DNA-induced Dimerization of the \textit{Escherichia coli} Rep Helicase

ALLOSTERIC EFFECTS OF SINGLE-STRANDED AND DUPEX DNA*

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The \textit{Escherichia coli} Rep helicase is a stable monomer \((M_r = 72,802)\) in the absence of DNA; however, binding of single-stranded (ss) or duplex (ds) DNA induces Rep monomers to dimerize. Furthermore, a chemically cross-linked Rep dimer retains both its DNA-dependent ATPase and helicase activities, suggesting that the functionally active Rep helicase is a dimer (Chao, K., and Lohman, T. M. (1991) \textit{J. Mol. Biol.} 221, 1165–1181). Using a modified "double-filter" nitrocellulose filter binding assay, we have examined quantitatively the equilibrium binding of Rep to a series of ss-oligodeoxynucleotides, \(d(pN)_n, \quad (8 \leq n \leq 20)\) and two 16-base pair duplex oligodeoxynucleotides, which are short enough so that only a single Rep monomer can bind to each oligonucleotide. This strategy enabled us to examine the linkage between DNA binding and dimerization. We also present a statistical thermodynamic model to describe the DNA-induced Rep dimerization in the presence of ss- and/or ds-oligodeoxynucleotides. We observe quantitative agreement between this model and the experimental binding isotherms and have analyzed these isotherms to obtain the seven independent interaction constants that describe Rep-DNA binding and Rep dimerization. We find that Rep monomers (P) can bind either ss-DNA or ds-DNA (D) to form PS or PD, respectively, which can then dimerize to form P2S, P2D. Furthermore, both protomers of the DNA-induced Rep dimer can bind DNA to form either P2S, P2D or the mixed dimer species P2SD and ss- and ds-DNA compete for the same sites on the Rep protein. When bound to DNA, the Rep dimerization constants are \(-1-2 \times 10^6 \text{ M}^{-1}\) (6 mM NaCl, pH 7.5, 4°C), which are greater than the dimerization constant for free Rep monomers by at least 104-fold. The Rep-ss-DNA interaction constants are independent of base composition and sequence, consistent with its role as a nonspecific DNA-binding protein.

Allosteric effects are associated with ss- and ds-DNA binding to the half-saturated Rep dimers, \(i.e.,\) the affinity of either ss- or ds-DNA to the free protomer of a half-saturated Rep dimer is clearly influenced by the conformation of DNA bound to the first protomer. These allosteric effects further support the proposal that the Rep dimer is functionally important and that the Rep-DNA species P2S, P2D may serve as useful models for intermediates that occur during DNA unwinding. The ability of Rep to form the ternary complexes P2S and P2D has important implications for how a Rep dimer may interact with a DNA replication fork and unwind DNA.

Duplex DNA is the stable form of the majority of DNA within a cell. However, duplex DNA must be unwound, at least transiently, to produce the single-stranded (ss) DNA intermediates that are needed for processes such as replication, repair, and recombination. This DNA unwinding is catalyzed by a class of DNA-binding enzymes referred to as DNA helicases in a reaction that is coupled to ATP binding and hydrolysis. Helicases have been identified and isolated from a variety of prokaryotic and eukaryotic sources (for recent reviews see Matson and Kaiser-Rogers, 1990; Thomens and Hubsher, 1990; Matson, 1991; Lohman, 1992) and they appear to be ubiquitous. Although the unambiguous assignment of the function in vivo of any particular helicase can be difficult due to their large number (at least 10 different helicases have been identified in \textit{Escherichia coli}) and the possibility for functional complementation (Fassler \textit{et al}., 1985; Lee and Kornberg, 1991; Taucher-Scholz \textit{et al}., 1983; Washburn and Kushner, 1991), helicases as a class are essential to most DNA metabolic processes.

The \textit{E. coli} Rep protein was one of the first helicases to have been characterized genetically (Denhardt \textit{et al}., 1967; Lane and Denhardt, 1974, 1975b) and biochemically (Scott \textit{et al}., 1977). Its role in DNA replication was established since genetic studies indicate that \textit{E. coli} rep mutants exhibit reduced rates of chromosomal replication fork movement and cannot support replication of ss-DNA bacteriophages such as \(\phi X174\), P2, fl, and M13 (Denhardt \textit{et al}., 1967; Lane and Denhardt, 1974, 1975). Although essential for the unwinding of duplex replicative form phage DNA during ss-DNA phage replication (Calderon \textit{et al}., 1970; Lane and Denhardt, 1975), \textit{rep} is not essential in \textit{E. coli} (Colasanti and Denhardt, 1987). This probably reflects the participation of other essential helicases in replication, such as the DnaB protein (Lebowitz and McMacken, 1986). Other studies suggest that \textit{rep} may also function in DNA repair (Calderon \textit{et al}., 1970; Bridges and von Wright, 1981). Interestingly, \textit{rep/uvrD} double mu-

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tants are lethal in *E. coli* (Washburn and Kushner, 1991).

The Rep helicase can unwind duplex DNA in *vitro* in the absence of DNA synthesis (Yarranton and Gefter, 1979; Kornberg *et al.*, 1978; Araí and Kornberg, 1981). In the absence of a phage accessory protein such as the eX174 cis A or fl gene II protein, Rep requires a 3’ ss-DNA region flanking the duplex DNA in order to initiate unwinding (Yarranton and Gefter, 1979; Runyon and Lohnan, 1989; Lohnan *et al.*, 1989). Chao and Lohnan (1991) have recently shown that although Rep remains monomeric (*M* = 72,802, Gilchrist and Denhardt, 1987) up to concentrations of at least 8 µM (monomer) even in the presence of nucleotide cofactors, binding of either ss- or duplex (ds) DNA induces Rep to dimerize. Furthermore, a chemically cross-linked Rep dimer retains its ss-DNA-dependent ATPase and DNA helicase activities, suggesting strongly that the active form of the Rep helicase is dimeric (Chao and Lohnan, 1991).

A dimeric structure in a helicase has important implications for its mechanism of unwinding DNA (Chao and Lohnan, 1991; Lohnan, 1992). For example, it has been suggested that unwinding of duplex DNA might proceed via intermediates in which the helicase would interact simultaneously with both ss- and ds-DNA at an unwinding fork (Yarranton and Gefter, 1979). Such a model would require a minimum of two separate DNA-binding sites, a requirement that can be readily satisfied by a dimeric helicase if each protomer of the dimer can bind DNA. Interestingly, essentially all helicases for which this property has been examined have an oligomeric assembly state, usually hexameric or dimeric. Hence, this may yet prove to be a property common to all helicases. It is plausible that such oligomeric structures provide helicases with the multiple DNA-binding sites which may be important for their mechanisms of DNA unwinding (Lohnan, 1992).

In order to assess the importance of DNA-induced dimerization in the mechanism of Rep-catalyzed DNA unwinding, it is important to determine the stoichiometry of Rep-DNA binding and to resolve the thermodynamic linkages in the energetics of DNA binding and protein dimerization. Using a series of ss- and ds-DNA oligonucleotides that are long enough to bind only a single Rep monomer to each oligonucleotide, we were able to determine all the equilibrium interaction constants for DNA binding and Rep dimerization independently. Such an approach required the development of a statistical thermodynamic model to describe the multiple binding and dimerization equilibria so that the individual interaction constants can be determined from experimental binding titrations. The results of this study suggest how Rep may bind to an unwinding fork at intermediate stages during DNA unwinding and provides the basis for additional studies on the modulation of DNA binding and Rep dimerization by ATP binding and hydrolysis.

**MATERIALS AND METHODS**

**Reagents and Buffers**—All solutions were made with reagent grade chemicals using Milli-Q H2O, i.e. distilled H2O that was subsequently deionized and further purified through a Milli-Q Water Purification System (Millipore Corporation, Bedford, MA). Standard titration buffer is 20 mM Tris-HCl, pH 7.5, at 4 °C, 6 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM NaN3EDTA, 10% (v/v) glycerol (spectrophotometric grade, Aldrich). Kinase buffer contained 50 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol (DTT), 5 mM MgCl2, 0.1 mM 2-mercaptoethanol, C6s buffer contained 100 mM Tris-HCl, pH 7.5, at 25 °C, 10 mM MgCl2, 10 mM 2-mercaptoethanol. C6s buffer contained 100 mM Tris-HCl, pH 7.5, at 25 °C, 10 mM triethylammonium bicarbonate (TEAB), pH 7.5, 1 mM NaN3EDTA. All buffers were made from 0.5 M Tris stocks titrated to the appropriate pH at the indicated temperature. The pH reported for each buffer was measured at the final buffer concentration and the experiment. ATP was obtained from Calbiochem (La Jolla, CA). [γ-32P]ATP (300 Ci/mmol) was obtained from Du Pont-New England Nuclear.

**Proteins and Enzymes**—*E. coli* Rep protein was purified to >99% purity from *E. coli* MZ-1/pRepO (Colasanti and Denhardt, 1987) as described (Lohnan *et al.*, 1989; Chao and Lohnan, 1991). Its concentration was determined spectrophotometrically, using an extinction coefficient of *ε* = 8.47 x 10^4 M^-1 cm^-1 (Lohnan *et al.*, 1989). T4 polynucleotide kinase was purchased from U. S. Biochemical Corporation (Cleveland, OH).

**Oligodeoxynucleotides**—All oligodeoxynucleotides were synthesized by the University of Washington School of Medicine Protein Chemistry Laboratory (St. Louis, MO) using an ABI model 380A Automated DNA Synthesizer (Applied Biosystems Inc., Foster City, CA). Single-stranded oligodeoxynucleotides, dTn, dT10, dTn, dTΔn, dTΔ10, dTΔ2n, dTΔΔn, dC6, dC34, KL3 (5’-GACTGTTACCGTACTGAGT), and KL4 (5’-ACTCAGTGTTACCGTACTGAGT) were purified to >99% purity as described (Lohnan and Bujalowski, 1989). A hairpin oligodeoxynucleotide, HP (5’-GACTGTTACCGTACTGAGT), was purified by electrophoresis through a 15% acrylamide, 1.5% bisacrylamide, 8 M urea gel in TBE at 60 °C. The DNA was recovered by electrophoresion using an Elutrap apparatus (Schleicher & Schuell) and further purified on a Maxi-Clean C6s cartridge (Altech Associates, Deerfield, IL) as described (Wong *et al.*, 1991). The duplex oligodeoxynucleotide, KL34, was formed by annealing KL3 and KL4 (200 µM each) at room temperature in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl and was purified away from unannealed ss-DNA by electrophoresis through a nondenaturing 24% acrylamide, 0.5% bisacrylamide gel in TBE. DNA was recovered by electrophoresion using an Elutrap apparatus (Schleicher & Schuell) and further purified on a Maxi-Clean C6s cartridge (Altech Associates, Deerfield, IL) as described (Wong *et al.*, 1991).

