Escherichia coli Single-Strand Deoxyribonucleic Acid Binding Protein: Stability, Specificity, and Kinetics of Complexes with Oligonucleotides and Deoxyribonucleic Acid†

Gerhard Krauss,* Herbert Sindermann,‡ Ulrich Schomburg, and Guenter Maass

ABSTRACT: The complex formation between the single-strand DNA binding protein (ssB protein) from *Escherichia coli* and oligonucleotides and single-stranded DNA has been studied by using fluorescence titrations, ultracentrifugation measurements, and fast kinetic techniques. Determination of the stoichiometries of oligo(dT)–ssB complexes shows that each of the four subunits of the ssB protein represents a binding site for an oligonucleotide about eight residues long. Occupation of all four binding sites with oligo(dT) or poly(dT) leads to 80% quenching of the intrinsic protein fluorescence. The binding sites are nearly equivalent and independent. For $d(pT)_{16}$, the intrinsic binding constant is 6×10^5 M^{-1}, and for $d(pT)_{30-40}$, which is long enough to extend continuously over the ssB tetramer, the binding constant is higher than 5×10^8 M^{-1}. Oligoadenylates bind about 2 orders of magnitude weaker than the corresponding oligo(dT) species. The binding of oligo(dT) is very weakly dependent on ionic strength, in contrast to the oligo(dA)–ssB complex formation. For $d(pT)_8$, $d(pT)_{16}$, and $d(pT)_{30-40}$, the complex formation can be described by a simple one-step reaction. The strength of the interaction is mainly expressed in the rate constant of dissociation. In the cooperative complexes with poly(dT) or poly(dA), all four binding sites on the ssB tetramer are also occupied. It is concluded that single-stranded DNA is coiled around the ssB molecule. Fluorescence melting experiments of the complexes show that the conformation of the single-stranded DNA has a strong influence on the stability of the complexes.

Deoxyribonucleic acid binding proteins which bind preferentially to single-stranded DNA have been isolated from a variety of organisms and tissues. The best knowledge as to possible physiological functions of these proteins comes from viral systems and from *Escherichia coli* [for a review, see Kornberg (1980)]. The single-strand DNA-binding protein from *E. coli* (ssB protein)[1] has been found to be involved in the main pathways of reproduction and maintenance of the genetic information. Meyer et al. (1979) have shown that the ssB protein is essential for the replication in *E. coli*, and it participates also in recombination and repair processes (Glassberg et al., 1979). A direct influence of the ssB protein on enzymatic activities of DNA specific proteins has been demonstrated in several cases; e.g., ssB protein increases the fidelity of several DNA polymerases (Kunkel et al., 1979). Also, the strand annealing activity of the recA protein is stimulated by the ssB protein (McEntee et al., 1980).

The ssB protein exists as a tetramer in solution with a subunit weight of about 20000 (Weiner et al., 1975). It binds cooperatively to single-stranded DNA and does not bind to double-stranded DNA. A more detailed understanding of the physiological function of the ssB protein requires a knowledge of the main structural features of its complex with ssDNA. Although several physicochemical investigations on the interaction between the ssB protein and ssDNA have been carried out (Ruyechan & Wetmur, 1975, 1976; Anderson & Coleman, 1975; Molineux et al., 1975), no unequivocal picture has emerged. This holds mainly for the stability and dynamics of the complexes and the nature of interactions involved. We have decided to investigate the isolated binding of the ssB protein to oligonucleotides of defined composition and length in order to discriminate protein–nucleic acid interactions from protein–protein interactions encountered in the cooperative binding to longer polynucleotides. Using fluorescence titrations, ultracentrifugation, and fast kinetics, we have obtained results on the stability, specificity, and dynamics of the complexes. Our results suggest that all four subunits of the ssB protein are involved in the binding process and that the conformation of the ssDNA has a strong effect on the stability of the complex with the nucleic acid.

Experimental Procedures

Materials

All reagents and solvents were of the highest purity available from commercial sources. Sepharose 4B-CL and BrCN-activated Sepharose were obtained from Pharmacia. Heparin was a product of Serva. Coupling of heparin to BrCN-activated Sepharose 4B-CL was performed according to the instructions of Pharmacia. Cibacron blue 3GA was a gift of Ciba-Geigy (Basel). Coupling of this dye to Sepharose 4B-Cl was done according to Böhme et al. (1972).

The following buffer systems were used: standard buffer, 20 mM potassium phosphate, pH 7.4, 0.2 M KCl, and 1 mM EDTA; buffer A, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride, and 10% (w/v) sucrose; buffer B, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 10% (w/v) glycerol; buffer C, 50 mM imidazole hydrochloride pH 6.9, 1 mM EDTA, 1 mM 2-mercaptoethanol, 20% (v/v) glycerol, and 250 mM NaCl; buffer D, 20 mM potassium phosphate, pH 7.4, 1 mM EDTA, 1 mM 2-mercaptoethanol, 20% (v/v) glycerol, and 50 mM NaCl.

† From the Zentrum Biochemie, Abteilung Biophysikalische Chemie, Medizinische Hochschule Hannover, D 3000 Hannover 61, West Germany. *Received January 26, 1981.* This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

‡ Present address: Schroedel Verlag, Hannover, West Germany.

