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Equilibrium Binding of *Escherichia coli* Single-Strand Binding Protein to Single-Stranded Nucleic Acids in the $(SSB)_{65}$ Binding Mode. Cation and Anion Effects and Polynucleotide Specificity†

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ABSTRACT: The *Escherichia coli* single-strand binding (SSB) protein binds single-stranded (ss) nucleic acids in at least four distinct binding modes depending on the salt conditions [Lohman, T. M., & Overman, L. B. (1985) *J. Biol. Chem. 260*, 3594; Bujalowski, W., & Lohman, T. M. (1986) *Biochemistry 25*, 7799]. Equilibrium binding constants for the interaction of the *E. coli* SSB protein with poly(A), poly(U), poly(dA), and poly(dT) have been measured over a range of monovalent salt concentrations and types under conditions which favor only the high site size, $(SSB)_{65}$ binding mode, which covers 65 nucleotides per SSB tetramer. The binding isotherms are analyzed by using a statistical thermodynamic model ("tetramer/octamer" model) that assumes cooperative binding of SSB is limited to the formation of octamers [Bujalowski, W., & Lohman, T. M. (1987) *J. Mol. Biol. 195*, 897] rather than the indefinite clustering of tetramers. The dependence of the intrinsic association equilibrium constant, K_{obsd}, and cooperativity parameter, $\omega_{T/O}$, on salt concentration has been determined by titrations which monitor the fluorescence quenching of the SSB protein upon complex formation. In the $(SSB)_{65}$ binding mode, SSB binds with only moderate cooperativity to ss nucleic acids [Lohman, T. M., Overman, L. B., & Datta, S. (1986) *J. Mol. Biol. 187*, 603]. The cooperativity parameter derived from the tetramer/octamer model, which represents the equilibrium constant for formation of a nucleic acid bound SSB octamer from two nucleic acid bound tetramers, has a value of $\omega_{T/O} = 410 \pm 120$ and is independent of salt concentration and type for poly(dA), poly(U), and poly(A) (25 °C, pH 8.1). However, K_{obsd} decreases steeply with increasing salt concentration, such that $\partial \log K_{obsd}/\partial \log [NaCl] = -7.4 \pm 0.5$ for poly(U), -6.1 ± 0.6 for poly(dA), and -6.2 ± 0.3 for poly(A) (25.0 °C, pH 8.1). The SSB–poly(dT) affinity is too high to measure in buffers containing even 5 M NaCl; however, in 1.8–2.5 M NaBr, we measure $\partial \log K_{obsd}/\partial \log [NaBr] = -5.7 \pm 0.7$, with a lower value of $\omega_{T/O} = 130 \pm 70$. The polynucleotide specificity of the $(SSB)_{65}$ binding mode (0.20 M NaCl, 25.0 °C, pH 8.1) is $K_{obsd}(dT) > K_{obsd}(dC) \gg K_{obsd}(ss\ M13\ DNA) > K_{obsd}(I) > K_{obsd}(U) = 8K_{obsd}(dA) = 87K_{obsd}(A) \gg K_{obsd}(C)$. A dramatic effect of anion type on both the salt dependence and magnitude of K_{obsd} is also observed such that for poly(U) $\partial \log K_{obsd}/\partial \log [potassium\ glutamate] = -5.7 \pm 0.4$, $\partial \log K_{obsd}/\partial \log [NaF] = -4.3 \pm 0.4$, $\partial \log K_{obsd}/\partial \log [NaCH_3COO] = -6.5 \pm 0.2$, and $\partial \log K_{obsd}/\partial \log [NaBr] = -6.7 \pm 0.6$; in 0.35 M monovalent cation, $K_{obsd}(Glu) = 5.6K_{obsd}(F) = 11K_{obsd}(CH_3COO) = (1.1 \times 10^3)K_{obsd}(Cl) = (1.1 \times 10^4)K_{obsd}(Br)$. These data indicate that significant electrostatic interactions occur in the $(SSB)_{65}$ complexes formed with all polynucleotides, resulting in a *net release* of both cations and anions, although there are also contributions due to cation and anion uptake.

The *Escherichia coli* single-strand binding (SSB) protein is required for replication of the *E. coli* chromosome and some single-stranded (ss) phages (Sigal et al., 1972; Wickner & Hurwitz, 1974; Meyer et al., 1979) and for recombination and repair processes (Johnson, 1977; McEntee et al., 1980; Glassberg et al., 1979). Although the *E. coli* SSB protein is a member of the same class of helix-destabilizing proteins as the bacteriophage T4 gene 32 protein and both proteins bind with high affinity to ss DNA, recent studies have shown that these two proteins interact very differently with DNA (Chrysogelos & Griffith, 1982; Lohman & Overman, 1985; Griffith et al., 1984; Lohman et al., 1986a; Bujalowski & Lohman, 1987b). One major difference between these two helix-destabilizing proteins is that the *E. coli* SSB protein can bind to ss DNA in multiple (at least four) binding modes which differ in the number of nucleotides occluded per bound SSB tetramer (i.e., the site size, n) (Lohman & Overman, 1985; Bujalowski & Lohman, 1986) and display distinct morphologies in the electron microscope (Griffith et al., 1984). Below 10 mM NaCl, $n = 35 \pm 3$ nucleotides per bound SSB tetramer $[(SSB)_{35}]$; in the region from 50 mM $\leq$ [NaCl] $\leq$ 0.1 M NaCl, $n = 56 \pm 3$ $[(SSB)_{56}]$, whereas above 0.2 M NaCl, $n = 65 \pm 3$ $[(SSB)_{65}]$ (25.0 °C, pH 8.1); in $MgCl_2$ buffers, the site size transitions are shifted to significantly lower salt concentrations, and the $(SSB)_{56}$ mode is stable over a much wider range of $MgCl_2$ concentrations (4–50 mM) than in NaCl. At 37 °C, a fourth binding mode with a site size of

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40 ± 2 nucleotides per SSB tetramer is also observed (Bujalowski & Lohman, 1986).