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quartz cuvettes (NSG Precision Cells, Inc., Farmingdale, NY). After each addition of titrant, a polyethylene stirring rod (NSG Precision Cells, Inc., Farmingdale, NY) was used to mix the solution. The observed fluorescence after the ith addition of Rep, $F_{i \text{obs}}$, was taken 2.0 min after the addition of protein to allow the solution to equilibrate. Equilibrium was assumed to have been reached in this time based on the observation that the fluorescence signal no longer increased. A parallel titration was performed by addition of Rep to a second cuvette containing only buffer without DNA to obtain the value of the background fluorescence, $F_{\text{blank}}$. The corrected fluorescence emission intensity, $F_{\text{corr}}$, at each concentration of Rep is then given by Equation 1

$$F_{\text{corr}} = (F_{\text{obs}} - F_{\text{blank}})(V_i/V_0)$$

(1)

where $V_i$ is total volume after the ith addition and $V_0$ is the initial volume at the start of the titration. Neither photobleaching nor non-filter effects were observed in these experiments.

We assumed that the fluorescence enhancement, $F_{\text{corr}}$, is directly proportional to the fraction of DNA bound, an assumption which was validated by direct comparison with binding isotherms obtained by nitrocellulose filter binding (see "Results"). Therefore, the fraction of bound DNA, $S_i/S_0$, after the ith addition of Rep can be calculated from Equation 2

$$S_i/S_0 = (F_{\text{corr}} - F_{\text{corr}0})/(F_{\text{corr}} - F_{\text{corr}0})$$

(2)

where $S_i$ and $S_0$ denote the concentrations of bound and total ssDNA, respectively, and $F_{\text{corr}}$ and $F_{\text{corr}0}$ denote the corrected fluorescence emission intensities in the absence of Rep and in the presence of a saturating concentration of Rep, respectively.

"Double-filter" Nitrocellulose Filter Binding Assay—A modification of the standard nitrocellulose filter binding method (Riggs et al., 1970) was used to obtain equilibrium binding isotherms for the interactions of Rep with a series of ss- and ds-oligodeoxynucleotides.$^2$ The modified double-filter procedure involves the use of sheets of nitrocellulose and DEAE paper and a 96-well dot-blot apparatus in order to take advantage of direct β-emission imaging technology. This procedure is significantly more rapid since it eliminates the manipulation of large numbers of filters and the long times required to quantitate each filter separately by liquid scintillation counting and improves significantly the accuracy and precision of the method.

Modification of a Dot-blott Apparatus for Use in Nitrocellulose Filter Binding—Filter binding was carried out using a modified 96-well dot-blot apparatus (MiniFlow I, Schleicher & Schuell, Keene, NH) as described.$^3$ On the top plate of the unmodified apparatus, a rubber O-ring surrounds each of the 96 wells. These O-rings provide a tight seal between the wells and the membrane. However, when used in this configuration for filter binding, we observed significant lateral diffusion of radiolabel on the membranes. This diffusion was eliminated by installing an inverted top plate below the membrane, such that each sample well has an O-ring seal above and below the membrane.

By using this modified apparatus, an entire binding isotherm consisting of a duplicate or triplicate set of 20–24 concentration points of 20–25 μl each can be obtained on a single 4.5 × 5-inch filter.

Samples were applied to 6–9 wells at a time. Immediately before samples were loaded to each subset of 6–9 wells, the designated wells were flushed with 100 μl of ice-cold binding buffer using a Repipet, Jr. fixed volume dispenser (Labindustries, Inc. Berkeley, CA). Vacuum was applied just long enough to draw the flush solution through the membrane.

Samples were then loaded and vacuum reapplied to draw the samples through. As soon as the samples have been drawn through the membrane, the wells were washed immediately with another 100 μl of ice-cold binding buffer. A flow rate of 5–6 ml/min was maintained using a house vacuum line (12–15 inch Hg). Timing constraints limited the number of samples processed to 6–9 wells each time. First, it was necessary to load the sample as soon as possible after the initial cooling flush to ensure that the temperature of the membrane did not increase significantly, and second, it is imperative that the wells be rinsed as quickly as possible following sample application in order to minimize the background retention.

The entire membrane was then imaged by direct β-emission detection using a Betascope 603 Blot Analyzer (Betagen, Waltham, MA).$^4$ Labeled DNA retained by the nitrocellulose at each concentration point was quantitated by "boxing" and integrating the β-emission detected within the area of the corresponding "dot" by digital manipulation of the image using software supplied by the manufacturer. This allowed the simultaneous quantitation of an entire set of titrations points in 30 min.

Pretreatment of Nitrocellulose Membranes—Nitrocellulose membranes were obtained as dry sheets (4.5 × 5 inch, Schleicher & Schuell) to fit the MiniFlow I apparatus. To reduce the binding of free ss-DNA to the nitrocellulose filters, the filters were presoaked for 10 min in 0.4 M KOH followed by continuous rinsing in Milli-Q H2O until the pH returned to neutral (Smolarsky and Tal, 1979). Filters were then equilibrated in standard titration binding buffer at 4 °C for a minimum of 1 h prior to use.

Preparation of Samples for Nitrocellulose Filter Binding—All samples for nitrocellulose filter binding were prepared in standard buffer (20 mM Tris, pH 7.5, 6 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol) at 4 °C. Titrations were performed either at constant Rep concentration or at varying DNA concentration, or vice versa. The titrant whose concentration was being varied was serially diluted to span the necessary concentration range. Typically, titrations at constant DNA concentration required serial dilution of the Rep by factors of 0.75–0.85 while titrations at constant Rep concentration required serial dilution of the DNA by factors of 0.65. Titrations at a constant Rep concentration ≥1 μM required a slightly different protocol in mixing to prevent precipitation problems. Here, the Rep was prepared in 5 × buffer containing 50% glycerol in one-fifth final volume. The remaining four-fifths volume was contributed by the addition of DNA which was in Milli-Q H2O. Samples were therefore repeated pipetting and then incubated at 4 °C for 10 min. Equilibrium was reached within this time based on the observation in control experiments that the extent of binding did not change beyond this time. All samples were maintained at 4 °C constantly throughout the titration.

Direct quantitation of the amount of DNA that passes through the nitrocellulose membrane, which is related to the unbound DNA (see Equation 4), improves the precision and the accuracy of the method. First, the total DNA in each titration sample can be determined by summing the radioactivity retained on the nitrocellulose and DEAE filters. This allows one to compensate for pipetting errors, since for each titration point the DNA bound by the nitrocellulose filter can be normalized to the total amount of DNA that was loaded on the filters. Second, since the radioactivity retained by the DEAE filter is directly related to the amount of free DNA, it provides a more accurate parameter for the determination of nonspecific background counts retained by the nitrocellulose. Traditionally, the amount of background DNA retained on the nitrocellulose has been determined by performing a parallel set of titrations in the absence of protein. These background values would then be subtracted directly from those obtained in the presence of protein. However, this method of correcting for background assumes that the nonspecifically bound counts are a function of the total DNA concentration rather than of the free DNA concentration. However, when protein is present during a titration, the relationship between total and free DNA is not linear, hence the traditional method would usually overestimate the amount of DNA bound nonspecifically to the nitrocellulose and thus each point in the titration would be overcorrected for this background.

Therefore, the background counts derived from loading the total DNA on a filter without protein would overestimate the actual background counts.

To circumvent this problem, a linear standard curve is constructed by plotting the DNA counts retained on the nitrocellulose filter as a function of the DNA counts retained on the DEAE filter in the absence of protein. The slope, $s$, of this standard curve can be used to determine the amount of background DNA as a function of the concentration of free DNA. In the presence of protein, the protein-bound DNA counts $C_{\text{prot}}$, and the free DNA counts, $C_{\text{free}}$, for the ith titration point, are therefore given by Equations 3 and 4

$$C_{\text{bound}} = C_{\text{NCS}} - sC_{\text{DEAE}}$$

(3)

$$C_{\text{prot}} = (1 + s)C_{\text{DEAE}}$$

(4)

$^2$I. Wong and T. M. Lohman, manuscript in preparation.
where \( C_{\text{NCL}} \) and \( C_{\text{DEAE}} \) are the counts retained on the nitrocellulose and DEAE membranes, respectively. The concentration of DNA bound to protein, \([\text{DNA}]_{\text{bound}}\), is then given by Equation 5.

\[
[\text{DNA}]_{\text{bound}} = \frac{[\text{DNA}]_{\text{total}}(C_{\text{NCL}} + C_{\text{DEAE}})}{(C_{\text{NCL}} + C_{\text{DEAE}})}
\]

Equations 3–5 are strictly valid only when the protein–nucleic acid complexes are retained on nitrocellulose with 100% efficiency as is true for the Rep-oligodeoxynucleotide complexes studied here. The DEAE membranes were regenerated by soaking in three changes of 1 M NaCl for 10 min each followed by a 1 min rinse in 0.5 M NaOH followed by rinsing in Milli-Q H2O until the pH has returned to neutral.