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; ssDNA, single-strand(ed) DNA; ssB protein, single-strand(ed) DNA binding protein; this protein has also been named *E. coli* helix-destabilizing protein I (Eco HD I), *E. coli* DNA-binding protein I, and *E. coli* DNA-unwinding protein; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/81/0420-5346$01.25/0

Preparation of ssB Protein. ssB protein was purified by using a modified version of a procedure described by Meyer et al. (1980). A suspension of 500 g of *E. coli* cells is made in buffer A. Cells are broken in a Manton-Gaulin laboratory homogenizer (3 passages). The suspension is cooled to 4 °C and centrifuged at 12000*g* for 1 h. The supernatant is precipitated by addition of solid ammonium sulfate (250 g/L). After stirring for 1 h at 4 °C, the solution is centrifuged at 10000*g* for 30 min. The precipitate is subjected to a successive backwash procedure using 200 and 160 g/L ammonium sulfate in buffer A. The remaining precipitate is suspended in 150 mL of buffer B and is dialyzed overnight against 10% (w/v) poly(ethylene glycol) 10000 in buffer B. The concentrated solution is diluted to 250 mL with buffer B and is applied to a blue Sepharose column (Cibacron blue 3GA coupled to Sepharose 4B-CL). The column is washed with 4 volumes of buffer B containing 1 M NaCl. Elution of the ssB protein is achieved by 2 M NaCl plus 5 M urea in buffer B. The eluate is extensively dialyzed against buffer C and adjusted to a protein concentration of 0.7–0.8 A_{280nm} units/mL. This solution is subjected to heat precipitation by incubating 3-mL portions for 2 min in a boiling water bath. After cooling to 4 °C, the solution is centrifuged for 10 min at 10000*g*. The supernatant is dialyzed against buffer D. The dialyzed solution is applied to a heparin Sepharose column (0.9 × 5 cm). Buffer D plus 250 mM NaCl elutes pure ssB protein. The yield is about 10 mg of ssB protein per kg of *E. coli* paste. $A^{280nm}_{1\%,1cm}$ of the pure ssB protein is 0.98, as determined by refraction measurements. In $NaDodSO_4$ electrophoresis, the protein migrates as a single band of M_r 20 000 ± 1000. A native molecular weight of 80 000 ± 5000 is obtained from electrophoresis in gradient gels (3–20% acrylamide) with the buffer system of Fisher & Korn (1977). The $s_{20,w}$ of the ssB protein is 4.4 S. It can be judged from these experiments that the amount of monomeric or dimeric protein in our preparations is less than 5%. The ssB protein has a strong tendency for aggregation and is subject to sudden precipitation in concentrated solution (>1.5 mg/mL) and at low ionic strength. For this reason, most of the binding experiments were performed in the presence of 0.2 M KCL. HU protein was prepared according to Rouviere-Yaniv & Kjeldgaard (1979).

Nucleotides and DNA. $d(pT)_6$, $d(pT)_8$, $d(pT)_{16}$, poly(dT), $d(pA)_8$, $d(pA)_{16}$, and $d(pA)_{40-60}$ were purchased from P-L Biochemicals, while fd DNA was obtained from Miles. The $d(pT)_{30-40}$ was synthesized as prescribed by Cozzarelli et al. (1969) by using highly purified terminal transferase from calf thymus (purification procedure to be published). Molarities of $d(pA)_{40-60}$ and $d(pT)_{30-40}$ refer to a chain length of 50 and 35, respectively.

Methods

Ultracentrifugation. Sedimentation velocity runs were performed similarly as described by Krauss et al. (1975). The number of oligonucleotides bound per ssB protein was determined as follows. Scans were taken at 280 nm. Due to the good separation of the boundaries, it was not necessary to include measurements of the absorption ratio at 250 and 280 nm. The slow moving boundary in Figure 2 represents the free oligonucleotide; the faster one contains the ssB–oligonucleotide complex and residual free ssB protein. Absorption of the boundaries was corrected for sectorial dilution. The known absorption of total ssB protein was subtracted from the absorption of the fast boundary to yield the absorption of bound oligonucleotide. Absorption measurements were calibrated by performing runs of known concentrations of ssB protein and oligonucleotide alone. Furthermore, it could be shown from absorption titrations that the absorption of the oligonucleotides did not change upon complexation with ssB protein. The ratio of bound oligonucleotide to total ssB protein yields the stoichiometry of the complexes, provided the amount of free ssB protein is negligible, due to the concentrations used. This requirement has been met in all experiments.

Fluorescence Titrations. Titrations of the ssB protein were monitored by the tryptophan fluorescence in a Schoeffel RRS 1000 spectrofluorometer. Excitation was at 296 nm and emission was observed at 340 nm. Under these conditions, inner-filter effects due to the UV absorption of the added nucleotides can be neglected. Cuvettes with dimensions of 0.4 × 1 cm or 0.3 × 0.3 cm with starting volumes of 350 and 200 μL, respectively, were used. The added nucleotide solution had the same ssB concentration as the starting solution. The cuvettes were thermostated at ±0.05 °C. Binding constants were evaluated by a nonlinear curve-fitting procedure (Peters & Pingoud, 1979). In the case of one binding site, the fitting was performed according to

$$P + O \rightleftharpoons P{\cdot}O$$

where P is the free protein, O is the free oligonucleotide, and P·O is the complex. For two sites, the following binding equations were used:

$$P + O \rightleftharpoons P{\cdot}O \qquad K_1 = [P{\cdot}O]/([P][O])$$

$$P{\cdot}O + O \rightleftharpoons P{\cdot}O_2 \qquad K_2 = [P{\cdot}O_2]/([P{\cdot}O][O])$$

Both K_1 and K_2 and the fluorescence quenches F_1 and F_2 that accompany the binding of the two oligonucleotides could be obtained from the fitting procedure. The number of oligonucleotides bound per ssB protein was determined from sedimentation analysis as outlined above.

Fluorescence Melting Experiments. The fluorescence of ssB protein was monitored as a function of temperature in the presence and absence of oligo- and polynucleotides. The temperature was raised in steps of 5 °C. The fluorescence intensities measured were normalized to the same value at 85 °C and were plotted against $1/T$ (Figure 1). The data for the complex were linearly extrapolated to the high temperature range in order to obtain the fluorescence intensity of fully complexed ssB protein at high temperatures. By use of the denominations of Figure 1, the ratio θ of free to total binding sites on the ssB protein is given by $\theta = b/(a + b)$. In all melting experiments, the oligo- or polynucleotide was present in excess over the ssB protein in order to ensure full saturation of the ssB protein in the lower temperature range.

