleotide by low concentrations of protein.

To confirm this binding constant, we fit the titration curves (Fig. 4b). The calculated fractional saturation (Equation 3) was transformed into anisotropy using results in Fig. 5a and a value for the plateau of the titration curve, \(\theta = 1\), of \(A = 0.18\). RecA concentration was determined using Equation 2, taking into account dilution of total nucleotide, NT, during titration. Theoretical curves using \(n = 3\) nt/RecA protein and \(K = 1.5 \pm 0.5 \times 10^{6} \text{m}^{-1}\) fit experimental data well (Fig. 4b).

Reverse titrations of various concentrations of RecA protein with fluorescent oligonucleotide were also carried out, and the binding constant was determined. Binding isotherms (Fig. 6) exhibited a plateau of \(\theta_{\text{obs}} = 0.175 \pm 0.01\) during the initial part of the titration curve. Plots of (RT, NT) corresponding to a constant anisotropy were linear for \(\theta_{\text{obs}}\) between 0.05 and 0.16 (data not shown), and the analysis of the reverse titration revealed the same relation between anisotropy and fractional saturation, \(\theta\), as that in Fig. 5a. Values of RN1 calculated from the intercepts of these plots were small, as expected for high concentrations of oligonucleotide, and their uncertainty was too large to determine a model-free binding isotherm.

However experimental curves for the reverse titration could be directly fit by the equilibrium used to describe the forward titration by RecA protein. The calculated fractional saturation was determined using Equation 3 and transformed into anisotropy as above; oligonucleotide concentration, NT, was calculated by Equation 2. It is worth noting that NT is proportional to binding site size, n (Equation 1). Simulated titrations showed that K intervened primarily in the shape of the curve, whereas \(n\) displaced it to lower or higher oligonucleotide concentrations (data not shown). Hence both parameters were varied to fit experimental data. Satisfactory agreement was obtained by \(n = 2.8 \pm 0.2\) nt/RecA and \(K = 2.3 \pm 0.7 \times 10^{6} \text{m}^{-1}\), where uncertainties are the range of values used to obtain the best visual fits (Fig. 6). Hence forward and reverse titrations could be described by a simple two-state model (Fig. 4a; Reaction I and Equations 2 and 3) \((K = 1.5 \pm 0.5 \times 10^{6} \text{m}^{-1})\); the binding site size determined by the reverse titration provided an independent confirmation of the results of stoichiometric binding experiments (Fig. 2).

The NaCl dependence of the equilibrium constant was estimated from the data for 6 and 9 \(\mu\text{m}\) oligonucleotide 2 in Fig. 3a. The signal at various salt concentrations was corrected for the anisotropy of unbound oligonucleotides using \(A_{0} = 0.03\) (see “Experimental Procedures”), and the resulting anisotropy was converted to fractional saturation using Fig. 5a. The binding constant was calculated by assuming that the resulting anisotropy was due to oligonucleotide binding at site II only (\(K = \theta/(1 - \theta))/[n\cdot(\text{RT} - \theta(\theta\cdot\text{NT}))]\). The slope of the linear parts of the plots of log(K) versus log([NaCl]), \(-3.5 \pm 0.3\), is a function of the electrostatic interactions per protein monomer contrib-
Fig. 5. a, fluorescence anisotropy as a function of fractional saturation. \( \theta \) was calculated from protein titration curves of Fig. 4 using the method of macromolecular binding density function analysis (25), with binding site size \( n = 3 \) nt/protein. b, model-free binding isotherm for protein titration used in a. The calculated isotherm (●) is presented with theoretical fits using a simple two-state thermodynamic model (\( n = 3 \) nRecA protein) (see “Results”). Upper curve, \( K = 1.5 \times 10^6 \text{ M}^{-1} \); middle curve, \( K = 1.25 \times 10^6 \text{ M}^{-1} \); lower curve, \( K = 1.0 \times 10^6 \text{ M}^{-1} \).