Non-linear Least Squares Analysis and Simulation of Binding Isotherms for the DNA-induced Rep Dimerization Model—The equilibrium interaction constants (see “Theory”) and their error estimates were derived from analysis of equilibrium binding isotherms using the non-linear least squares analysis method of Johnson and Frasier (1985) on a Hewlett-Packard 9000 computer. The error estimates represent 67% confidence limits. In cases where the error estimates were asymmetric, we have given the range based on the larger value. The function used to model the extent of binding in these analyses is described under “Appendix.” The experimental data points for a binding isotherm were weighted equally. However, in order to resolve the three interaction constants (e.g. \( K_{1s}, L_{ss}, \) and \( K_{ss} \)) it was necessary to perform a simultaneous analysis of multiple isotherms (see “Results”). Theoretical binding isotherms, based on the statistical thermodynamic model presented under “Theory,” were simulated using KaleidaGraph (Synergy Software, Reading, PA) on an Apple Macintosh IIfx computer.

THEORY

Statistical Thermodynamic Model for DNA-induced Rep Dimerization—Equilibrium binding of Rep to DNA was examined using oligodeoxynucleotides that are sufficiently short to preclude contiguous binding of Rep monomers or dimers to a single oligodeoxynucleotide (e.g. \( d(pN)\), with \( 8 \leq n \leq 20 \)) (Chao and Lohman, 1991). Binding equilibria were analyzed using the DNA-induced Rep dimerization model shown in Fig. 1, the details of which are discussed under “Appendix.” The assumptions of the model are: 1) each Rep monomer (P) possesses a single DNA-binding site; 2) each DNA-binding site can bind either ss-DNA (S) or ds-DNA (D); 3) Rep dimers can form when at least one subunit is bound to DNA; 4) both subunits of a Rep dimer (P2) can bind DNA; 5) in the presence of both ss- and ds-DNA, five possible dimeric Rep species can form: the half-saturated dimers, P2S and P2D, or the fully saturated dimers, P2S2, P2D2 or the mixed ligated dimer, P2SD; 6) dimerization of Rep is negligible in the absence of DNA since \( L_2 \leq 10^4 \text{ M}^{-1} \) (Chao and Lohman, 1991).

For clarity we will first discuss the simplified case in which only one type of DNA is present (either S or D). The case in which only ss-DNA is present is represented by the boxed portion of Fig. 1. In general, the six equilibria defined in Equations 6a–6f describe this case

\[
P + S \leftrightarrow PS \quad K_{1s}
\]

\[
PS + P \leftrightarrow P2S \quad L_{ss}
\]

\[
P + P \leftrightarrow P2 \quad L_2
\]

\[
PS + PS \leftrightarrow P2S2 \quad L_{ssS}
\]

\[
P2 + S \leftrightarrow P2S \quad K_{ss}
\]

\[
P2S + S \leftrightarrow P2S2 \quad K_{ssS}
\]

where \( K \) is the macroscopic equilibrium binding constant and \( L \) is the macroscopic Rep dimerization constant (see Equations A-13 under “Appendix” for the relationship to the intrinsic interaction constants). The first subscript of both \( K \) and \( L \) indicates the assembly state of the Rep protein, either monomeric (1) or dimeric (2). The second subscript indicates the type of DNA (single-stranded, S, or duplex, D) that is bound to the Rep monomer or dimer. The presence of a third subscript (S or D) indicates that a second oligodeoxynucleotide is bound to a Rep dimer.

However, only three of these equilibrium constants are needed to describe the Rep-ss oligodeoxynucleotide binding for the following reasons. 1) Since the Rep dimer does not form in the absence of DNA at Rep monomer concentrations less than 8 \( \mu \text{M} \), \( L \leq 10^4 \text{ M}^{-1} \) (Chao and Lohman, 1991), the two equilibria represented in Equations 6c and 6e can be eliminated from consideration under the conditions of our experiments. 2) The remaining four equilibrium constants are not all independent since they are related by the thermodynamic cycle at the top of Fig. 1. We have chosen to describe the equilibrium binding of Rep to short ss oligodeoxynucleotides using the three equilibrium constants \( K_{1s}, L_{ss}, \) and \( K_{ssS} \). \( L_{ssS} \) can then be calculated from Equation 7.

\[
L_{ssS} = L_{ss}K_{ssS}/K_{ss}
\]

We express the experimentally determined extents of DNA binding in either one of two forms: 1) the moles of ss-DNA bound/Rep monomer, \( S_b/P_f \) or 2) the fraction of total ss-DNA bound, \( S_b/S_f \), depending on whether the protein or DNA is held constant throughout the experiment. For the binding of ss-DNA to Rep in the absence of duplex DNA, the expressions for these quantities are given in Equations 8 and 9.

\[
S_b/P_f = (K_{1s}S_f[1 + L_{ss}P_f(1 + 2K_{1ss}S_f)])/(1 + K_{1s}S_f(1 + 2L_{ss}P_f(1 + K_{1ss}S_f)))
\]

\[
S_b/S_f = (K_{1s}P_f(1 + L_{ss}P_f(1 + 2K_{1ss}S_f)))/(1 + K_{1s}P_f(1 + L_{ss}P_f(1 + 2K_{1ss}S_f)))
\]

For clarity we have designated explicitly the free ss-DNA and free Rep monomer concentrations as \( S_f \) and \( P_f \). The situation in the presence of only duplex DNA is exactly analogous requiring an analogous set of three equilibrium constants, \( K_{1D}, L_{DD}, \) and \( K_{DD} \), as illustrated by the bottom
half of Fig. 1. Expressions for \( D_s/D_p \) and \( D_s/D_D \) can be obtained from Equations 8 and 9, by replacing \( K_{1s}, L_{2s}, K_{2s}, \) and \( S \) with \( K_{1d}, L_{2d}, K_{2d}, \) and \( D \), respectively.

In the presence of both ss-DNA and ds-DNA, we must consider the formation of the mixed ligation state Rep dimer, \( P_s\) or \( P_d \), in which ss-DNA is bound to one subunit of the Rep dimer while ds-DNA is bound simultaneously to the other Rep subunit as shown in Fig. 1. \( P_s\) or \( P_d \) can be formed either by binding of \( S \) to \( P \), with binding constant \( K_{1s} \) or by binding of \( D \) to \( P \), with binding constant \( K_{1d} \) or \( K_{1d} \); however, only one of these equilibria is needed to define the equilibrium involving \( P_s\) or \( P_d \). We have chosen to use \( K_{2s} \), from which \( K_{2s} \) can be calculated using Equation 10.

\[
K_{2s} = K_{2s}(K_{1s} L_{2s}/K_{1d} L_{2d})
\]  

(10)

The species, \( P_s\) or \( P_d \), can also be formed by the interaction of \( P \) and \( D \), described by dimerization constant \( L_{2s} \) (see "Appendix"). Therefore, a total of seven independent equilibrium constants \( (K_{1s}, L_{2s}, K_{2s}, K_{1d}, L_{2d}, K_{2d}, \) and \( K_{2d} \)) are needed in order to describe the multiple interactions of Rep in the presence of both ss- and ds-oligodeoxynucleotides that are short enough to prevent multiple binding of Rep monomers to a single oligodeoxynucleotide. The expressions for \( S_s/\) or \( D_s/\) and \( D_s/\) in the presence of both \( S \) and \( D \) are given under "Appendix" (Equations A-11).

To determine these seven interaction constants experimentally, we have adopted the following strategy. First, binding isotherms determined using only ss-DNA are used to determine \( K_{1s}, L_{2s}, \) and \( K_{2s} \). Second, binding isotherms determined using only duplex DNA are used to determine \( K_{1d}, L_{2d}, \) and \( K_{2d} \). Third, to determine the last interaction constant, \( K_{2s} \), experiments are performed to measure the competitive binding of ss-DNA and duplex DNA for Rep. By constraining the six interaction constants \( (K_{1s}, L_{2s}, K_{2s}, K_{1d}, L_{2d}, \) and \( K_{2d} \)) to their values determined from the two sets of independent experiments, the competitive experiments can be analyzed to determine \( K_{2s} \).

RESULTS

**Nitrocellulose Filter Assay to Obtain Equilibrium Binding Isotherms for Rep-Oligodeoxynucleotide Interactions**—Nitrocellulose filter binding has been used extensively to measure equilibrium binding constants for sequence specific protein-DNA interactions (Riggs et al., 1970; Winter et al., 1981; Barkley et al., 1981). However, its use to measure nonspecific DNA-protein interactions is not as widespread due mainly to the fact that for such nonspecific interactions multiple protein molecules can usually bind to each DNA molecule (Clore et al., 1982; Woodbury and von Hippel, 1983). To avoid this problem, we used 16-nucleotide (or base pair) long synthetic oligodeoxynucleotides that are long enough to bind only one Rep monomer/DNA (Chao and Lohman, 1991).

The utility of nitrocellulose filter binding as a quantitative method can also be dependent upon the particular protein-nucleic acid system under study due to a number of system-dependent variables that must first be determined (e.g. efficiency of filter retention and background retention of DNA). Furthermore, the use of this method to study a nonspecific DNA-binding protein such as Rep that also undergoes a DNA-induced dimerization raises additional questions such as whether the efficiency of filter retention differs for DNA that is bound to Rep monomers or dimers. The efficiency of retention of protein-bound DNA was determined to be 100 ± 5% by direct measurement over a wide range of Rep to ss-DNA ratios, under the standard conditions used here (Chao, 1991). Consistent with this finding, we observe that the fraction of DNA bound by Rep always reaches unity at saturation for both ss- and duplex oligodeoxynucleotides (e.g. see Figs. 5A and 7A).

As a further control we also compared the isotherms obtained by nitrocellulose filter binding with those obtained by an independent, spectroscopic method. The fluorescently labeled ss-oligodeoxynucleotide, 5' dT7ATs, prepared as described under "Materials and Methods," shows a 2.75-fold enhancement of its etheno-A fluorescence (\( \lambda_e = 320 \) nm; \( \lambda_{emax} = 400 \) nm) upon binding Rep in our standard conditions at 4°C. Using this fluorescence enhancement to monitor binding, we obtained isotherms from fluorescence titrations performed at two different concentrations of dT7ATs, 0.2 and 1.0 \( \mu \)M and compared them directly with isotherms obtained by nitrocellulose filter binding of radiolabeled dT7ATs. The isotherms obtained by both methods are superimposable at both concentrations; the isotherms obtained at 1.0 \( \mu \)M dT7ATs are compared directly in Fig. 2. We conclude that the nitrocellulose filter binding method can be used to determine true equilibrium binding isotherms for the interaction of Rep with ss- and duplex oligodeoxynucleotides (see below) under the conditions reported here.