Temperature Jump Experiments. Temperature jump experiments were performed by monitoring the fluorescence of the protein as described by Riesner et al. (1976) in cells of 1.2 or 0.2 mL (Coutts et al., 1975). The heights of the temperature jumps were between 3 and 5 °C, to a final temperature of 8 °C. The signal was stored in a transient digital recorder and fitted to one or more exponentials with the computer.

Stopped-Flow Experiments. Rapid mixing experiments were carried out and evaluated as described by Pingoud et al. (1973, 1975).

Results

Subunit Structure and Number of Binding Sites. In view of the tetrameric subunit structure of the ssB protein, it is of interest to establish the number of oligonucleotides that can be bound to the ssB protein. Sedimentation experiments in the analytical ultracentrifuge have proven to be a reliable method for the determination of the stoichiometries of protein–nucleic acid complexes (Krauss et al., 1975). This type

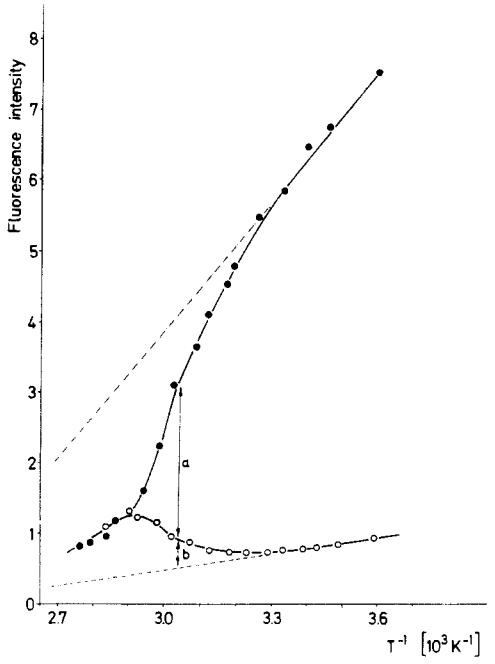

FIGURE 1: Fluorescence of ssB protein and the ssB–poly(dT) complex as a function of temperature. (●) ssB protein alone. (○) ssB protein (2.2 μM) and nucleotides poly(dT) (190 μM). (–·–) Extrapolation of the ssB fluorescence to higher temperatures. (---) Extrapolation of the fluorescence of the ssB–poly(dT) complex to higher temperatures. The fluorescence of ssB and ssB–poly(dT) was normalized to the same value at 85 °C. Sections a and b were used to construct the melting curves of the ssB complexes in Figure 8.

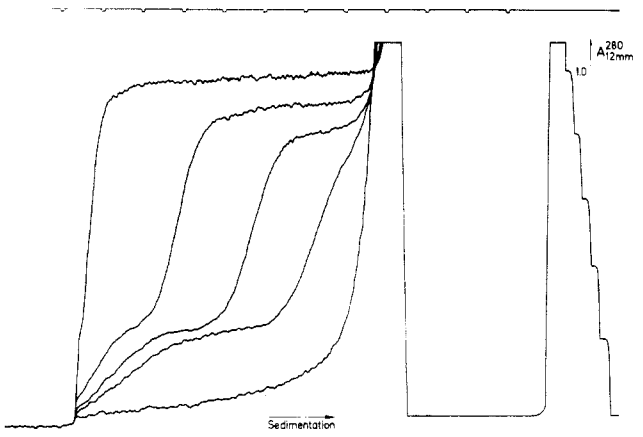

FIGURE 2: UV absorption profiles at 280 nm of a sedimentation velocity run of 6 μM ssB protein and 16 μM $d(pT)_{16}$ (60 000 rpm). Stair steps to the right divide the full range of 1 A_{280} unit into 0.2 A_{280} unit. In standard buffer at 20 °C.

of experiment has the advantage that the complex formation can be observed directly by photometric scanning. As an example, Figure 2 shows the binding between $d(pT)_{16}$ and ssB protein. The results of the binding experiments are summarized in Table I.

The sedimentation coefficient of the ssB protein fully saturated with $(dpT)_{16}$ is 5.2 S as compared to 4.4 S for the ssB protein alone. This clearly demonstrates that the tetrameric subunit structure of the protein is preserved upon binding of the oligonucleotide. The same conclusion can be drawn from binding experiments in the presence of $d(pT)_8$, $d(pA)_{40-60}$, and $d(pT)_{30-40}$. These results confirm earlier reports of Anderson & Coleman (1976), Ruyechan & Wetmur (1975), and Bandyopadhyay & Wu (1978). The data in Table I indicate that the ssB protein contains two binding sites for oligonucleotides of chain length 16 and one binding site for oligonucleotides

Table I: Physical Characteristics of ssB Complexes

	stoichiometry[a] (mol of oligonucleotide per mol of ssB tetramer)	sedimentation coefficient, $s_{20,w}$ (S)	intrinsic binding constant (M^{-1})	change of fluorescence
$d(pT)_8$	3.2 ± 0.1	ND[c]	2×10^4	−80 ± 2%[b]
$d(pT)_{16}$	2.1	5.2 ± 0.1	6×10^5	−82%
$d(pT)_{30-40}$	1.1	5.3	$>3 \times 10^8$	−82%
$d(pA)_{40-60}$	1.1	5.1	4×10^6	−62%
$d(pA)_{16}$			5×10^3	

[a] From analytical ultracentrifugation. Values represent the highest measurable stoichiometries. [b] Obtained by computer fit to titration data. [c] ND, not determined.