The transitions between these different SSB binding modes are modulated by salt concentration and type (Lohman & Overman, 1985; Bujalowski & Lohman, 1986) as well as by the protein binding density on the DNA (Griffith et al., 1984; Bujalowski et al., 1988). The observation that increasing salt concentration promotes the transition from the lower to the higher site size binding modes, as well as the presence of specific cation effects, indicates that direct uptake of cations accompanies the formation of the higher site size binding modes. Specific anion effects on the $(SSB)_{56}$ to $(SSB)_{65}$ transition indicate that anions are also taken up in this process (Bujalowski et al., 1988). In the electron microscope, high site size complexes appear as "beaded" structures, where each bead seems to be composed of 140–160 nucleotides of DNA wrapped around an octamer of SSB monomers, whereas low site size complexes have a smoother morphology (Griffith et al., 1984). Wrapping of ss DNA around individual tetramers has also been proposed (Krauss et al., 1981). At low NaCl concentrations (≤0.2 M), the binding density of the SSB–ss DNA complexes also affects the relative populations of each binding mode such that the high site size, beaded complex is favored at low binding densities and the low site size complex is favored at high binding densities (Griffith et al., 1984; Bujalowski et al., 1988). The conditions under which the beaded structures (SSB octamers) are observed correlate with conditions which favor the $(SSB)_{56}$ and/or the $(SSB)_{65}$ binding modes (Griffith et al., 1984; Lohman & Overman, 1985; Bujalowski & Lohman, 1986). Quantitative analysis of binding isotherms strongly suggests that, when bound to ss nucleic acids, the $(SSB)_{65}$ complex exists as an equilibrium between isolated SSB tetramers and limited aggregates of SSB octamers (Bujalowski & Lohman, 1987b).

Another major difference between the T4 gene 32 protein and the *E. coli* SSB protein concerns the cooperative binding to ss nucleic acids. Recent studies have shown that *E. coli* SSB protein does not form long clusters of protein when bound to ss DNA or RNA *at equilibrium*; that is, SSB has a low degree of cooperativity (Lohman et al., 1986a). In the $(SSB)_{65}$ mode, cooperativity seems to result in the formation of SSB clusters which are limited to dimers of tetramers (Chrysogelos & Griffith, 1982), and the "limited" cooperativity parameter for formation of octamers has a value of 420 ± 80 (0.20 M NaCl, 25 °C, pH 8.1) (Bujalowski & Lohman, 1987b). However, under *nonequilibrium* conditions, primarily at low NaCl concentrations, a *metastable* situation can exist in which highly extensive clustering of SSB molecules occurs along ss DNA (Lohman et al., 1986a). The low equilibrium value of the limited cooperativity parameter for the *E. coli* SSB protein in the $(SSB)_{65}$ mode differs dramatically from the high degree of equilibrium cooperativity that describes the T4 gene 32 protein ["unlimited" nearest-neighbor cooperativity parameter of $(\sim 1\text{–}5) \times 10^3$)] (Kowalczykowski et al., 1981).

Quantitative studies of the effects of salt concentration on protein–nucleic acid equilibria can provide useful information about the number of electrostatic interactions in the complex, as well as an estimate of the relative electrostatic component of the binding free energy (Record et al., 1976, 1978). The effects of salt concentration on the kinetics of protein–nucleic acid interactions can also be used to probe the mechanism of the interaction (Lohman et al., 1978; Lohman, 1986). The effects of salt concentration on the equilibrium binding of SSB protein to single-stranded polynucleotides have not been thoroughly examined. Ruyechan and Wetmur (1976) reported that the equilibrium constant for SSB–oligonucleotide interactions [$d(pC)_6$ and other hexanucleotides] is independent of [NaCl] in the range from 40 mM to 0.20 M NaCl; however, the equilibrium constant decreased significantly above 0.20 M NaCl. In another study of SSB–oligonucleotide interactions, Krauss et al. (1981) observed only a slight dependence on [KCl] for SSB–$d(pT)_{16}$ binding, whereas the interaction of SSB with $d(pA)_{40\text{–}60}$ was found to be much more dependent upon [KCl]. Attempts to determine the salt dependence of SSB binding to poly(dT) have also been made (Molineux et al., 1975; Williams et al., 1983); however, since the binding of SSB to poly(dT) is stoichiometric even in 5 M NaCl (Lohman & Overman, 1985), these studies provide only a gross estimate of the *lower limit* for the binding affinity of SSB to poly(dT) and underestimate the salt dependence. The general conclusions that seem to have been drawn from these composite studies are that *E. coli* SSB protein–polynucleotide interactions are not very salt dependent; as a result, the inference has been made that only a small electrostatic component to the binding free energy exists for SSB–ss nucleic acid interactions (Chase & Williams, 1986; Clore et al., 1986).