Also shown in Fig. 2 is an isotherm determined at 0.1 \( \mu \)M dT7ATs using the nitrocellulose filter binding method. Both isotherms, determined at DNA concentrations differing by a factor of 10, are well described by the DNA-induced Rep dimerization model (see "Theory") with the same interaction constants: \( K_{1s} = 3.9 \times 10^6 \) M\(^{-1}\), \( L_{2s} = 1.1 \times 10^8 \) M\(^{-2}\), and \( K_{2s} = 2.0 \times 10^8 \) M\(^{-1}\). For comparison we have included in Fig. 2 the best-fit isotherm obtained by assuming that a single Rep monomer binds to one molecule of dT7ATs to form a simple 1:1 complex (dashed line; see Equation A-5). Clearly a simple 1:1 binding model fails completely to describe the experimen-
tal binding isotherm obtained at 0.1 \( \mu M \) and also 1 \( \mu M \) dT7eAT8 (curve not shown).

**Binding Isotherms Determined at Constant Rep Concentration**—In Fig. 3, we show Rep-d(T16) binding isotherms determined at constant Rep concentration (0.2 \( \mu M \) monomer) plotted as the fractional saturation of DNA-binding sites, \( \text{Sb}/\text{P}_{7} \), versus the total d(T16) concentration, \( \text{S}_{7} \). Isotherms were obtained under standard conditions (6 mM NaCl, pH 7.5, 4 \( ^\circ \)C) in the presence or absence of 25 mM MgCl2. In the absence of Mg2\(^{+}\) (Fig. 3A), a clear saturation point was reached at 1 d(T16) molecule bound/Rep monomer. Isotherms obtained at lower Mg2\(^{+}\) concentrations (≤5 mM Mg2\(^{+}\)) do reach well-defined saturation end points of \( \text{Sb}/\text{P}_{7} = 1.2 \). More interestingly, while the binding isotherm in the absence of Mg2\(^{+}\) (Fig. 3A) appears monotonic, the binding isotherm in the presence of 25 mM Mg2\(^{+}\) (Fig. 3B) exhibits biphasic character with a distinct intermediate plateau region at 0.5 d(T16) bound/Rep monomer. Since the binding of d(T16) induces dimerization of the Rep protein, the stoichiometry at this plateau reflects the formation of a stable intermediate with 1 d(T16) bound/Rep dimer, P4S. Thus under these conditions, the two subunits of the Rep dimer are filled sequentially.

**Determination of \( K_{\text{ss}} \), \( K_{\text{ssS}} \), and \( L_{\text{ss}} \) for Rep Binding to ss-oligodeoxynucleotides**—As discussed under “Theory,” three independent interaction constants are needed to describe the DNA-binding and DNA-induced Rep dimerization equilibria in the presence of a single conformation of oligodeoxynucleotide, e.g. \( K_{\text{ss}} \), \( K_{\text{ssS}} \), and \( L_{\text{ss}} \) for ss-DNA. However, since these parameters are correlated, particularly \( K_{\text{ss}} \) and \( L_{\text{ss}} \), it is necessary to perform multiple titrations spanning a wide range of both protein and DNA concentrations in order to resolve all three constants. One binding isotherm obtained at a single concentration of Rep or DNA does not contain sufficient information to resolve all three constants unambiguously. Since the three equilibria described by \( K_{\text{ss}} \), \( K_{\text{ssS}} \), and \( L_{\text{ss}} \) show different dependences on DNA and Rep concentrations, information on both the Rep and DNA concentration dependence of the binding isotherms must be obtained. This requires multiple titrations since an individual titration provides information on the concentration dependence of only one truant.

We have used two approaches to resolve all three interaction constants. In the first approach, the requisite Rep and DNA concentration ranges can be spanned by performing a series of titrations of a single type, e.g. titrating a constant DNA concentration with Rep at several DNA concentrations. However, this approach is relatively inefficient. While each titration provides substantial information on the concentration dependence of one species (Rep), information at only one DNA concentration is obtained. Using this approach, we found typically that it was necessary to perform titrations at a minimum of three, but often more, DNA concentrations. An example of this approach is shown in Fig. 4, which shows the fraction of DNA bound, \( \text{Sb}/\text{S}_{7} \), plotted as a function of total Rep monomer concentration, \( \text{P}_{7} \), for four titrations performed over a 50-fold range of dT7eAT8 concentrations.

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**Fig. 3.** Rep-d(T16) binding isotherms obtained at constant Rep concentration. Isotherms were determined by nitrocellulose filter binding methods at constant Rep concentration (0.2 \( \mu M \) monomer) and increasing dT16 concentrations. A, isotherm determined under standard conditions. B, isotherm determined under standard conditions plus 25 mM MgCl2. This isotherm displays significant biphasic character with an intermediate plateau at 0.5 dT16 bound/Rep monomer (1 dT16 bound/Rep dimer). Both isotherms were well-fit by the DNA-induced Rep dimerization model (solid lines) (see Equation 8 in text).

**Fig. 4.** Determination of interaction constants based on multiple titrations of dT16 with Rep protein at fixed DNA concentrations. In order to resolve the individual interaction constants, \( K_{\text{ss}} \), \( K_{\text{ssS}} \), and \( L_{\text{ss}} \), for the Rep-dT16 interaction, four titrations of dT16AT8 with Rep were performed at constant dT7eAT8 concentrations of 0.1 ( ), 0.2 ( ), 1.0 ( ) and 5.0 \( \mu M \) ( ) in standard conditions (6 mM NaCl, pH 7.5, 4 \( ^\circ \)C). Simultaneous non-linear least squares analysis of all four titrations yielded values of \( K_{\text{ss}} = 3.9(\pm 0.5) \times 10^{7} \) M\(^{-1}\), \( L_{\text{ss}} = 1.1(\pm 0.2) \times 10^{8} \) M\(^{-1}\), and \( K_{\text{ssS}} = 2.0(\pm 1.1) \times 10^{9} \) M\(^{-1}\). Solid lines through the experimental isotherms are simulated isotherms based on these same interaction constants for the DNA-induced Rep dimerization model (Equation 9).
(0.1, 0.2, 1.0, and 5.0 μM). Simultaneous analysis of these isotherms by non-linear least squares methods was able to resolve all three interaction constants: $K_{18} = 3.9(±0.5) \times 10^6$ M$^{-1}$, $L_{28} = 1.1(±0.2) \times 10^6$ M$^{-1}$, $K_{SSS} = 2.0(±1.1) \times 10^6$ M$^{-1}$.

The second approach, which we have adopted for routine use, proved to be a more efficient method for resolving the three interaction constants since it involved performing only two titrations: one at constant DNA concentration and the other at constant Rep concentration, such as those shown in Fig. 5. This combinatorial approach hinges on the fact that the two different types of titrations sample the concentration dependence of a different titrant, hence the concentration dependence of both titrants can be obtained from the combination of these two isotherms. The interaction constants determined by this approach based on non-linear least squares analysis of the two isotherms shown in Fig. 5 are: $K_{18} = 4.5(±1.0) \times 10^6$ M$^{-1}$, $L_{28} = 1.3(±0.3) \times 10^6$ M$^{-1}$, $K_{SSS} = 3.8(±1.2) \times 10^6$ M$^{-1}$ as listed in Table I. These values are in excellent agreement with those determined from the series of experiments shown in Fig. 4. The curves describing the isotherms in Fig. 5 are theoretical isotherms using the DNA-induced dimerization model and these interaction constants.

**Table I**

| Rep-oligodeoxynucleotide equilibrium interaction constants | $\times 10^6$ M$^{-1}$ |
|-----------------------------------------------------------|------------------------|
| $P + S \leftrightarrow PS$                                | $K_{18}$ 4.5 (±1.0)    |
| $P + P \leftrightarrow P_S$                               | $L_{28}$ 130 (±30)     |
| $P_S + S \leftrightarrow P_S$                             | $K_{SSS}$ 3.8 (±1.2)   |
| $P + D \leftrightarrow P_D$                               | $K_{KD}$ 0.53 (±0.13)  |
| $P_D + P \leftrightarrow P_D$                             | $L_{DD}$ 270 (±90)     |
| $P_D + D \leftrightarrow P_D,D$                           | $K_{DDP}$ 0.050 (±0.011) |
| $P_S + S \leftrightarrow P_S$                             | $L_{SS}$ 110           |
| $PD + P \leftrightarrow P_D$                              | $L_{DS}$ 19           |
| $PS + PD \leftrightarrow P_S$                             | $L_{SD}$ 60           |
| $2P \leftrightarrow P_2$                                  | $L_{2P}$ ≤0.01        |
| $P_2 + S \leftrightarrow P_2S$                            | $K_{SSS}' ≥6.0 \times 10^6$ |
| $P_2 + D \leftrightarrow P_2D$                             | $K_{SSS}' ≥9.0 \times 10^6$ |

$^a S = dT_{16}$, $D = 16$-bp duplex hairpin (HP).

$^b K_{DSS} = (K_{18}L_{28}K_{KD})/(K_{DD}L_{2D})$.

$^c L_{2S} = (L_{2S}K_{SSS})/K_{DD}$.

$^d L_{SS} = (L_{SS}K_{SSS})/K_{DD}$.

$^e K_{S DD} = (K_{18}L_{28})/L_{DD}$.

$^f K_{SD} = (K_{18}L_{28})/L_{2D}$.