Fig. 6. Fluorescence anisotropy as a function of oligonucleotide concentration. 0.5 \( \mu \text{M} \) (●), 1 \( \mu \text{M} \) (▲), 1.5 \( \mu \text{M} \) (▲), 3 \( \mu \text{M} \) (●), or 5 \( \mu \text{M} \) (●) RecA protein was titrated with fluorescent oligonucleotide 1; reaction conditions were as described in the legend to Fig. 4. Experimental isotherm (symbols) were fit as in Fig. 5 using \( K = 2.3 \pm 0.7 \times 10^6 \text{ M}^{-1} \), \( n = 2.8 \pm 0.2 \text{ nt/RecA} \), and assuming an anisotropy for oligonucleotide alone of 0.025.

Discussion

Fluorescence anisotropy of 5'-fluorescein-labeled oligonucleotides appears to be a useful method to investigate the reaction of DNA with RecA protein under conditions where DNA strand exchange can occur. Using this technique we found that the stoichiometry of the nucleoprotein complex formed between fluorescent oligonucleotide 1 and RecA protein was 5.8 ± 0.3 nt/RecA, independent of cofactor (Fig. 2), in agreement with previous results for polynucleotides (1, 3–5, 7) and oligonucleotides (8); the same stoichiometry was observed in experiments using titration by RecA (Fig. 2) and titration by oligonucleotide (Fig. 6). In addition, the oligonucleotide-RecA complex formed with excess protein was disrupted by NaCl in the absence of cofactor, whereas it was stable in the presence of ATPγS (Fig. 2), in agreement with experiments with polynucleotide-RecA complexes (26, 27). These results show that the observed fluorescence anisotropy changes reflect DNA binding in the nucleoprotein filament. Finally, the oligonucleotide with fluorescein at its 5'-extremity was a substrate for RecA-catalyzed strand exchange in vitro (Fig. 1). The reaction required protein, ATPγS, and sequence homology, as expected. RecA protein carried out strand exchange with fluorescein-labeled oligonucleotide with an efficiency comparable with that with unlabeled oligonucleotides (8, 9). Hence the fluorescein label had no significant effect on protein-DNA interactions necessary for strand exchange. These results show that fluorescence anisotropy of this oligonucleotide can be used to study protein-DNA interactions in a productive RecA nucleoprotein filament.

Fluorescence anisotropy is not proportional to fluorophore concentration (29), and it was important to experimentally determine the relationship between spectroscopic signal and fractional saturation, \( \theta \). Macromolecular binding density function analysis (25) is a useful method to address this question...


**Table II**

Equilibrium constants for the titration of RecA protein with oligonucleotide I (100 μM ATPγS, 150 mM NaCl, TMDG buffer, pH 7.5) at various temperatures

| Temperature | K  |
|-------------|----|
| 31 °C       | 1.5 × 10^6 |
| 34 °C       | 1.3 × 10^6 |
| 37 °C       | 5.0 × 10^6 |
| 40 °C       | 4.5 ± 1.5 × 10^6 |

(24). The starting point of this approach is that the signal is a function of fractional saturation. RecA has two binding sites, and straightforward application of this method requires that the anisotropies of fluorescent oligonucleotide in sites I and II be the same. The following observation suggested that this was the case. The filament with two fluorescent oligonucleotides, RN2, (which is present when the anisotropy signal saturates in tight binding conditions) rearranged upon further addition of RecA to produce a filament with a single oligonucleotide in site I, RN1 (Fig. 2); the identification of this species as RN1 was consistent with its stability in NaCl and MgCl₂ (Figs. 2d and 3). The constant signal above saturating RecA concentrations indicates that fluorescent oligonucleotide had the same anisotropy in both filaments RN1 and RN2. Macromolecular binding density function analysis showed that the slight sigmoidal shape in the experimental binding isotherms (Fig. 4b) was a result of the nonlinear relation between A and θ (Fig. 5a) and did not reflect a property of the binding process, such as cooperativity (30).