The binding studies cited above were undertaken before it was recognized that the *E. coli* SSB protein can bind to ss nucleic acids in several binding modes; hence, the measurements were generally performed under conditions which favor a mixture of SSB binding modes. We have identified solution conditions in which a single SSB–ss nucleic acid binding mode, the $(SSB)_{65}$ mode, is stable and can be studied at equilibrium. Previous studies of the $(SSB)_{65}$ complex with poly(U) indicated that these interactions are highly salt dependent (Lohman et al., 1986a; Overman & Lohman, 1986). In this report, we examine the equilibrium binding properties of the SSB protein in the $(SSB)_{65}$ mode with a number of polynucleotides, and we show that binding to all polynucleotides is *highly* salt dependent. In buffers containing NaCl (or KCl) as the major low molecular weight salt, the intrinsic association binding constant decreases by a factor of $\sim 10^7$ upon raising the [NaCl] by a factor of 10 (at 25 °C, pH 8.1), although the cooperativity is independent of salt concentration. Hence, we conclude that electrostatic interactions play a major role in the interaction of *E. coli* SSB protein with ss nucleic acids and by this criteria SSB is similar to the majority of nucleic acid binding proteins examined thus far. However, the molecular basis for these effects is complex since cation release and uptake as well as anion release and uptake contribute to the observed salt dependence, although there is a *net release* of both anions and cations upon formation of the $(SSB)_{65}$ complex.

Theoretical Section

Salt Effects on Protein–Nucleic Acid Equilibria. The interactions of proteins with nucleic acids, both equilibrium binding and kinetics, are usually extremely sensitive to changes in the salt concentration in solution. The molecular bases for the sensitivity of both the observed equilibrium binding constant and the observed kinetic rate constants for these interactions have been discussed in detail (Record et al., 1976, 1978; Lohman et al., 1978; Lohman, 1986). The major effect originates from the fact that linear nucleic acids are polyanions possessing very high linear charge densities; hence, condensed cations accumulate in the vicinity of the nucleic acid in order to partially neutralize the high charge density on the nucleic acid (Manning, 1969; Anderson et al., 1978). The symbol ψ refers to the number of counterions thermodynamically associated *per nucleic acid phosphate* (both condensed and screening ions), and this value is dependent only upon the linear

density of the nucleic acid and is independent of the bulk salt concentration (Record et al., 1976; Manning, 1969). Values of ψ have been estimated for a variety of linear nucleic acids (Record et al., 1976).

If we consider the equilibrium binding of a protein, P, with a nucleic acid site, D, to form a complex, PD, the observed, intrinsic equilibrium association constant, K_{obsd}, is defined in terms of only the macromolecular species, i.e.

$$P + D \leftrightarrow PD \quad (1)$$

$$K_{obsd} = [PD]/[P][D] \quad (2)$$

In our discussion of the various contributions to the salt dependence of K_{obsd}, we consider only those terms that we currently recognize as relevant to the formation of the $(SSB)_{65}$ complex. If a protein with a positively charged binding site (or any oligocation of charge $+z$) binds to a nucleic acid site in the presence of excess univalent salt (MX), then some counterions ($z\psi M^+$) will be released from the nucleic acid, in a thermodynamic sense, since they are no longer needed to partially neutralize the phosphates involved in the protein binding reaction (Record et al., 1976). If the protein also binds anions or cations and this ion binding is perturbed by the interaction of the protein with the nucleic acid, there can also be a release (or uptake) of cations (M^+) and/or anions (X^-), originating from the protein, upon formation of the complex.[1] As we show in this paper, there is a *net release* of both ΔC cations and ΔA anions upon formation of the $(SSB)_{65}$ complex (see eq 3); however, an uptake of cations and anions also contributes to the observed salt dependence (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988). In general, a net release or uptake of Δw water molecules (preferential hydration) can also accompany the formation of the complex (Tanford, 1969; Record et al., 1978). Accounting for these interactions, but neglecting possible protonation events, the thermodynamic description for the process shown in eq 1, in the presence of excess monovalent salt, MX, can be written as

$$P + D \leftrightarrow PD + \Delta CM^+ + \Delta AX^- + \Delta wH_2O \quad (3)$$

Recognizing that ΔC has contributions due to the *release* of $z\psi$ cations from the nucleic acid, as well as the *uptake* of b cations (Lohman & Overman, 1985; Bujalowski et al., 1988), and ignoring activity coefficients of the small ions, the dependence of K_{obsd} on monovalent salt concentration (at constant T, P, and pH) can be written as (Record et al., 1978)

$$-\frac{\partial \log K_{obsd}}{\partial \log [MX]} = (z\psi - b) + \Delta A - \frac{2[MX]\Delta w}{[H_2O]} \quad (4)$$

In eq 4, we have not decomposed the anion release term, ΔA, into its contributions from both uptake and release of anions.

In general, the terms b and ΔA in eq 4 will each be a function of the salt concentration, [MX], whereas the cation release from the nucleic acid, $z\psi$, is expected to be independent of salt concentration (Manning, 1969; Record et al., 1976; Anderson et al., 1978). Explicit expressions for the terms b and ΔA, in terms of ion binding constants and ion concentrations, can be obtained if one knows or assumes a model for the interaction of the ions with the protein (de Haseth et al., 1977; Record et al., 1978). For any given protein–nucleic acid interaction, not all of the ligands will bind preferentially to the protein or nucleic acid; hence eq 3 and 4 can usually be simplified. For example, except at salt concentrations >0.5 M, the term due to preferential hydration is usually negligible when compared to the ion release terms. At a minimum, as we show here, the remaining three terms are necessary to describe the salt dependence of the equilibrium binding of *E. coli* SSB protein to ss nucleic acids to form the $(SSB)_{65}$ complex.