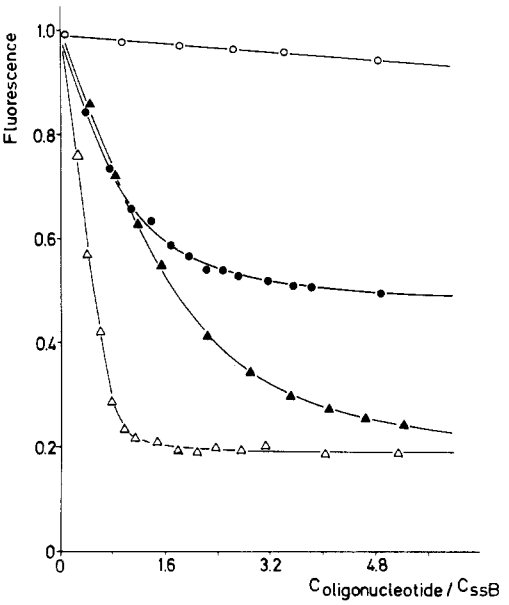

FIGURE 3: Titration of ssB protein with oligonucleotides. (○) $d(pA)_{16}$ and 2.3 μM ssB protein. (●) $d(pA)_{40-60}$ and 0.95 μM ssB protein. (▲) $d(pT)_{16}$ and 3 μM ssB protein. (△) $d(pT)_{35-40}$ and 0.08 μM ssB protein. The drawn lines represent the computer fit as obtained for the binding constants of Table I.

with a chain length of 30–60 residues. With the exception of $d(pT)_8$, the data given in Table I represent saturating values that could not be raised by increasing concentrations. For $d(pT)_8$, the analysis of the binding data obtained at different concentrations shows that the value of 3.2 $d(pT)_8$ molecules bound per ssB protein does not represent full saturation of all binding sites. We could not achieve occupation of all binding sites due to the difficulty in preparing highly concentrated solutions of the ssB protein. Considering the stoichiometries found for the larger oligonucleotides and taking into account the tetrameric subunit structure of the ssB protein, we feel justified to interpret the binding data for $d(pT)_8$ as a binding to one site on each subunit. The sedimentation coefficient of the complex with the oligonucleotides of chain length 30–40 is close to that determined for the complex of the ssB protein with $d(pT)_{16}$ molecules. This shows that oligonucleotides of chain length 30–40 are not long enough to favor cooperative protein–protein interactions.

Binding Constants. The tryptophan fluorescence of the ssB protein is strongly quenched upon binding of single-stranded nucleic acids. Saturation of the ssB protein with $d(pT)_{16}$ leads to about 80% quenching. The same value is obtained upon binding of $d(pT)_{30-40}$ or poly(dT) (Table I). By contrast, only

Table II: Ionic Strength Dependence of ssB Complexation[a]

oligonucleotide	KCl concn (mM)	intrinsic binding constant (M^{-1})
$d(pT)_{16}$	50	$(1.4 \pm 0.3) \times 10^6$
	200	$(6 \pm 2) \times 10^5$
400	400	$(6 \pm 2) \times 10^5$
	750	$(2.6 \pm 0.5) \times 10^5$
	200 mM KCl, 10 mM $MgCl_2$	$(6 \pm 2) \times 10^5$
$d(pA)_{40-60}$	50	1.5×10^7
	200	3×10^6
	400	1.5×10^5

[a] From fluorescence titrations in standard buffer, pH 7.4, at 8 °C.

60% quenching is observed during binding of oligoadenylates (Figure 3). These quenching magnitudes agree with those measured by Molineux et al. (1975). For all oligonucleotides investigated, the binding data could be interpreted by assuming equivalent and independent binding sites. However, the fluorescence titrations are not sensitive enough to exclude binding sites with slightly different affinities and fluorescence quenching. This holds particularly for the binding of $d(pT)_8$, where the ultracentrifuge measurements indicated four binding sites. In this case, one would have to fit four different binding constants and quenching parameters in the evaluation.

ssB–$d(pT)_8$ complexation results have been evaluated on the assumption of four independent and equivalent binding sites. We were unable to directly measure the value of quenching at saturation; however, the value of 80% from the fitting procedure corresponds to that for all other T oligonucleotides investigated. The data for $d(pT)_{16}$ have been evaluated according to a simple sequential binding scheme given above with $K_I = 3 \times 10^5$ M^{-1}, $F_I = 50\%$ and $K_{II} = 1.2 \times 10^6$ M^{-1}, $F_{II} = 30\%$. The two binding constants are separated by a factor of 4, which corresponds to the statistical factor that has to be considered in this type of evaluation. An intrinsic binding constant of 6×10^5 M^{-1} is obtained.

The affinity of $d(pT)_{30-40}$ to the ssB protein is so high that we could not determine accurate values for the binding constant in the fluorescence titrations. Even at the lowest concentrations possible, nearly stoichiometric titrations resulted. We can estimate the binding constant to be 3×10^8 M^{-1}. For the cooperative binding of the ssB protein to longer polydeoxynucleotides, binding constants of 10^{10}–10^{11} M^{-1} had been reported (Molineux et al., 1975; Ruyechan & Wetmur, 1975), with a cooperativity factor of 10^5–10^6. Our data suggest, however, that the contribution of cooperativity to the strength of these complexes amounts to not more than 2–3 orders of magnitude.

The binding of A oligonucleotides differs with respect to affinity and fluorescence quenching from that of the T oligonucleotides. The fluorescence quenching measured by saturating the ssB protein with $d(pA)_{40-60}$ amounts only to about 60%. The A oligonucleotides are bound at least 2 orders of magnitude weaker than the corresponding T oligonucleotides.

Ionic Strength Dependence of Complex Formation. Binding constants for $d(pT)_{16}$ measured at various ionic conditions are summarized in Table II. The complex formation between $d(pT)_{16}$ and the ssB protein depends only slightly on ionic strength, indicating that ionic interactions contribute only to a very small extent of the free energy of binding. Ruyechan & Wetmur (1976) have reported that a plateau of binding exists between 0.04 and 0.2 M KCl and that binding is weakened at salt concentrations above 0.2 M. However, in these investigations, shorter oligonucleotides have been utilized, and no quantitative data are given for the higher salt conditions. On the contrary, the same authors and Weiner et al. (1975) have shown that binding to single-stranded DNA occurs also at high salt. Mg^{2+} up to concentrations of 20 mM has no effect on the complex formation (Table IV). Ruyechan & Wetmur (1975) report a strong effect of Mg^{2+} on the binding. This discrepancy between our results cannot be explained at present.