During homologous recombination, RecA protein reacts with two DNA molecules, and it was proposed many years ago that the protein therefore has two functional DNA binding sites. Early studies showed that site I binds ssDNA to form a presynaptic filament and that site II binds complementary dsDNA (6). In this model, site II would contain ssDNA after strand exchange, and tight binding of ss product DNA at this site may drive the reaction (9). The amino acids corresponding to site II have been investigated. We recently observed that mutant RecA protein, RecA<sub>MG207Q</sub>, with a single amino acid substitution in the L2 loop, lacked one of the two ssDNA binding sites of wild type protein; the remaining site was functional, and biochemical activities indicated that the mutant protein possessed an active primary site (20). Taken together with structural studies, cross-linking experiments, and properties of mutant RecA proteins (10–21), these results suggest that the L2 loop may be at or near the secondary binding site. The reaction between DNA and peptides representing the L2 loop has been studied (31, 32). Here we report a thermodynamic analysis of ssDNA binding at site II of the protein.

Site II can be distinguished from site I by its weaker DNA binding properties. It has previously been reported that, in the presence of ATPγS, ss oligonucleotides and *E. coli* ssDNA binding protein, SSB, displace ssDNA from site II of the filament but not from site I (8, 9). We report here evidence that ss oligonucleotide binding at site II is also more sensitive to salt than is binding at site I. Titration of RecA-oligonucleotide complexes with various salts revealed two binding modes, which differed by their stability at high salt concentrations (Figs. 2 and 3; Table I). Oligonucleotide binding at site I was shown to be insensitive to monovalent salts. Because RecA has two binding sites, 3 nt/protein monomer (Fig. 2; Refs. 1 and 7), the salt-sensitive reaction corresponds to binding at site II.

Quantitative binding of oligonucleotide to RecA at site I in the presence of ATPγS was not disrupted by 150 mM NaCl (Figs. 2 and 3a; Table I); therefore, in these solution conditions, when oligonucleotide concentration is sufficiently high so that free oligonucleotide is present, the concentration of unreacted RecA can be ignored, and equilibrium DNA binding can be described as a reaction between ssDNA and the presynaptic complex (Fig. 4a). Fractional saturation was calculated from a two-state model for this equilibrium (Equation 3), assuming (1/K) ≪ N. Most of the titrations occurred in these limits, and a value for the binding constant of K = 1.5 ± 0.5 × 10<sup>6</sup> M<sup>−1</sup> gave a good fit for 3–15 μM oligonucleotide titrated with RecA at 25 °C (Figs. 4b and 5b). A similar binding constant was found for the reverse titration of RecA with oligonucleotide (Fig. 6).

This reaction was favored by enthalpy and opposed by entropy. Similar results are reported for the reaction of single-stranded DNA-binding protein gp32 with DNA (34). In contrast, both entropy and enthalpy contribute to the reaction of ssDNA with the herpes ssDNA-binding protein ICP8 (24). Displacement of positively charged counter-ions from the polynucleotide by positively charged proteins increases entropy and can stabilize protein-DNA interactions (28). Negative entropy indicates that this mechanism does not contribute significantly to our reaction. In support of this hypothesis, it is worth noting that the binding of ssDNA with the L2 loop peptide has only a small electrostatic contribution and is stabilized primarily by nonelectrostatic interactions (31).

Likewise, the different capacities of Cl₃ anions and CH₃COO⁻ anions to inhibit the reaction cannot be explained by displacement of positively charged counter-ions. Rather, these salt effects probably involve displacement of anions from the protein (33, 34). These results imply that, in the intact presynaptic complex, anion-sensitive protein conformations or protein-protein interactions may be important for tight binding of ssDNA at site II.

The techniques developed in this study should be useful for investigating the thermodynamic basis of homologous recombination. The simplest model of this reaction would be an equilibrium between a reactive filament (with ssDNA at site I
and dsDNA at site II) and a product filament (with dsDNA at site I and ssDNA at site II). We report here the equilibrium constant for the reaction of ssDNA at site II of a nucleoprotein filament in which ssDNA also occupied site I. Work is in progress to extend this approach to RecA nucleoprotein filaments that are potential intermediates of the strand exchange reaction.

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