In the case of the binding of simple oligocations, that do not bind anions, the coefficients b and ΔA equal zero (Lohman et al., 1980). Therefore, the salt dependence for this simple ligand originates solely from the release of condensed counterions from the nucleic acid upon formation of the complex, and the dependence of K_{obsd} on [MX] simplifies to eq 5. In this case, one can easily calculate the number of ionic interactions formed in the equilibrium complex, z, if the thermodynamic extent of counterion association to the nucleic acid, ψ, is known. The sole driving force for a reaction of this type is the increase in entropy due to the release of $z\psi$ counterions into the bulk solution containing a low concentration of counterions (a free energy of dilution), rather than the ionic interaction itself (Record et al., 1976; Lohman et al., 1980). For this simple case, one can also estimate the relative contributions of electrostatic vs nonelectrostatic interactions to the free energy of binding upon comparing an extrapolation of the linear log–log plot to 1 M $[M^+]$ (standard state) with the value obtained for simple model ligands (Record et al., 1976; Lohman et al., 1980). The binding of charged oligopeptides to DNA is well described by this simple case (Lohman et al., 1980), and the binding of *lac* repressor protein to nonspecific DNA approximates this case very closely (de Haseth et al., 1977). Obviously, for more complicated cases such as the *E. coli* SSB protein discussed here, it is necessary to dissect the salt dependence of the equilibrium constant into its various contributions in order to accurately estimate the number of ionic interactions, z, formed in the protein–nucleic acid complex.

$$\partial \log K_{obsd}/\partial \log [M^+] = -z\psi \quad (5)$$

Materials and Methods

Reagents and Buffers. All chemicals were reagent grade; all solutions were made with distilled and deionized (Milli-Q) water. The standard buffer was buffer T (pH 8.1) which is 10 mM tris(hydroxymethyl)aminomethane (Tris) and 0.1 mM trisodium ethylenediaminetetraacetate (Na_3EDTA). The NaCl, NaF, $NaCH_3COO$, NaBr, KCl, and potassium glutamate concentrations in buffer T were as indicated in the text. No differences in SSB binding parameters were detected when buffer H (pH 8.1) [10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) and 0.1 mM Na_3EDTA] was used in place of buffer T.

E. coli SSB Protein. The SSB protein was prepared as previously described from a strain containing the plasmid pTL119A-5, which is temperature inducible for SSB overproduction (Lohman et al., 1986b). The concentration of SSB was determined spectrophotometrically using the extinction coefficient $\epsilon_{280} = 1.5$ mL mg^{-1} cm^{-1} [1.13×10^5 M^{-1} (tetramer) cm^{-1}] in buffer T + 0.20 M NaCl (Lohman & Overman, 1985). Unfortunately, there have been a number of widely different extinction coefficients reported for the *E. coli* SSB protein; however, the most recent estimates agree with a value of 1.5 ± 0.1 mL mg^{-1} cm^{-1} (Lohman & Overman, 1985; Williams et al., 1983). SSB protein stocks were stored at −20 °C in buffer containing 50% glycerol (Lohman et al., 1986b)

[1] Note that there is *no* requirement that the number of cations and anions released or taken up in the reaction be constrained by electroneutrality; hence, in general, there can be a net release of both x anions and y cations, with $x \neq y$.

and prepared for use in binding studies by dialyzing extensively vs several changes of buffer (usually buffer T) containing at least 0.2 M NaCl. SSB protein stocks, once in buffers without 50% glycerol, were stored at 4 °C but were used for a maximum of 3 weeks. This precaution was taken, since after approximately 4 weeks of storage at 4 °C, a small amount of degradation product (<5%) is occasionally observed in the SSB sample when analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Sedimentation coefficients for SSB protein were determined by sedimentation velocity in a Beckman Model E analytical ultracentrifuge equipped with a multiplexer and photoelectric scanner. In order to correct the sedimentation coefficients to $s_{20,w}$, a value of 0.718 cm^3/g was used for the partial specific volume of the SSB protein; this value was calculated from the amino acid composition of the protein (Sancar et al., 1981; Williams et al., 1983). Viscosities and densities of the salt solutions, which were used to calculate $s_{20,w}$, were from Lobo and Quaresma (1975) and Weast (1971).

Nucleic Acids. The synthetic homopolynucleotides were purchased from Pharmacia–PL Biochemicals, Boehringer-Mannheim Biochemicals, and Miles. No detectable differences in binding isotherms were observed for polynucleotide lots of different average molecular weights for $s_{20,w} \geq 6.5$ S (Lohman & Overman, 1985). Stocks of poly(U) had $s_{20,w} = 8.1$ and 9.5 S, corresponding to average lengths of 900 ± 100 and 1100 ± 200 nucleotides, respectively. A fractionated sample of poly(U) having $s_{20,w} = 14$ S gave identical results in binding assays (Lohman et al., 1986a). Poly(dA) had $s_{20,w} = 6.6 \pm 0.1$ S, corresponding to an average length of 400 ± 20 nucleotides. The poly(A) and poly(C) stocks had $s_{20,w} = 7.8 \pm 0.2$ S, corresponding to an average length of 560 ± 30 nucleotides. Poly(dT) had $s_{20,w} = 11.8$ S, corresponding to an average length of ~1400 nucleotides. The average lengths were estimated from the sedimentation coefficients using experiments with poly(A) and poly(U) as calibrations (Eisenberg & Felsenfield, 1967; Inners & Felsenfeld, 1970). All polynucleotides were extensively dialyzed, and concentrations were determined spectrophotometrically in buffer T + 0.10 M NaCl, using the following extinction coefficients (per mole of nucleotide): polyU), $\epsilon_{260} = 9.2 \times 10^3$ M^{-1} cm^{-1}; poly(dA), $\epsilon_{257} = 1.00 \times 10^4$ M^{-1} cm^{-1}; poly(A), $\epsilon_{257} = 1.03 \times 10^4$ M^{-1} cm^{-1}; poly(dC), $\epsilon_{270} = 7.2 \times 10^3$ M^{-1} cm^{-1}; poly(C), $\epsilon_{267} = 6.5 \times 10^3$ M^{-1} cm^{-1}; poly(dT), $\epsilon_{260} = 8.1 \times 10^3$ M^{-1} cm^{-1}; poly(I), $\epsilon_{248} = 1.0 \times 10^4$ M^{-1} cm^{-1} (Kowalczykowski et al., 1981). Sedimentation coefficients were determined by sedimentation velocity as described above.