**Effects of Single-stranded DNA Length**—The number of nucleotides occluded by a Rep monomer bound to ss-DNA (apparent site size) has been estimated to be 16 ± 2 nucleotides in experiments with ss-homopolynucleotides (Chao, 1991; Chao and Lohman, 1991). This apparent site size provides an estimate of the number of nucleotides occluded/Rep monomer; however, it does not provide an estimate of the length of the oligodeoxynucleotide, $m$, needed so that all contacts are made with the protein (Kelly et al., 1976; Draper and von Hippel, 1978). In order to examine this, we have measured $K_{18}$, $K_{SSS}$, and $L_{2S}$, using nitrocellulose filter binding as described above, for a series of oligodeoxynucleotides, $d(pT)_m$, with $n = 8, 12, 14, 16, and 20$ in standard buffer (6 mM NaCl, pH 7.5, 4 °C). Each of these oligodeoxynucleotides binds to a stoichiometry of one oligonucleotide/Rep monomer. The interaction constants are plotted in Fig. 6 as a function of ss-DNA length and are given in Table II. While all three interaction constants increase with increasing ss-DNA length, the two Rep-DNA-binding constants, $K_{18}$ and $K_{SSS}$, exhibit abrupt 5- and 16-fold increases, respectively, upon increasing the ss-DNA length from $n = 12$ to $n = 14$ nucleotides. In contrast, the Rep dimerization constant $L_{2S}$ shows only a gradual increase from 6.6(±1.6) $\times 10^6$ M$^{-1}$ to 2.7(±0.9) $\times 10^6$ M$^{-1}$ over the range of lengths tested from $n = 8$ to 20. These results suggest that $d(pT)_14$ is not long enough to make all of the contacts within the Rep-binding site, whereas all contacts are made with $d(pT)_20$. Therefore, we conclude that $12 < m \leq 14$, which is consistent with the observed occluded site size of 16 ± 2 nucleotides on ss-DNA.

An interesting feature of Fig. 6 is that $K_{SSS}$, the apparent equilibrium constant for binding a second ss-oligodeoxynucleotide to the half-ligated Rep dimer, $P_S$, decreases by a factor of 4 upon increasing the ss-DNA length from $n = 16$ to $n = 20$. This effect is not observed for $K_{18}$, the affinity of a Rep monomer for an oligodeoxynucleotide, which actually displays a slight increase. Therefore, it seems unlikely that the decrease in $K_{SSS}$ reflects a direct effect on the intrinsic Rep-ss-DNA binding affinity. Since $d(pT)_20$ is slightly longer than the occluded site size, it seems more likely that when bound to one subunit in the $P_S$:Rep complex, it can also partially occupy the unfilled DNA-binding site on the second Rep subunit thereby inhibiting the binding of a second molecule of $dT_{20}$ at

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**Fig. 5.** Determination of Rep-$dT_{14}$ interaction constants based on simultaneous analysis of two different isotherms obtained at constant DNA and constant Rep concentrations. All three interaction constants, $K_{18}$, $L_{28}$, and $K_{SSS}$, can be resolved from the simultaneous analysis of two titrations determined under identical conditions (6 mM NaCl, pH 7.5, 4 °C): titration of $dT_{14}$ with Rep at constant $dT_{14}$ concentration (0.1 μM) (A); titration of Rep with $dT_{14}$ at constant Rep concentration (0.2 μM monomer) (B). Simultaneous non-linear least squares analysis of the two isotherms, based on the DNA-induced Rep dimerization model yields $K_{18} = 4.5(±1.0) \times 10^6$ M$^{-1}$, $L_{28} = 1.3(±0.3) \times 10^6$ M$^{-1}$, and $K_{SSS} = 3.8(±1.2) \times 10^6$ M$^{-1}$. The solid lines are simulated isotherms based on Equation 9 (panel A) and Equation 8 (panel B) and these parameters.

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**Fig. 6.** Determination of Rep-DNA interaction constants for a series of oligodeoxynucleotides, $d(pT)_m$, with $n = 8, 12, 14, 16, and 20$ in standard buffer (6 mM NaCl, pH 7.5, 4 °C). Each of these oligodeoxynucleotides binds with a stoichiometry of one oligonucleotide/Rep monomer. The interaction constants are plotted in Fig. 6 as a function of ss-DNA length and are given in Table II. While all three interaction constants increase with increasing ss-DNA length, the two Rep-DNA-binding constants, $K_{18}$ and $K_{SSS}$, exhibit abrupt 5- and 16-fold increases, respectively, upon increasing the ss-DNA length from $n = 12$ to $n = 14$ nucleotides. In contrast, the Rep dimerization constant $L_{2S}$ shows only a gradual increase from 6.6(±1.6) $\times 10^6$ M$^{-1}$ to 2.7(±0.9) $\times 10^6$ M$^{-1}$ over the range of lengths tested from $n = 8$ to 20. These results suggest that $d(pT)_14$ is not long enough to make all of the contacts within the Rep-binding site, whereas all contacts are made with $d(pT)_20$. Therefore, we conclude that $12 < m \leq 14$, which is consistent with the observed occluded site size of 16 ± 2 nucleotides on ss-DNA.
that site. In light of these results, we performed experiments comparing ss- and ds-DNA with oligodeoxynucleotides that are 16 nucleotides or base pairs in length.

Effects of DNA Sequence and Base Composition on Rep-ss-DNA Interactions—The E. coli Rep helicase is a nonspecific DNA-binding protein that can unwind duplex DNA independent of its sequence. We examined the interactions of Rep with several 16-nucleotide long ss-oligodeoxynucleotides in order to determine the effect of base composition on the Rep equilibrium binding and dimerization constants. Three of these, d(pT)\textsubscript{16}, d(pA)\textsubscript{16}, and d(pC)\textsubscript{16}, are homo-oligodeoxynucleotides and a fourth, KL3, has a mixed sequence with 50% GC content (see “Materials and Methods”). Table III indicates that the interaction constants, \(K_{IS}, K_{SS}, L_{IS},\) and \(L_{SS}\), for d(pT)\textsubscript{16}, d(pC)\textsubscript{16}, and the mixed sequence oligodeoxynucleotide KL3 are identical within experimental error. Binding of d(pA)\textsubscript{16} to Rep shows a 2-3-fold lower affinity (both for \(K_{IS}\) and \(K_{SS}\)) relative to the other oligodeoxynucleotides examined, although the Rep dimerization is unaffected. Based on these data, we conclude that the equilibrium binding of Rep to ss-DNA is independent of ss-DNA sequence under the conditions of these experiments (6 mM NaCl, pH 7.5, 4°C) and that experiments performed with oligodeoxynucleotides reflect the general binding properties of Rep to ss-DNA. This might have been anticipated for a protein that binds nonspecifically to ss-DNA, although most well-characterized ss-DNA-binding proteins, such as the E. coli SSB protein (Overman et al., 1988), the T4 phage gene 32 protein (Newport et al., 1981), and the fd phage gene 5 protein (Sang and Gray, 1989) display a surprisingly large dependence of affinity on ss-DNA base composition.

**Table II**

| ss-DNA length-dependence of Rep interaction constants (6 mM NaCl, pH 7.5, 4°C) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| \(n\) | \(K_{IS}\) | \(K_{SS}\) | \(L_{IS}\) | \(L_{SS}\) |
|-----------------|-------------------|-------------------|-------------------|-------------------|
| 8 | 0.19 (±0.04) | 0.043 (±0.007) | 66 (±15) | 14 |
| 12 | 0.47 (±0.56) | 0.15 (±0.02) | 69 (±11) | 22 |
| 14 | 2.1 (±0.6) | 2.4 (±0.19) | 100 (±50) | 114 |
| 16 | 4.5 (±1.0) | 3.8 (±1.2) | 130 (±30) | 110 |
| 20 | 6.5 (±2.3) | 0.97 (±0.19) | 240 (±120) | 36 |

* \(L_{SS} = (L_{IS}K_{SS})/K_{IS}\).*
DNA-induced Rep Dimerization

In order to determine whether ss-DNA and ds-DNA bind to the same or separate DNA-binding sites, we examined the characteristics of the competitive binding of d(pT)_{16} and the HP duplex to Rep. These experiments were performed under standard buffer conditions (6 mM NaCl, pH 7.5, 4 °C) starting with preformed Rep-d(pT)_{16} complexes (4 μM Rep monomer plus 4 μM d(pT)_{16}) which were then titrated with HP duplex. Two parallel experiments were performed under identical conditions. In one case, preformed Rep-^{32}P-d(pT)_{16} complexes were titrated with unlabeled HP duplex, while the extent of ^{32}P-(d(pT))_{16} bound to Rep was monitored. In the second experiment, preformed Rep-d(pT)_{16} complexes were titrated with ^{32}P-HP duplex and the binding of the ^{32}P-HP duplex to Rep was monitored. The results of both experiments are shown in Fig. 8A and indicate that ss-DNA binding to the same sites on Rep monomers and dimers.

The data in Fig. 8A were also analyzed to determine the extent to which the mixed ligation species P_{2SD} can form under these conditions. Simulation of these competition curves based on the DNA-induced Rep dimerization model requires independent knowledge of the seven interaction constants, K_{2sD}, K_{1sD}, L_{2S}, K_{1D}, K_{2DD}, and L_{2D} (see "Theory" and "Appendix"). However, six of these, K_{1sD}, K_{2sD}, L_{2S} and K_{1D}, K_{2DD}, and L_{2D} have already been determined from the independent binding experiments performed separately with d(pT)_{16} and the hairpin dimer oligodeoxynucleotides. Therefore, the competition experiments can be analyzed to obtain the remaining interaction constant, K_{2DD}. Using the values of K_{1s}, K_{2sS}, L_{2S} and K_{1D}, K_{2DD}, and L_{2D} listed in Table I, analysis of the competition experiments in Fig. 8A yields a best-fit value of K_{2DD} = 3.3±(1.3) × 10^{6} M^{-1} (6 mM NaCl, pH 7.5, 4 °C). From Equation 10, we calculate a value for K_{2DS} = 1.0 × 10^{8} M^{-1}. The smooth curves describing the competition experiments in Fig. 8A are simulated curves based on the DNA-induced Rep dimerization model and these seven interaction constants (see Equations A-11). For comparison, Fig. 8A also shows simulated curves for a value of K_{2SD} = 5 × 10^{10} M^{-1} (dashed lines) indicating the sensitivity of these competition isotherms to the value of K_{2DD}. The agreement between the simulated curves based on the DNA-induced Rep dimerization model and the experimental competition isotherms lends additional support to the conclusion that this model provides an excellent quantitative description of the binding of both duplex and ss-oligodeoxynucleotides to the Rep protein. Fig. 8B shows the predicted population distributions of the three doubly ligated Rep dimer species, P_{2SD}, P_{2DD}, and P_{2SD}, based on Equations A-3 and the seven interaction constants.