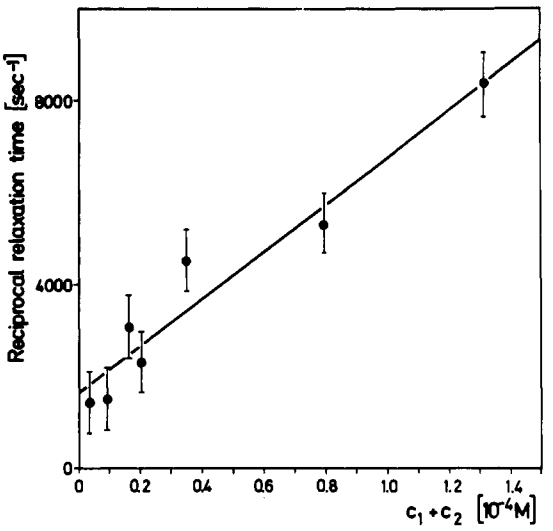

FIGURE 4: Binding of $d(pT)_8$ to the ssB protein. Dependence of the reciprocal relaxation times on the sum of the concentrations of free reactants. c_1, concentration of free $d(pT)_8$; c_2, concentration of free ssB binding sites. The error bars indicate the experimental error obtained for about five temperature jumps with the same solution.

Table III: Intrinsic Rate Constants for the Binding of Oligonucleotides to ssB Protein As Determined by Temperature Jump and Stopped-Flow Experiments

oligonucleotide	binding model	k_R (M^{-1} s^{-1})	k_D (s^{-1})
$d(pT)_8^8$	four independent, equivalent sites	7×10^7	1700
$d(pT)_{16}$	two independent, equivalent sites	5×10^7	40
$d(pT)_{30-40}$	one site	3×10^8	≤ 1

Kinetics of Oligonucleotide Binding. A temperature jump on the ssB–$d(pT)_8$ complex yields only one detectable relaxation time. The concentration dependence of this relaxation time is shown in Figure 4. Over the accessible concentration range, this relaxation time is linearly dependent on the concentrations of the reactants; therefore, the $d(pT)_8$ binding can be described by a simple one-step binding to equivalent and independent sites. The rate constants are given in Table III. The binding constant calculated from the kinetic data is, considering the magnitude of error, in agreement with the value obtained from the fluorescence titrations.

In most temperature jump experiments on the binding of $d(pT)_{16}$, only one relaxation time is observable (Figure 5). In the higher concentration range, however, the oscilloscope traces can be fitted slightly better by using two exponentials. Since the second process is visible only at high concentrations and since the time scale of the two processes is very close, we could not evaluate the two processes separately. For the plot in Figure 6, fitting has been performed to one exponential at all concentrations. The rate constants obtained are given in Table III. The presence of a second relaxation time points to a slight nonequivalence of the binding sites. Evidently, in this case, the kinetics are more sensitive than the equilibrium measurements.

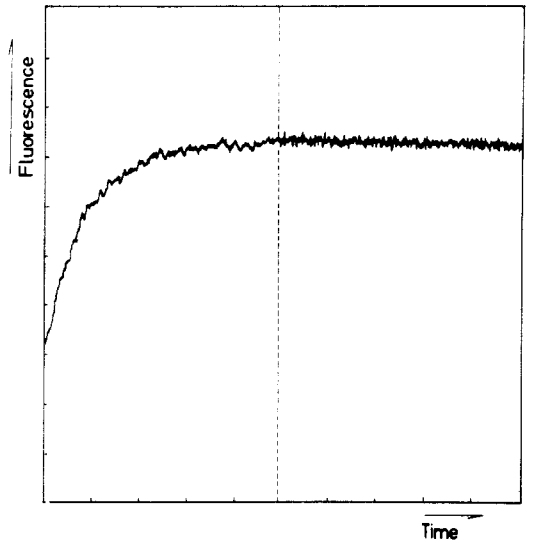

FIGURE 5: Relaxation curve of the binding of $d(pT)_{16}$ to the ssB protein. Final temperature 8 °C; 1.3 μM ssB protein and 6.8 μM $d(pT)_{16}$ in standard buffer. Oscilloscope settings: 4 V total intensity, 100 mV/unit, and 500 μs rise time; time base of the first five units is 5 ms/unit and that of the last five units is 50 ms/unit.

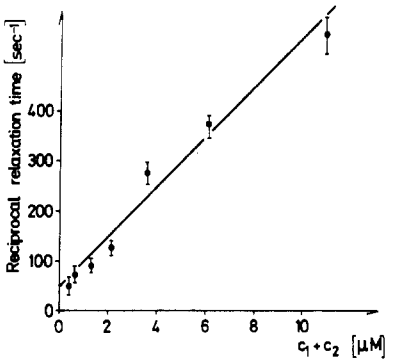

FIGURE 6: Binding of $d(pT)_{16}$ to the ssB protein. Dependence of the reciprocal relaxation times on the sum of concentrations of free reactants. c_1, concentration of free $d(pT)_{16}$; c_2, concentration of free ssB binding sites. For error bars, see Figure 4.

In temperature jump experiments on the binding of $d(pT)_{30-40}$ to the ssB protein, we could not detect relaxation effects even at 10 nM ssB and $d(pT)_{30-40}$. The inability to detect relaxation effects under these conditions is essentially a consequence of the high binding constant. In stopped-flow experiments, however, the binding between $d(pT)_{30-40}$ and the ssB protein is easily detectable. In such mixing experiments, the $d(pT)_{30-40}$ concentration was always in 5–7-fold excess over ssB protein, so that the reaction curves can be described by single exponentials. The concentration dependence of these exponentials is given in Figure 7. From this plot, only the rate constant of recombination can be determined with accuracy. The dissociation rate, estimated from k_R and the lower limit of the binding constant, is slower than 1 s^{-1}. We find no evidence for slow conformational transitions accompanying the binding process.