Construction of Binding Isotherms from Fluorescence Measurements and Determination of the Maximum Extent of Fluorescence Quenching, Q_{max}, for the $(SSB)_{65}$ Binding Mode. Titrations of SSB with ss homopolynucleotides ("reverse" titrations) were performed while monitoring the quenching of the intrinsic tryptophan fluorescence of SSB (λ_{ex} = 282 or 296 nm; λ_{em} = 347 nm) in an SLM 8000 spectrofluorometer as described previously (Lohman et al., 1986a). The temperature was controlled at 25.0 (±0.1) °C, and all measurements were corrected for dilution, photobleaching, and inner filter effects (Birdsall et al., 1983) as described (Lohman & Overman, 1985; Bujalowski & Lohman, 1987a).

A general method of analysis (Bujalowski & Lohman, 1987a) was used to obtain an absolute measure of the average binding density $\sum \nu_i$ (moles of bound SSB tetramer per mole of nucleotide), as a function of free SSB tetramer concentration, L_F, from a titration of SSB protein (ligand) with polynucleotide (macromolecule), in which the signal change originates from the SSB protein. This method requires no assumptions concerning the number of modes of bound protein or the relationship between the fractional signal change (fluorescence quenching, Q_{obsd}) and the fraction of bound ligand. In addition, this method allows one to rigorously *determine*, rather than assume, the relationship between the fractional change in signal from the protein and the fraction of bound protein. Let L_T be the total SSB tetramer concentration and D_T be the total nucleotide concentration for which we measure the value Q_{obsd}. The method uses the fact that, at equilibrium, the value of the "binding density function", $Q_{obsd}(L_T/D_T)$, is dependent only upon the binding density, ν (or binding density distribution, $\sum \nu_i$, if multiple states "i" of bound protein exist). As a result, when several titrations are performed at different protein concentrations and the data are plotted as $Q_{obsd}(L_T/D_T)$ vs log D_T, then for all titrations, the values of L_F and $\sum \nu_i$ are constant at any constant value of $Q_{obsd}(L_T/D_T)$. From the set of values of L_T and D_T, determined from each titration at a constant $Q_{obsd}(L_T/D_T)$, one can calculate $\sum \nu_i$ and L_F by using the relationship $L_T = L_F + (\sum \nu_i)D_T$. A plot of L_T vs D_T yields L_F from the intercept and $\sum \nu_i$ from the slope. Repeating this at a number of values of $Q_{obsd}(L_T/D_T)$ yields $\sum \nu_i$ as a function of L_F from which a binding isotherm can be constructed. The concentration of bound SSB tetramer can be calculated from $L_B = (\sum \nu_i)D_T$.

Using this method, Bujalowski and Lohman (1987a) have shown that the fractional quenching of the SSB tryptophan fluorescence upon binding to ss nucleic acids in the $(SSB)_{65}$ binding mode is equal to the fraction of bound SSB protein, in 0.2 M NaCl (25 °C, pH 8.1); i.e., $L_B/L_T = Q_{obsd}/Q_{max}$. Once it has been rigorously *proven* that L_B/L_T is directly proportional to Q_{obsd}, then one can determine the value of Q_{max}, the maximum extent of fluorescence quenching when SSB is fully saturated with nucleic acid, from a linear extrapolation of a plot of Q_{obsd} vs L_B/L_T to $L_B/L_T = 1$ (Bujalowski & Lohman, 1987a; see also Figure 1). Then one can simply calculate the concentration of bound and free SSB, L_B and L_F, respectively, and the binding density, ν, using eq 6–8.

$$L_B = L_T(Q_{obsd}/Q_{max}) \quad (6)$$

$$L_F = (1 - Q_{obsd}/Q_{max})L_T \quad (7)$$

$$\nu = (Q_{obsd}/Q_{max})(L_T/D_T) \quad (8)$$

We emphasize that the direct proportionality, $L_B/L_T = Q_{obsd}/Q_{max}$, does not generally apply to all systems under all conditions. In fact, experiments reported here indicate that this direct proportionality does not hold for SSB protein binding to polynucleotides in NaBr (see Figure 6). In addition, when a mixture of SSB binding modes is present, the direct proportionality also does not hold (T. M. Lohman, unpublished results). Hence, in constructing binding isotherms, one must use caution in making assumptions concerning the relationship between a signal change from the ligand and the fraction of bound ligand. However, even when no simple relationship exists between the fractional signal change from the protein and the fraction of bound protein, one can still rigorously determine the average degree of binding and hence obtain an experimental binding isotherm using the general method of analysis described by Bujalowski and Lohman (1987a).

"Limited" Cooperativity (Tetramer/Octamer) Model for Analysis of E. coli SSB–ss Nucleic Acid Binding in the $(SSB)_{65}$ Binding Mode. A statistical thermodynamic model (tetramer/octamer model) (Bujalowski & Lohman, 1987b), which quantitatively accounts for the observation that *E. coli* SSB protein, when bound in its "beaded", $(SSB)_{65}$ mode, forms protein clusters that are limited in size to dimers of tetramers