**DISCUSSION**

The *E. coli* Rep helicase is a stable monomer up to concentrations of at least 8 μM monomer, even in the presence of...
DNA-induced Rep Dimerization

The major results of our studies are as follows.

1) Monomers of Rep can bind both ss- and duplex DNA, both of which induce Rep dimerization. Under the conditions used in these studies (6 mM NaCl, pH 7.5, 4 °C), ss-DNA binds with higher affinity than duplex DNA (see Table I). We have been able to resolve all seven interaction constants needed to describe Rep binding to ss- and duplex oligodeoxynucleotides and the DNA-induced Rep dimerization as defined by the statistical thermodynamic model in Fig. 1.

2) Rep monomers are induced to dimerize upon binding ss- or ds-DNA. The dimerization constant for DNA-bound Rep increases by at least a factor of ~10^4 (LogS, LogD and LogSS ~1–3 × 10^8 M^-1) (6 mM NaCl, pH 7.5, 4 °C), since its maximum value in the absence of DNA is L ≤ 10^4 M^-1 (Chao and Lohman, 1991). Fig. 9 shows a plot of the fraction of Rep protein in the dimeric form as a function of total Rep concentration in the presence of an equal molar concentration of d(pT)IG. Fully 90% of the Rep protein is in a dimeric form at Rep and d(pT)IG concentrations of 100 nM. Since Rep must be bound to DNA in order to function as a helicase these results are consistent with a dimer as the active form of the Rep helicase.

3) Rep dimerization enhances dramatically its affinity for both ss- and ds-DNA.

4) Both protomers of a Rep dimer can bind ss- or ds-DNA to the same sites.

5) We observe dramatic allosteric effects in this system. The affinity for DNA of one protomer of a Rep dimer is

![Graph showing competitive binding of ss- and ds-oligodeoxynucleotides to the Rep protein.](image-url)

**Fig. 8.** Competitive binding of ss- and ds-oligodeoxynucleotides to the Rep protein. A, competitive binding of ss- and ds-oligodeoxynucleotides to Rep was determined under standard buffer conditions (6 mM NaCl, pH 7.5, 4 °C). A preformed complex of Rep (4 μM monomer) and d(pT)IG (4 μM) was titrated with increasing concentrations of the 16-bp hairpin duplex, HP. Two separate experiments were performed in parallel under identical conditions using either [2P]-d(pT)IG (B) or [3P]-HP (C) to monitor the extent of Rep binding to ss- versus ds-DNA, respectively, as a function of HP concentration using nitrocellulose filter binding. Solid lines represent theoretical isotherms simulated using Equations A-11 and the values of Kss, KSS, Lss, Ksd, Kdd, and LSD listed in Table I and a binding constant for the formation of the mixed ligation state, Ksd = 3.3±1.3 × 10^6 M^-1. Competitive isotherms were simulated for a series of values of Ksd, holding the other six parameters constant, until agreement was obtained between the experimental and simulated isotherms. The dashed curves represent simulated isotherms for Ksd = 5 × 10^8 M^-1, showing the sensitivity of the competitive isotherms to the value of Ksd. The population distributions of the three possible doubly-ligated Rep dimers, PssPsd, PsdPsd, and PssDsd, were calculated for the exact Rep, ss- and ds-DNA concentrations of the experiments in panel A, using the values of the interaction constants listed in Table I (6 mM NaCl, pH 7.5, 4 °C). Significant accumulation of the mixed ligation state, PssDsd, occurs under these conditions.

nucleotide cofactors (Lohman et al., 1989; Arai et al., 1981); however, the protein is induced to dimerize upon binding either ss- or duplex DNA (Chao and Lohman, 1991). Furthermore, a chemically cross-linked Rep dimer retains its ss-DNA-dependent ATPase and DNA helicase activity (Chao and Lohman, 1991), which suggests strongly that the dimer is the active form of the Rep helicase. Higher order assembly states beyond the dimer have not been observed. However, these previous studies did not determine whether both subunits of the Rep dimer are able to bind DNA simultaneously. In an attempt to understand how a DNA-induced Rep dimer might function to unwind duplex DNA, we sought to determine the stoichiometries and affinities of the various Rep-DNA complexes, with the ultimate goal of determining whether these might be influenced by ATP binding and subsequent hydrolysis. For this purpose we have developed a statistical thermodynamic model that describes DNA binding to Rep monomers and the coupled DNA-induced Rep dimerization. Using a modified double-filter nitrocellulose filter binding method, which improves significantly the quality of binding isotherms and the ease of data acquisition, we have examined the equilibrium binding of Rep to a series of single- and double-stranded oligodeoxynucleotides. These studies were performed with oligodeoxynucleotides that were sufficiently short so that only one Rep monomer can bind to each oligodeoxynucleotide, thus enabling us to examine true DNA-induced dimerization in the absence of adventitious binding of two monomers to a single DNA molecule.

![Graph showing fraction of Rep protein found in the dimeric form as a function of Rep concentration.](image-url)

**Fig. 9.** Fraction of Rep protein found in the dimeric form as a function of Rep concentration. The fraction of Rep protein that is induced to form dimers upon binding d(pT)IG is shown plotted as a function of Rep concentration under conditions such that the total concentrations of Rep (monomer) and d(pT)IG are equal at each Rep concentration. This fraction of Rep dimer is defined as (2Pss + 2Psd)/(PS + 2Pss + 2Psd) and is calculated using the interaction constants in Table I (6 mM NaCl, pH 7.5, 4 °C).
dependent on the type of DNA (ss or duplex) that occupies
the other protomer.

6) The minimum length of ss-DNA needed to form all
contacts with a Rep monomer is $12 < m < 14$ nucleotides.

7) Little dependence of Rep-ss DNA affinity on base com-
position or sequence is observed (see Table III), consistent
with the role of Rep as a nonspecific DNA-binding protein
and its ability to unwind duplex DNA independent of se-
quence.

We discuss these points in more detail below.

Analysis of Binding Isotherms to Determine Equilibrium
Interaction Constants—Quantitative determination of the
various interaction constants that describe the multiple Rep-
DNA equilibria is essential for obtaining a molecular under-
standing of the functional energetics of the DNA unwinding
process. We have found that only two titrations, one at
constant DNA and one at constant Rep concentration, are
necessary in principle to determine the three independent
equilibrium constants that describe the interaction of Rep
with a single conformation of oligodeoxynucleotide, e.g. ss-
DNA. The reason for this is apparent from inspection of Fig.
10, which shows the population distributions of the three
DNA-bound Rep species (e.g. PS, P,S, P,S) as calculated
from Equations A-3 (see "Appendix") and the values of $K_{1s},
L_{as},$ and $K_{ess}$ in Table I. These population distributions were
calculated for the conditions and concentrations correspond-
ing to the two binding isotherms in Fig. 5. It is clear from Fig.

![Fig. 10. Relative population distribution of Rep-d(pT)$_{16}$
complexes. The population distributions of the three species of Rep-
d(pT)$_{16}$ complexes, PS, P,S, and P,S, shown as solid lines, were
calculated using the interaction constants listed in Table I for titra-
tions at 0.1 $\mu$m dT$_{16}$ (panel A) and 0.2 $\mu$m Rep (panel B), correspond-
ing to the experimental titrations shown in Fig. 5. Dashed lines show
the total isotherms ($S_{a}/S_1$ in panel A and $S_{a}/P_1$ in panel B).]

![Fig. 11. Relative population distribution of Rep-duplex ol-
godeoxynucleotide complexes. The population distributions of the three species of Rep-hairpin duplex complexes, PD$_D$, P,D, and
P,P,D, shown as solid lines, were calculated using the interaction
constants listed in Table I for titrations at 0.1 $\mu$m HP (panel A) and
0.2 $\mu$m Rep (panel B), corresponding to the experimental titrations
shown in Fig. 7. Dashed lines show the total isotherms ($D_6/D_7$ in
panel A and $D_6/P_7$ in panel B). The species P,D was not shown in
panel A due to its negligible accumulation under these conditions.]
merization model (Fig. 1) indicates that this is the minimal scheme needed to describe the multiple equilibria for this system.

The use of the double-filter nitrocellulose filter binding assay has facilitated greatly our ability to resolve the seven independent interaction constants that describe the DNA-induced Rep dimerization. The introduction of a DEAE membrane and the use of a dot-blot apparatus, coupled with direct counting of the radioactivity on both the nitrocellulose and DEAE membranes are modifications that should be generally applicable for use with any protein-nucleic acid interaction that can be studied by nitrocellulose filter binding.

Rep Dimers Bind DNA with Substantially Higher Affinity Than Rep Monomers—Chao and Lohman (1991) have shown that the affinity of ss-DNA for a chemically cross-linked Rep dimer is substantially higher than for a Rep monomer. We cannot measure directly the affinity of an unliganded Rep dimer for DNA, since unliganded Rep dimers are not populated at the concentrations used in our experiments (L0 ≤ 10^4 M^{-1}; Chao and Lohman, 1991). However, we can estimate lower limits for K_Ds and K_Dd, the macroscopic binding constants for ss- and ds-oligodeoxynucleotide binding to an isolated Rep dimer, based on the estimate of L0 and the relationship K_Ds = K_DsL0/L0. We estimate that both KDs/Ks and KDd/KD ≥ 7 × 10^7, i.e. a nearly 10,000-fold increase in intrinsic binding affinity results from dimerization (see Equations A-13 in “Appendix” for definitions of the intrinsic constants). The fact that Rep dimerization is induced upon binding DNA is reflected directly in the magnitude of the ratio of these binding constants.

A comparison of the intrinsic constants for binding (pT)_{i6} to a subunit of a preformed Rep dimer versus the second protomer of a half-saturated Rep dimer shows that Ks/ks ≥ 4 × 10^7. This indicates a dramatic negative cooperativity for binding (pT)_{i6} to the second protomer of the Rep dimer. A similar comparison for ds-DNA indicates kDsd/kDd25 × 10^7. These substantial differences may be functionally important, since they indicate that a Rep dimer can remain tightly bound to DNA with one protomer while the other protomer can exchange DNA as, for example, during an unwinding reaction.