Cooperative Binding to Polynucleotides. It has been shown that ssB protein binds cooperatively to single-stranded DNA (Anderson & Coleman, 1975; Ruyechan & Wetmur, 1975). The stoichiometry and the fluorescence quenching of binding to various polynucleotides as determined by fluorescence titrations and analytical ultracentrifugation are given in Table IV. Comparisons with results of noncooperative binding to oligonucleotides indicate that the cooperative binding does not lead to additional quenching of tryptophan fluorescence in the

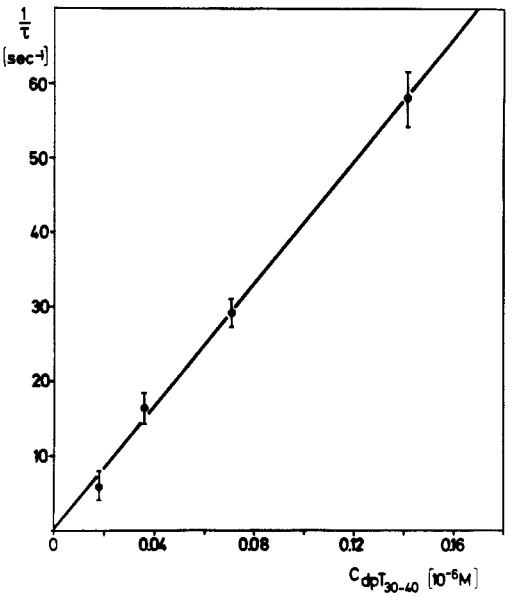

FIGURE 7: Binding of $d(pT)_{30-40}$ to the ssB protein. Concentration dependence of the exponentials observed in stopped-flow experiments. $d(pT)_{30-40}$ was always present in 6–7-fold excess over ssB protein. In standard buffer at 8 °C. Error bars indicate the experimental error obtained for three mixing experiments with the same solution.

Table IV: Stoichiometry and Fluorescence Quenching of the Complex between Polynucleotides and ssB Protein in Standard Buffer, pH 7.2, at 8 °C

polynucleotide	fluorescence quenching (%)	nucleotides covered[a] per ssB tetramer
poly[d(pT)]	80 ± 2	35 ± 2
poly(dA)	60	37 ± 2
fd DNA	76	36 ± 2
poly[r(pU)]	50	ND[b]

[a] From fluorescence titrations. [b] ND, not determined.

ssB protein. The poly(dA)–ssB complex exhibits the lowest fluorescence quenching while that from fd-DNA binding is slightly lower than that of poly(dT), perhaps because of the A residues in fd-DNA.

The number of nucleotides bound per ssB tetramer is in the range 30–40 for all polynucleotides investigated. This value agrees with that obtained from the studies on oligonucleotides. Both stoichiometric and fluorescence results indicate that there is no gross rearrangement in the complex upon going from the isolated binding mode to the cooperative binding mode.

Fluorescence Melting Curves. The temperature dependence of the fluorescence of the ssB protein in the presence and absence of poly(dT) is illustrated in Figure 1. The dissociation of the complex at elevated temperatures is indicated by an increase in fluorescence. The great heat stability of the ssB protein allows measurement up to 90 °C since melting curves are reversible over this range. From Figure 1, the amount of free and bound binding sites on the ssB protein may be calculated, permitting the melting curves to be normalized with regard to the ratio free/bound as shown in Figure 8.

The position of the midpoints and the widths of the transitions are functions of the intrinsic binding constant, the cooperativity constant, the reaction enthalpy, and the length of the polynucleotides. Since we do not have quantitative information about the cooperativity constant and the reaction enthalpies, a detailed analysis of these melting curves has not yet been performed. From inspection, it is evident that the most stable complex is that between fd-DNA and ssB protein,

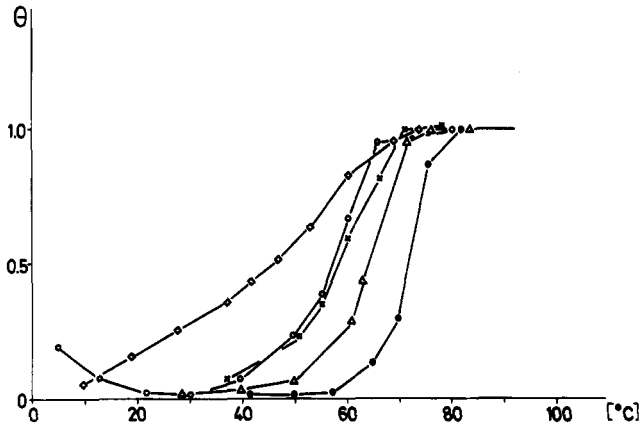

FIGURE 8: Melting curves of ssB complexes. Curves were constructed as described under Methods. θ = ratio of free to total ssB binding sites. (◇) $d(pT)_{16}$–ssB protein. (○) Poly(dA)–ssB protein. (△) Poly(dT)–ssB protein. (●) fd DNA–ssB protein. (×) ssB protein alone. For ssB, θ = degree of transition to the conformation present at high temperatures. In all experiments: 2.2 μM ssB protein and 1.9×10^{-4} M nucleotides of ssDNA or homopolymer.