(octamers), was used to analyze the SSB equilibrium binding data presented in this paper, rather than a model which assumes that SSB clusters of unlimited length can form [e.g., see McGhee and von Hippel (1974) and Schellman (1974)]. The SSB tetramer seems to be the species that binds ss nucleic acids, since the SSB protein exists as a very stable tetramer in solution over a wide range of SSB protein concentrations, from 10^{-8} to 10^{-5} M (tetramer), and salt concentrations (Weiner et al., 1975; Williams et al., 1983; Sancar et al., 1981; Lohman & Overman, 1985), and the tetramer binds oligonucleotides (Bandyophadhyay & Wu, 1978; Krauss et al., 1981). The tetramer/octamer model assumes that (1) tetramers of SSB can dimerize to form octamers on the nucleic acid lattice; (2) SSB cooperativity is limited to the formation of octamers of SSB (dimers of tetramers); i.e., bound SSB tetramers do not interact with both nearest-neighbor tetramers simultaneously; (3) SSB octamers do not interact with adjacent tetramers or octamers; thus, we designate this a "limited" cooperativity; and (4) non-nearest-neighbor interactions do not occur. The model also accounts for the overlap of potential protein binding sites on the nucleic acid and requires only three parameters to describe the binding isotherm; the site size for protein binding, n, the cooperativity parameter, $\omega_{T/O}$, and the intrinsic equilibrium constant for isolated binding of SSB tetramers, K_{obsd}. In the tetramer/octamer model, the cooperativity parameter, $\omega_{T/O}$, represents the equilibrium constant for formation of a nucleic acid bound SSB octamer from two isolated, nucleic acid bound SSB tetramers. This model provides a better description of equilibrium data for SSB protein–ss polynucleotide binding in the $(SSB)_{65}$ binding mode over the entire range of binding densities than a model which assumes unlimited nearest-neighbor cooperative interactions (Bujalowski & Lohman, 1987b). The closed-form expression for the tetramer/octamer binding isotherm is given below in eq 9 and 10 for easy reference.

In addition to the difference in the numerical value and physical interpretation of the cooperativity parameter obtained from an analysis of binding isotherms using the tetramer/octamer model (Bujalowski & Lohman, 1987b) rather than the unlimited cooperativity model (McGhee & von Hippel, 1974; Schellman, 1974), we note that the values of the intrinsic binding constant, K_{obsd}, obtained from analysis of identical data by the two models differ by a factor of 2. This is due to an assumption, in the tetramer/octamer model, that two configurations of the protein–nucleic acid complex coexist, possessing equivalent binding constants, whereas the McGhee and von Hippel (1974) model, as originally developed, assumes only unidirectional binding of the protein. Therefore, the intrinsic binding constants which we report here, based on the tetramer/octamer model (Bujalowski & Lohman, 1987b), are lower by a factor of 2 than those reported previously (Lohman et al., 1986a) using the unlimited cooperativity model (McGhee & von Hippel, 1974); i.e., $K_{MvH} = 2K_{T/O}$. We also note that the quantitative salt dependences reported here do not change if the isotherms are analyzed by the unlimited cooperativity model of McGhee and von Hippel (1974).

Determination of Binding Parameters by Computer Fitting of Theoretical Isotherms to the Experimental Titrations. The experimental binding isotherms were generally plotted in the form according to Scatchard (1949) or as the fraction of bound SSB protein, L_B/L_T, vs D_T/L_T, where D_T is the total nucleotide concentration and L_T is the total SSB tetramer concentration. Estimates of the intrinsic binding constant for isolated binding of SSB tetramers, K_{obsd}, and the limited nearest-neighbor cooperativity parameter, $\omega_{T/O}$, were obtained by using the tetramer/octamer model. From the closed-form expression for the tetramer/octamer binding isotherm, the free ligand concentration can be expressed as (Bujalowski & Lohman, 1987b)

$$L_F = \nu(q^{2n-1})/\{K_{obsd}[q^2 - 2\nu q + (1 - \omega)\nu^2]^n\} \quad (9)$$

where

$$q = [1 - (n - 1)\nu] + \{[1 - (n - 1)\nu]^2 - \nu(1 - \omega)[2 - (2n - 1)\nu]\}^{1/2} \quad (10)$$

and $\omega_{T/O}$ has been designated as ω in eq 9 and 10. Theoretical isotherms were generated in the following manner. The site size, $n = 65$ nucleotides per tetramer, is known, and test values of K_{obsd} and $\omega_{T/O}$ are specified. For a given total ligand concentration, L_T, one can calculate L_F for a given value of ν using eq 9 and 10 and thereby obtain L_B ($=L_T - L_F$), L_B/L_T, and D_T ($=L_B/\nu$). By successively incrementing the value of ν, and repeating the calculations, a theoretical "reverse" titration is generated that can be directly compared to the experimental isotherm. Initial estimates of K_{obsd} and $\omega_{T/O}$ were obtained by visual comparison of the experimental isotherms with the computer-generated theoretical isotherms based on the tetramer/octamer model (Bujalowski & Lohman, 1987b). Refined estimates of the binding parameters were obtained by using a nonlinear least-squares analysis of the data (SYSTAT, Inc., Evanston, IL) fitted to the tetramer/octamer model. Note that since the site size, n, is accurately known from independent measurements (65 nucleotides per tetramer), the analysis represents only a two-parameter fit (K_{obsd} and $\omega_{T/O}$). Similar procedures, using closed-form expressions for the unlimited cooperativity model of McGhee and von Hippel (1974), have been used previously to analyze SSB binding isotherms in this fashion (Lohman et al., 1986a).

Determination of the Salt Concentration Dependence of the Binding Constant for the $(SSB)_{65}$ Binding Mode from "Salt-Back" Titrations. If and only if Q_{max}, n, $\omega_{T/O}$, and the fluorescence of free SSB protein are known and *independent of salt concentration*, and $L_B/L_T = Q_{obsd}/Q_{max}$, then the equilibrium binding constant, K_{obsd}, can be calculated from the value of Q_{obsd} measured at each salt concentration in the following manner. Equations 6–8 can be used to calculate ν and L_F at each salt concentration. Consequently, K_{obsd} can be calculated at each salt concentration by using the expression given in eq 11 (Bujalowski & Lohman, 1987b), where q is

$$K_{obsd} = (\nu/L_F)(q^{2n-1})/\{[q^2 - 2\nu q + (1 - \omega)\nu^2]^n\} \quad (11)$$

given in eq 10. By this method, a very accurate determination of the dependence of K_{obsd} on salt concentration can be obtained from a single experiment. We emphasize that this procedure *cannot* be used with confidence unless it has been rigorously proven that Q_{max}, n, and $\omega_{T/O}$ are independent of salt concentration and $L_B/L_T = Q_{obsd}/Q_{max}$.