Allosteric Effects on DNA Binding to the Rep Dimer—Our results indicate that two molecules of either ss- or ds-DNA can bind to a Rep dimer to form the ternary complexes P_{2S} or P_{2D} respectively. Furthermore, a Rep dimer can also form the mixed ligation species P_{SD}, in which one molecule each of ss- and ds-DNA is bound to the Rep dimer simultaneously. Furthermore, our results also show that ss- and ds-DNA both compete for the same binding sites on the Rep dimer. This differs from the conclusion reached by Arai et al. (1981) that individual Rep monomers possess distinct sites for ss- and ds-DNA, although their studies were carried out with polymeric ss- and ds-DNA.

Significant allosteric effects are also apparent in this system, i.e. the binding of ss- or ds-DNA to the second protomer of a half-saturated Rep dimer is clearly influenced by the type of DNA that occupies the first subunit (see Table 1). The following general conclusions can be drawn (6 mM NaCl, pH 7.5, 4 °C): (i) The affinity of either ss- or duplex DNA to the second protomer is always higher if the first protomer is filled with ss-DNA (e.g. Kss ≈ 4K_{DS} and K_{SD} ≈ 7K_{DD}); (ii) ss-DNA always binds to the second protomer with higher affinity than duplex DNA (Kss ≈ 12K_{SD} and K_{DS} ≈ 20K_{DD}). These allosteric effects suggest that different conformations of the Rep protein are stabilized by the type of bound DNA and this likely involves communication across the Rep dimer interface. The observation of such allosteric effects suggests to us the possibility that these ternary complexes may mimic intermediate complexes that can occur at an unwinding fork and that are important in DNA unwinding.

Implications for Rep-catalyzed DNA Unwinding—The ability of the Rep protein to dimerize has important implications for how this helicase might interact with an unwinding fork (Chao and Lohman, 1991). Since each Rep protomer can bind DNA, then at an unwinding fork, the dimer is likely to bind two different regions of DNA simultaneously to form ternary complexes. The formation of ternary structures in which the Rep dimer binds either two regions of ss-DNA (P_{S}S_{2}) or a region of ss-DNA and a region of duplex DNA (P_{S}D_{2}) would seem to be the most likely models for intermediates at a replication fork. Three possibilities for such ternary complexes are depicted in Fig. 12. In Fig. 12A, both Rep protomers are bound to ss-DNA, one on the leading strand and the other on the lagging strand, whereas in Fig. 12B, both Rep protomers are bound to ss-DNA, but on the same (leading) strand. Although both protomers might also be bound to the lagging strand (not shown), the apparent 3' to 5' polarity of unwinding observed for Rep (Yarranton and Getfer, 1979; Lohman et al., 1989; Runyon and Lohman, 1989) suggests that this binding mode is not productive. Finally, Fig. 12C depicts a mixed ligation binding mode in which one protomer is bound to ss-DNA on the leading strand while the other protomer is bound to ds-DNA ahead of the fork. We consider it unlikely that a Rep dimer would bind two regions of duplex DNA simultaneously at an unwinding fork due to the intrinsic stiffness of duplex B-form DNA (Hagerman, 1988); hence, we do not consider a P_{D}S_{2} species to be important functionally. The mixed ligation species, P_{SD}, is of special interest since the transient formation of a ternary complex consisting of Rep, ds-DNA, and ss-DNA has been postulated in models for unwinding (Lohman, 1992).

Oligomeric Nature of Helicases—The results reported here are consistent with previous results which have shown that a cross-linked Rep dimer retains helicase activity (Chao and Lohman, 1991) suggest that the functionally active form of the Rep helicase is a dimer. Furthermore, it has been suggested that the active forms of most helicases might be oligomeric (Loh-
man, 1992). Other examples of oligomeric helices include hexamers such as *E. coli* DnaB (Reha-Krantz and Hurwitz, 1978), SV40 large T antigen (Mastrangelo et al., 1989), *E. coli* Rho (Finger and Richardson, 1982), and dimers such as *E. coli* Helicase III (Yarranton et al., 1979), phage T7 gene 4 protein, a human (HeLa) helicase (Seo et al., 1991), and HSV-1 origin binding protein (Bruckner et al., 1991). The RecBCD protein, a helicase involved in recombination (Smith, 1990; Roman and Kowalczykowski, 1990a, 1990b) forms at least a heterotrimer, although one measurement of its native molecular weight is consistent with it being a hexamer, i.e. (RecBCD)3 (Dykstra et al., 1984). *E. coli* Helicase II (uprD)4 and the phage T4 gene 41 protein (Liu and Alberts, 1981) also oligomerize, although the final assembly state as well as its assembly state on the DNA has not yet been defined. In fact, for the few cases of helicases that appear to be “monomeric,” the assembly state of these helicases when bound to DNA has not been examined. Since Rep is clearly induced to dimerize only upon binding DNA (Chao and Lohman, 1991), this needs to be examined for other helicases that appear to be monomeric in the absence of DNA.

The fact that helicases appear generally to be oligomeric suggests that this is an important feature for their mechanism of action. Such oligomeric structures would provide a relatively simple means to generate multiple DNA-binding sites within a helicase (Lohman, 1992). The fact that both DNA-binding sites of a Rep dimer can accommodate either DNA or duplex DNA suggests unwinding mechanisms in which the two sites on the Rep dimer alternate binding of ss- and duplex DNA, modulated by the binding and subsequent hydrolysis of ATP (Lohman, 1992). Such unwinding mechanisms may be used generally by helicases that contain multiple DNA-binding sites provided by oligomeric structures.

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**APPENDIX**

**Statistical Thermodynamic Model for the DNA-induced Rep Dimerization**

**Definition of Equilibrium Interaction Constants**—The statistical thermodynamic model used to describe the DNA-induced Rep dimerization in the presence of both ss- and ds-oligodeoxynucleotides that are short enough to preclude multiple binding of Rep monomers to a single oligodeoxynucleotide is depicted in Fig. 1 and is defined explicitly by 11 equilibria among eight distinct states (see Fig. 1 and “Theory”). Therefore, seven independent equilibrium interaction constants are needed to describe this equilibrium and we have chosen the seven macroscopic interaction constants defined in Equations (A-1).

\[
P + S \leftrightarrow PS \quad K_{SS} = [PS]/[P][S] \quad (A-1a)
\]

\[
PS + P \leftrightarrow P_S S \quad L_{SS} = [PS]/[P][PS] \quad (A-1b)
\]

\[
P_S S + S \leftrightarrow P_S S_2 \quad K_{SS_2} = [P_S S_2]/[P_S S][S] \quad (A-1c)
\]

\[
P + D \leftrightarrow PD \quad K_{DD} = [PD]/[P][D] \quad (A-1d)
\]

\[
PD + P \leftrightarrow P_D D \quad L_{DD} = [P_D D]/[P][PD] \quad (A-1e)
\]

\[
P_D D + D \leftrightarrow P_D D_2 \quad K_{DD_2} = [P_D D_2]/[P_D D][D] \quad (A-1f)
\]

\[
P_S D + D \leftrightarrow P_S D D \quad K_{SD} = [PS D D]/[P_S D][D] \quad (A-1g)
\]

where S, P, and D represent ss-oligodeoxynucleotide, Rep monomer, and ds-oligodeoxynucleotide, respectively, and the free DNA and Rep monomers are designated explicitly with the subscript f.

Since Rep dimers do not form in the absence of DNA binding up to Rep monomer concentrations of at least 8 μM, indicating that \( L_2 \leq 10^8 \text{ M}^{-1} \) (Chao and Lohman, 1991), any equilibria involving free Rep dimers, \( P_D \), can be neglected under the conditions of our experiments, i.e. \( K_{SD} \) and \( L_2 \) as defined in Equations A-1h and A-1i need not be considered.

\[
P_F + S \leftrightarrow P_S S \quad K_{SS} = [PS]/[P][S] \quad (A-1h)
\]

\[
P + P \leftrightarrow P_2 \quad L_2 = [P_2]/[P]^2 \quad (A-1i)
\]

The remaining four interaction constants can be defined in terms of the seven parameters as in Equation A-2.

\[
PS + P_S \leftrightarrow P_S S_2 \quad L_{SS} = L_2 K_{SS_2}/K_{SS} \quad (A-2a)
\]

\[
PD + P_D \leftrightarrow P_D D \quad L_{DD} = L_2 K_{DD_2}/K_{DD} \quad (A-2b)
\]

\[
PS + PD \leftrightarrow P_S D D \quad L_{SD} = L_2 K_{SD}/K_{SD} \quad (A-2c)
\]

\[
P_S D + S \leftrightarrow P_S D D \quad K_{SD} = K_{SD} L_2 K_{DD_2}/K_{DD} \quad (A-2d)
\]

The concentrations of all distinct Rep ligation states can be written in terms of \( P_F \), \( S_F \), and \( D_F \) as in Equations (A-3).

\[
PS = K_D P_S S \quad (A-3a)
\]

\[
P_S = K_D L_2 P_S S \quad (A-3b)
\]

\[
P_S S = K_{SS} L_2 K_{SS_2} P_S S \quad (A-3c)
\]

\[
PD = K_D P_D D \quad (A-3d)
\]

\[
P_D D = K_D L_2 P_D D \quad (A-3e)
\]

\[
P_S D = K_{SD} L_2 K_{DD_2} P_S D \quad (A-3f)
\]

\[
P_S D D = K_{SD} L_2 K_{DD} P_S D D \quad (A-3g)
\]

**Simulation of the Binding Isotherm in the Absence of Competition**—In the presence of only one conformation of DNA (either ss or ds), the number of protein states reduces to 4 (e.g. P, PS, SPS, and P_S). In this case only three of the seven interaction constants are needed. We have used \( K_{SS} \), \( L_2 \), and \( K_{SD} \) to describe the ss-DNA binding equilibria and \( K_{DD} \) and \( K_{DD_2} \) for the ds-DNA binding equilibria. We describe below the methods for simulation of Rep binding isotherms obtained in the presence of only ss-DNA; however, exactly analogous equations apply in the case of only ds-DNA.