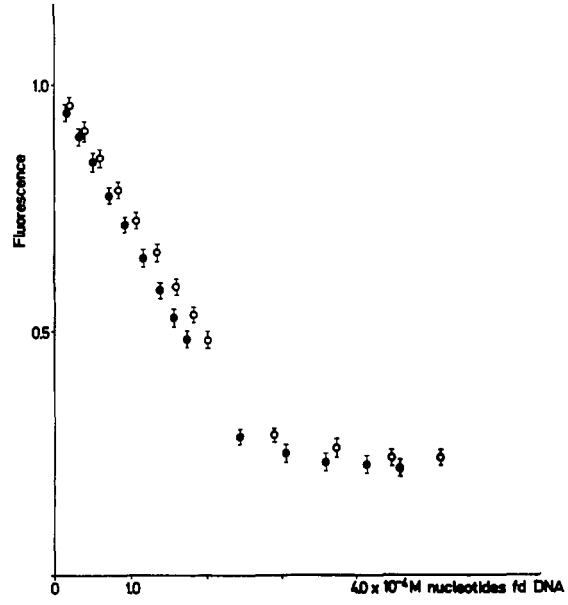

FIGURE 9: Competition between ssB protein and HU protein for fd DNA. (●) 7.2 μM ssB protein and fd DNA. (○) 7.2 μM ssB protein, 35 μM HU protein, and fd DNA. In standard buffer at 8 °C.

while the complex with poly(dA) is the least stable. It is interesting to note that below 20 °C the apparent extent of complexation between ssB protein and poly(dA) increases with increasing temperature. Temperature jump studies on the binding of poly(dA) to ssB protein in this temperature range clearly demonstrate the stronger binding with increasing temperature. These observations are interpreted as a shift of a stacking of various poly(dA) conformations toward a conformation that is more favorable for binding.

The ssB protein itself undergoes a conformational transition in the temperature range between 40 and 70 °C, as indicated from the deviation of linearity of the plot in Figure 1. This transition coincides with that for the poly(dA)–ssB complex, suggesting that poly(dA) does not protect the ssB protein against thermal denaturation. We do not yet have information about the nature of the conformational change in the ssB protein at higher temperatures. Presumably its conformation shifts to one where binding to ssDNA is no longer possible. The presence of poly(dT) or fd-DNA seems to shield the ssB protein against this thermally induced transconformation whereas poly(dA) is ineffective.

Competition between ssB Protein and HU Protein. The HU protein is assumed to exert histone-like functions in prokaryotes (Yaniv et al., 1979). It exhibits both single- and double-stranded DNA binding capacity. There have also been reports on a specific interaction between the ssB protein and the HU protein (Geider, 1978). However, results shown in Figure 9 indicate that there is no competition between HU and ssB for fd-DNA even when HU is present at a 5-fold molar excess over the ssB tetramer. Moreover, we do not see an influence of HU protein on the position and/or the width of the melting transitions of the ssB–fd-DNA complex. We suspect that the discrepancy between these results and those of Geider (1978) may be due to the different ionic conditions that have been used. For this to be true, however, the HU protein or the HU–ssB complex must exhibit a sensitivity to ionic strength not seen with ssB protein alone.

Discussion

Subunit Structure and Number of Binding Sites. Our measurements on the stoichiometry of oligo(dT)–ssB protein complexes show that the ssB protein has more than three binding sites for $d(pT)_8$, two binding sites for $d(pT)_{16}$, and only one binding site for $d(pT)_{30-40}$. We conclude from these values and from the amount of fluorescence quenching measured that (i) all four subunits of the ssB protein provide a binding site for oligonucleotides and (ii) all four binding sites are occupied in complexes with oligo$(dT)_n$, where n exceeds 30 nucleotides. The stretch of oligonucleotides that can be covered by the ssB tetramer is between 30 and 40 residues long. This number does not change between isolated and cooperative binding; therefore, the protein–protein interactions that occur in the cooperative complex must not influence the essential features of the protein–nucleic acid interaction. This interpretation also implies that ssDNA is in contact with all four subunits of the ssB tetramer during cooperative binding with neighboring ssB protein. Such a binding mode requires a folding of the DNA around the ssB protein. In support of this proposal of a coiling of the ssDNA is an observed 2.5-fold reduction of the contour length of fd DNA in complexes with ssB protein (Sigal et al., 1972). Due to the lack of detailed information about the arrangement of the subunit of the ssB protein in the complex, it is not possible to make predictions about the amount of single-strand coiling. Also it cannot be decided at the moment whether the four subunits participate equivalently in the complex formation. The kinetic experiments on the binding of $d(pT)_{16}$ seem to point to a slight nonequivalence of the subunits in these complexes.

Strength of Binding and Nature of Interactions. The binding constants measured for $d(pT)_{16}$ and $d(pT)_8$ are comparable to those reported earlier by Ruyechan & Wetmur (1976) and Molineux et al. (1975). We have now been able to establish a lower limit for the binding constant of an oligonucleotide that is long enough to extend continuously over the whole ssB tetramer. The binding constant for $d(pT)_{30-40}$ is higher than 5×10^8 M^{-1}. This value is close to that for the cooperative binding estimated on the basis of electron microscopic data (Ruyechan & Wetmur, 1975). Due to the very high association for the isolated binding, it was not possible to determine quantitatively the cooperative binding constant experimentally.

The A homopolymers are bound at least 2 orders of magnitude weaker than the corresponding T oligomers. This may be due to a base recognition capability of the ssB protein with a low intrinsic affinity for A, or it may be due to a special conformation of the dA strand. In support of the latter

possibility is the known tendency of A homopolymers to form strong stacking interactions in both the ribo and deoxyribo series (Pörschke, 1978; Dewey & Turner, 1980). Depending on temperature, different single-stranded stacked conformations of polyadenylates have been detected, the exact geometry of which is not yet known. A contribution of the single-strand conformation to the strength of binding is clearly shown by the annealing behavior of the poly(dA)–ssB complex at low temperatures. The increased binding observed upon raising the temperature may be explained by a shift in an equilibrium between a more stacked conformation toward a more flexible, less stacked one that is a better substrate for binding to the ssB protein.

The results on the ionic strength and base dependence of the binding constants provide information about the nature of the interactions involved in the complex formation. The very low ionic strength dependence of the oligo(dT)–ssB complexes indicates a relatively intimate interaction with the base and sugar moieties and a lesser interaction with the negatively charged phosphate residues. In favor of a direct interaction with the base is the strong fluorescence quenching observed for the binding of the T oligonucleotides. Such a strong fluorescence quenching could be due to an intercalation of Trp residues of the ssB protein with the bases (Toulmê & Hélène, 1977), while the greater stacking tendency of the A residue inhibits intercalation with the tryptophan residues, resulting in a weaker binding. The stronger ionic strength dependence of the A oligonucleotides suggests that as a consequence of reduced intercalation, electrostatic interactions become more important.