A typical "salt-back" titration involved formation of an SSB–polynucleotide complex by first titrating SSB with nucleic acid, under stoichiometric conditions, until an excess of nucleic acid was obtained, based on a site size of 65 nucleotides per SSB tetramer. This was followed by the addition of small aliquots (2–10 μL) of buffer T (pH 8.1) containing a high concentration of salt (4 M in the case of NaCl). The increase in SSB protein fluorescence, accompanying dissociation of the SSB–nucleic acid complex, was then monitored as a function of salt concentration. We note that the fluorescence of free SSB protein has been shown to be independent of NaCl concentration (Lohman & Overman, 1985) and that it is also independent of the concentrations of NaF, sodium acetate, and

KCl; however, it is dependent upon the NaBr and potassium glutamate concentrations.

RESULTS

Solution Conditions Favoring the $(SSB)_{65}$ Binding Mode and the Stability of the SSB Tetramer. In the equilibrium binding studies reported here, we have generally limited our measurements to solution conditions in which the $(SSB)_{65}$ binding mode solely exists (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988). The transition between the $(SSB)_{56}$ and the $(SSB)_{65}$ binding modes is dependent upon the cation and anion types and concentrations as well as the SSB binding density. At high binding densities, the $(SSB)_{65}$ mode is stable in ≥0.1 M NaBr, ≥0.2 M NaCl, and ≥0.8 M NaF (Bujalowski et al., 1988). However, at lower SSB protein binding densities, the transition is shifted to lower salt concentrations, so that for the SSB–poly(U) interaction, the $(SSB)_{65}$ binding mode is stable in ≥0.2 M NaF (L. B. Overman, unpublished experiments). Therefore, the experiments in NaF and $NaCH_3COO$ described here were performed at low SSB binding densities to favor the conditions in which the $(SSB)_{65}$ mode is stable. In the experiments in NaCl (≥0.2 M) and NaBr (≥0.1 M), the $(SSB)_{65}$ binding mode is stable over the entire binding density range (Bujalowski et al., 1988).

The *E. coli* SSB protein remains a stable tetramer under all of the solution conditions used in our equilibrium binding experiments. This was verified by measuring the sedimentation coefficient, $s_{20,w}$, of the SSB protein at several concentrations of the various salts: NaCl (0.20–3.0 M), NaF (0.2–0.63 M), NaBr (0.2–3.0 M), $NaCH_3COO$ (0.20–0.90 M), and potassium glutamate (0.2 M). The results indicate that the values of $s_{20,w}$ are independent of salt concentration and type, with $s_{20,w} = 4.4 \pm 0.3$ S. No dependence of $s_{20,w}$ on protein concentration was observed over the range from 0.6 to 4.9 μM (tetramer) in NaCl, although the SSB concentration was limited to 1.3 μM in glutamate, fluoride, and acetate due to the solubility limits in these salts. Protein solubility is not a problem in the binding experiments since these are performed at much lower protein concentrations [<50 μg/mL ~ 0.7 μM (tetramer)]. The $s_{20,w}$ values are the same, within experimental error, as those previously reported for the *E. coli* SSB tetramer (Weiner et al., 1975; Ruyechan & Wetmur, 1976; Lohman & Overman, 1985); hence, we conclude that the *E. coli* SSB protein, when not bound to nucleic acid, exists as a stable tetramer under the conditions of our equilibrium binding experiments. Therefore, the salt effects reported here reflect effects on the SSB protein–nucleic acid interactions rather than changes in the quaternary structure of the SSB protein.

Relationship between SSB Fluorescence Quenching and the Fraction of Bound SSB Protein and Determination of Q_{max} for the $(SSB)_{65}$ Binding Mode. Bujalowski and Lohman (1987a) have developed a general method for the absolute determination of the average degree of ligand binding per macromolecule, ν, when a spectroscopic signal from the ligand is used to monitor binding, which is briefly described under Materials and Methods. This method can be used to (1) rigorously determine the relationship between the fraction of ligand bound and the degree of fluorescence quenching and, (2) if this relationship is linear, accurately determine Q_{max}. Only when it can be rigorously shown that the fraction of bound protein is directly proportional to the observed quenching, Q_{obsd}, can one confidently use a single titration to calculate L_B using eq 6. However, even when the fractional signal change is not directly proportional to the fraction of bound ligand, one can still rigorously determine an experimental binding isotherm using this method (Bujalowski & Lohman, 1987a), as we show for SSB protein binding to poly(U) and poly(dT) in NaBr solutions (see Figures 6 and 7).