The extent of ss-oligodeoxynucleotide binding can be expressed either as the fraction of bound ss-DNA, \( S/S_T \), or as the fractional saturation of DNA-binding sites assuming one binding site/Rep monomer, \( S_F/P_F \). In practice, binding is normalized with respect to the species (protein or DNA) whose concentration is held constant during the titration. Thus, extent of binding is defined in terms of \( S_T \) and \( S_T/S_T \) are given in Equations A-4.

\[
S_T/P_T = (PS + P_S S + 2P_S S_2)/S_T + PS + 2P_S S + 2P_S S_2 = K_{SS} S_T (1 + L_2 P_F (1 + 2K_{SS_2} S_T))/[1 + K_{SS} S_T] (1 + 2L_2 P_F (1 + K_{SS_2} S_T)) \quad (A-4a)
\]

\[
S_T/S_T = (PS + P_S S + 2P_S S_2)/S_T + PS + P_S S + 2P_S S_2 = K_{SS} P_F (1 + L_2 P_F (1 + 2K_{SS_2} S_T))/[1 + K_{SS} P_F (1 + L_2 P_F (1 + 2K_{SS_2} S_T))] \quad (A-4b)
\]

In the absence of dimerization, Equation A-4a reduces to the

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simple Langmuir isotherm for 1:1 complex formation given in Equation A-5.

\[ S_i/P_T = (K_{iS_i})/(1 + K_{iS_i}) \] (A-5)

To simulate theoretical isotherms, we start with the mass conservation equations for \( S_i \) and \( P_r \) in Equations A-6.

\[ S_r = (1 + K_{sP_r} + K_{sS_P})S_i + 2K_{sLsS_P}P_T \] (A-6a)
\[ P_r = (1 + K_{sS_i}P_T + 2K_{sLsS_i} + K_{sLsS_P})P_T \] (A-6b)

From Equations A-6, explicit expressions for \( S_r \) as a function of \( P_r \) and \( S_T \) (Equation A-7a), and for \( P_r \) as a function of \( S_r \) and \( P_T \) (Equation A-7b) can be obtained.

\[ S_r = \left((-1 + K_{sP_r}(1 + L_{SP}) + (1 + K_{sP_T})/\left(1 + L_{SP})\right)^2 + 8K_{sLsS_P}S_T/\left(1 - L_{SP}/(1 + K_{sS_i})\right)\right)/4(1 + L_{SP}/(1 + K_{sS_i})) \] (A-7a)
\[ P_r = \left((-1 + K_{sS_i}) + (1 + K_{sS_i}) + 8(K_{sLsS_P}/(1 + K_{sS_i}))\right)/4(1 + L_{SP}/(1 + K_{sS_i})) \] (A-7b)

For titrations performed at constant DNA concentration, the value of \( S_r \) is known and remains invariant throughout the titration. We can therefore use Equation A-7a to calculate \( S_r \) explicitly for an assumed value of \( P_r \). For each \( S_T \) (or \( S_r \)) pair, the fraction of DNA bound, \( S_T/S_P \), and the total concentration of Rep monomer, \( P_T \), are easily calculated using Equations A-4b and A-6b, respectively. An entire binding isotherm can therefore be simulated by repeating this process iteratively while incrementing \( P_T \). To simulate binding isotherms for titrations at constant total Rep monomer concentration, \( P_T \), Equation A-7b can be used to calculate \( P_r \) explicitly for an assumed value of \( S_r \), and the binding isotherm is generated by solving for \( S_r/P_T \) and \( S_T \) using Equations A-4a and A-6a, respectively.

Using this algorithm, binding isotherms can be simulated with relative ease and compared graphically with isotherms obtained experimentally. However, using this algorithm, both the abscissa, either \( P_T \) or \( S_T \), and the ordinate, either \( S_T/P_T \) or \( S_T/S_T \), of the isotherm are derived values, i.e. the algorithm does not define an explicit relationship between the two. As a result, this algorithm, although intuitive, cannot be used to calculate directly the extent of binding for a given total concentration of tetrant, \( P_T \) or \( S_T \), whereas this capability is useful for computational purposes related to non-linear least squares analysis.

To derive the free concentrations of both tetrants, \( P_T \) and \( S_T \), from the known total concentrations, \( P_T \) and \( S_T \), requires simultaneous solution of the two quadratic equations given in Equations A-6a and A-6b. To do this, we use the Newton-Raphson method of root finding to solve iteratively for \( P_T \) and \( S_T \). The root-finding function, \( F(P_T) \), defined by rearrangement of Equation A-6b.

\[ F(P_T) = P_T + K_{sS_i}P_T + 2K_{sLsS_P}P_T^2 + 2K_{sLsS_P}S_T^2 - P_T \] (A-8)

Subtraction of Equation A-7b from A-7a and subsequent rearrangement yields the expression for \( S_i \) in Equation A-9.

\[ S_i = \left(P_T - P_T - S_T)/\left(1 - K_{sLsP_T}\right)\right) \] (A-9)

which can be substituted into Equation A-8. This substitution eliminates \( S_i \) and the need to search simultaneously for \( P_T \) and \( S_T \), and consequently greatly improves the robustness of the root-finding algorithm. A Fortran implementation of this algorithm, used in conjunction with the non-linear least squares fitting algorithm NONLIN (Johnson and Frasier, 1985), provides direct error estimates for the best-fit parameters.

Simulation of Binding Isotherms in the Presence of Both ss- and ds- oligonucleotides—In the presence of both \( S \) and \( D \), all seven interaction constants listed in Equations A-1 are needed to describe the system in a set of three simultaneous quadratic equations as defined by the mass conservation Equations A-10.

\[ S_r = S_i[1 + K_{sP_r}(1 + L_{SP})(1 + K_{sdD_i})] \] (A-10a)
\[ D_T = D_i[1 + K_{sP_r}(1 + L_{SP}) + K_{sLsS_P}S_T] \] (A-10b)
\[ P_r = P_i(1 + K_{sS_i} + K_{sD_i}) + [2K_{sLsS_P}(1 + K_{sS_i} + K_{sD_i}) + K_{sLsD_i}(1 + K_{sdD_i})] \] (A-10c)

The expressions for \( S_T/P_T \) and \( D_T/P_T \) are given in Equations A-11.

\[ S_T/P_T = S_iK_{s}[1 + L_{SP}(1 + 2K_{sdS_i}) + K_{sdD_i}] \] (A-11a)
\[ D_T/P_T = D_i[1 + L_{SP} + K_{sdD_i}] + K_{sdD_i}D_i(1 + K_{sdD_i}) \] (A-11b)
\[ (K_{sLsS_P}(1 + K_{sS_i} + K_{sD_i}) + K_{sLsD_i}(1 + K_{sdD_i})) ] \] (A-11c)

To simulate the competitive binding isotherms, we chose to increment \( D_i \) since our competition experiments were performed by varying the total concentration of ds-DNA at fixed concentrations of ss-DNA and Rep. For each value of \( D_i \), we then search simultaneously for \( S_i \) and \( P_T \) using a Newton-Raphson root-finding algorithm extended to two dimensions. The root-finding functions are defined from Equations A-10a and A-10c and are given in Equations A-12a and A-12b.

\[ F(S_i, P_T) = S_i[1 + K_{sP_T} \] (A-12a)
\[ (1 + K_{sP_T}(1 + K_{sdD_i} + 2K_{sdS_i}) - S_T \] (A-12b)
\[ P_T = P_i[1 + K_{sS_i}(1 + 2P_iL_{SP}(1 + K_{sS_i}) + K_{sD_i} \] (A-12b)

However, unlike the case described above for root finding in one dimension, numerical methods for root finding in multi-dimensions are typically not robust. In practice, convergence is guaranteed only in the immediate vicinity of the root. Therefore, very good initial guesses are required. By ensuring that each incremental change in \( D_i \) is sufficiently small, we are guaranteed to be always near a root. Therefore, the previous values of \( S_i \) and \( P_T \) would always provide good initial guesses for the search for the new values of \( S_i \) and \( P_T \). Starting at an initial value of \( D_i = 0 \), \( S_T \) and \( P_T \) are calculated as described above in the absence of ds-DNA. We then increase \( D_i \) in small (5%) increments starting with an initial value equal to 0.02\( S_i \). Each time \( D_i \) is incremented, new values of \( S_T \) and \( P_T \) are derived iteratively using the old values as initial guesses. Following convergence, \( S_T/P_T \), \( D_T/P_T \), and \( D_T \) are calculated using Equations A-11a, A-11b, and A-10b, respectively. \( D_i \) is then incremented logarithmically until the calculated value of \( D_T/P_T \) exceeds 0.95. A Fortran program was written to implement this algorithm. We were unable to solve directly for the binding isotherms from the experimentally relevant total concentrations of the three tetrants. To do so required the simultaneous solution of three quadratic equations. All attempts to search in three dimensions for \( D_i \), \( S_T \), and \( P_T \) by Newton-Raphson led invariably to singular matrices.
Intrinsic Interaction Constants—The interaction constants defined above are macroscopic constants and hence have statistical factors incorporated into them. However, the free energy changes associated with each process are related to the intrinsic interaction constants which we have designated by lower case $k$ and $l$. The relationships between the intrinsic and the macroscopic constants are given in Equations A-13.

\[
\begin{align*}
 k_{is} &= K_{is} \\
 k_{ss} &= K_{ss} \\
 k_{ss} &= 2K_{ss} \\
 l_{s} &= L_{s} \\
 l_{ss} &= L_{ss} \\
 k_{id} &= K_{id} \\
 k_{dd} &= K_{dd} \\
 k_{sd} &= K_{sd} \\
 l_{id} &= L_{id} \\
 l_{dd} &= L_{dd} \\
 l_{sd} &= L_{sd}
\end{align*}
\]

Note that all of the macroscopic duplex DNA-binding constants, $K_{id}$, $K_{dd}$, $K_{sd}$, and $K_{sd}$, contain an additional statistical factor of 2, relative to the single-stranded macroscopic binding constants, based on our assumption that a duplex DNA is likely to be able to bind in two orientations, whereas ss-DNA is likely to bind in a unique orientation with respect to its backbone polarity.

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