Kinetics. The rate constants of association for the binding of the ssB protein to the various oligo(dT) species are in the range 10^7–10^8 M^{-1} s^{-1}. From a comparison with other protein–nucleic acid association reactions, e.g., the binding of tRNAs to aminoacyl-tRNA synthetase (Krauss et al., 1973), it can be concluded that these k_R values are not far from those expected for diffusion-controlled reactions. The increasing strength of binding in going from the shorter to the longer oligonucleotides is expressed mainly in the dissociation rates, e.g., resulting in a k_D value slower than 1 s^{-1} for $d(pT)_{30-40}$. In relating these data to the physiological situation, one has to consider the fast rate of replication in *E. coli*. It can be calculated from the generation time of *E. coli* that the replication rate is in the range of 1000 nucleotides incorporated per s. This remarkably high rate requires a fast dissociation–association equilibrium or a fast sliding process on the DNA for all replication proteins. Thus, the slow dissociation rate measured for $d(pT)_{30-40}$ and the even slower dissociation rate predicted for the cooperative binding to poly(dT) certainly do not apply to the situation in vivo, where a multienzyme complex is engaged in replication and where the DNA contains all four bases. Dissociation in vivo may be facilitated by the interaction of the ssB protein with other replication proteins or by the presence of special conformations of the ssDNA that are unfavorable for the binding of the ssB protein. Such conformations could be induced by replication proteins engaged in binding to the ssDNA or by the sequence of the ssDNA. The importance of the conformation of the DNA is clearly demonstrated by the experiments with the A oligonucleotides. Although we have not yet measured the kinetics of the binding of the A oligonucleotides, it can be assumed that most probably the weaker binding of the A oligonucleotides as compared to the T oligonucleotides is reflected by a faster dissociation rate. Thus, A-rich stretches on the ssDNA may contribute to a faster dissociation of the ssB protein.

Acknowledgments

We thank Dr. F. Peters for supplying the computer programs and Dr. C. Urbanke for carrying out the ultracentrifugation measurements. We are indebted to Professor D. Blake for critically reading the manuscript and for helpful discussions. The expert technical assistance of M. Wehsling is gratefully acknowledged.

References

Anderson, R. A., & Coleman, J. E. (1975) *Biochemistry 14*, 5485–5491.

Bandyopadhyay, P. K., & Wu, Ch-W. (1978) *Biochemistry 17*, 4078–4085.

Böhme, H. J., Kopperschlaeger, G., Schulz, J., & Hofman, E. (1972) *J. Chromatogr. 69*, 209–214.

Coutts, S. M., Riesner, D., Römer, R., Rabl, C. R., & Maass, G. (1975) *Biophys. Chem. 3*, 275–289.

Cozzarelli, N. R., Kelly, R. B., & Kornberg, A. (1969) *J. Mol. Biol. 45*, 513–519.

Dewey, T. G., & Turner, D. H. (1980) *Biochemistry 19*, 1681–1685.

Fisher, P. A., & Korn, D. (1977) *J. Biol. Chem. 252*, 6528–6535.

Geider, K. (1978) *Eur. J. Biochem. 87*, 617–622.

Glassberg, J., Meyer, R. R., & Kornberg, A. (1979) *J. Bacteriol. 140*, 14–19.

Kornberg, A. (1980) in *DNA Replication*, p 227, W. H. Freeman, San Francisco.

Krauss, G., Römer, R., Riesner, D., & Maass, G. (1973) *FEBS Lett. 30*, 6–10.

Krauss, G., Pingoud, A., Boehme, D., Riesner, D., Peters, F., & Maass, G. (1975) *Eur. J. Biochem. 55*, 517–529.

Kunkel, Th. A., Meyer, R. R., & Loeb, L. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6331–6335.

McEntee, K., Weinstock, G. M., & Lehman, I. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 857–861.

Meyer, R. R., Glassberg, J., & Kornberg, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1702–1705.

Meyer, R. R., Glassberg, J., Scott, J. V., & Kornberg, A. (1980) *J. Biol. Chem. 255*, 2897–2901.

Molineux, I. J., Pauli, A., & Gefter, M. L. (1975) *Nucleic Acids Res. 2*, 1821–1828.

Peters, F., & Pingoud, A. (1979) *Int. J. Bio-Med. Comput. 10*, 401–415.

Pingoud, A., Riesner, D., Boehme, D., & Maass, G. (1973) *FEBS Lett. 30*, 1–5.

Pingoud, A., Boehme, D., Riesner, D., Kownatzki, R., & Maass, G. (1975) *Eur. J. Biochem. 56*, 617–622.

Pörschke, D. (1978) *Biopolymers 17*, 315–324.

Riesner, D., Pingoud, A., Boehme, D., Peters, F., & Maass, G. (1976) *Eur. J. Biochem. 68*, 71–80.

Rouviere-Yaniv, J., & Kjeldgaard, N. O. (1979) *FEBS Lett. 106*, 297–302.

Ruyechan, W. T., & Wetmur, G. (1975) *Biochemistry 14*, 5529–5534.

Ruyechan, W. T., & Wetmur, G. (1976) *Biochemistry 15*, 5057–5064.

Sigal, N., Delius, H., Kornberg, T., Gefter, B., & Alberts, B. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3537–3541.

Toulmê, J. J., & Hélène C. (1977) *J. Biol. Chem. 252*, 244–249.

Weiner, J. H., Bertsch, L. L., & Kornberg, A. (1975) *J. Biol. Chem. 250*, 1972–1980.

Yaniv, J. R., Yaniv, M., & Germond, J. E. (1979) *Cell (Cambridge, Mass.) 17*, 265–274.