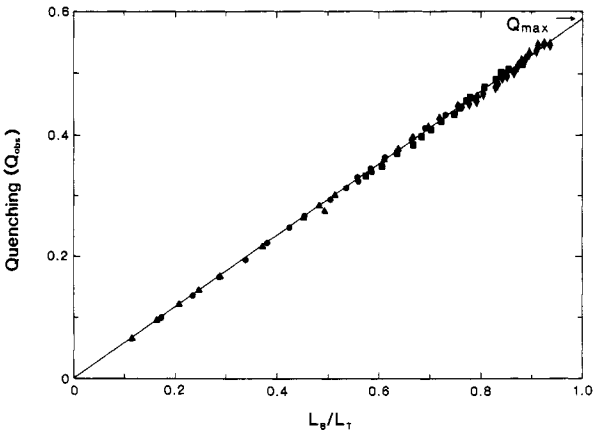

FIGURE 1: Determination of Q_{max} and demonstration that $Q_{obsd}/Q_{max} = L_B/L_T$ for SSB protein binding to poly(U) (buffer T, pH 8.1, 0.25 M NaCl, 25 °C). The method of Bujalowski and Lohman (1987a) was used to determine the fraction of bound SSB protein, L_B/L_T, as a function of the degree of quenching (Q_{obsd}), using data from four reverse titrations. The SSB tetramer concentrations are (▲) 0.24, (●) 0.32, (■) 0.47, and (♦) 0.94 μM. A linear extrapolation to $L_B/L_T = 1$, as indicated in the figure, yields $Q_{max} = 0.59 \pm 0.01$.

For the SSB–poly(U) interaction in 0.2 M NaCl (pH 8.1, 25 °C), Bujalowski and Lohman (1987a) have shown that $L_B/L_T = Q_{obsd}/Q_{max}$. In Figure 1, we have plotted Q_{obsd}, for the SSB–poly(U) interaction, as a function of the fraction of SSB bound in the $(SSB)_{65}$ binding mode, L_B/L_T, determined from the analysis of four titrations at different SSB concentrations in 0.25 M NaCl. Clearly, Q_{obsd} is directly proportional to L_B/L_T, and extrapolation to $L_B/L_T = 1$ yields a value of $Q_{max} = 0.59$. This linear relationship is independent of binding density under these conditions (Bujalowski & Lohman, 1987a). We have checked this relationship at a number of concentrations for the different salts used in this study and have found it to hold for all conditions in which the $(SSB)_{65}$ complex is formed, at pH 8.1, 25 °C, with the *exception* of the experiments in NaBr (see below and Figure 6). For the experiments in potassium glutamate (KGlu), the direct proportionality holds, although Q_{max} is also slightly dependent on the concentration of KGlu.

The determination of Q_{max} for a protein–nucleic acid interaction is often performed under "low salt" conditions where binding is stoichiometric. This procedure is not possible for the $(SSB)_{65}$ binding mode in NaCl solutions for complexes of SSB with poly(U), poly(dA), and poly(A), since the salt concentration must be reduced well below 0.2 M NaCl, promoting the formation of the lower site size SSB binding modes, one of which possesses different fluorescence properties than those of $(SSB)_{65}$ (Lohman & Overman, 1985; Lohman et al., 1986a). Therefore, for these polynucleotides, it was necessary to use the general method of Bujalowski and Lohman (1987a) to determine Q_{max} under the salt conditions of interest. Another method to obtain estimates of Q_{max} is that of Schwarz and Watanabe (1983); however, that method *assumes* that the fluorescence quenching is proportional to the fraction of bound ligand and that Q_{max} is independent of binding density; hence, incorrect isotherms can be obtained if that assumption is not valid, as is the case in our experiments in NaBr (see Figure 6).

Table I shows the values of Q_{max} determined for the interaction of SSB protein with poly(U), poly(dA), and poly(A)

Table I: Polynucleotide Specificity of SSB Protein in the $(SSB)_{65}$ Binding Mode (25 °C, pH 8.1)

polynucleotide	Q_{max}	$\partial \log K_{obsd}/\partial \log$ [NaCl]	$\log K_{obsd}$ (1.0 M NaCl)[a]	K_{obsd} (M^{-1}) (0.20 M NaCl)	$\omega_{T/O}$
poly(U)	0.59 (±0.01)	−7.4 (±0.5)	−0.4 (±0.2)	5.9 (±2.1) × 10^4	380 (±100)
poly(dA)	0.55 (±0.01)	−6.1 (±0.6)	−0.4 (±0.4)	7.3 (±0.3) × 10^3	350 (±250)
poly(A)	0.39 (±0.01)	−6.2 (±0.3)	−1.5 (±0.3)	6.8 (±1.4) × 10^2	450 (±250)

[a] Extrapolated.

Table II: Effect of Anion Type on the SSB–Polynucleotide Interaction in the $(SSB)_{65}$ Binding Mode (25 °C, pH 8.1)

polynucleotide	salt	Q_{max}	$\partial \log K_{obsd}/\partial \log$ [MX]	$\log K_{obsd}$ (1.0 M salt)[a]	K_{obsd} (M^{-1}) (0.35 M NaCl)	$\omega_{T/O}$[b]
			Data from Salt-Back Titrations			
poly(U)	KCl	0.59 (±0.01)	−7.1 (±0.1)	−0.5 (±0.1)	5.5 (±0.7) × 10^2[a]	413
poly(U)	NaCl	0.59 (±0.01)	−7.4 (±0.5)	−0.4 (±0.2)	9.4 (±0.6) × 10^2	413
poly(U)	$NaCH_3COO$	0.65 (±0.01)	−6.5 (±0.3)	2.0 (±0.1)	9.2 (±0.8) × 10^4	413
poly(U)	NaF	0.66 (±0.01)	−4.3 (±0.4)	3.3 (±0.3)	1.8 (±0.5) × 10^5[a]	413
			Data from Reverse Titrations			
poly(U)	KCl	0.59 (±0.01)	−7.2 (±0.5)	−0.7 (±0.3)	3.8 (±0.6) × 10^2[a]	460 (±100)
poly(U)	NaCl	0.59 (±0.01)	−7.0 (±1.1)	−0.2 (±0.7)	1.0 (±0.5) × 10^3	380 (±100)
poly(U)	$NaCH_3COO$	0.65 (±0.01)	−6.6(±0.4)	1.9 (±0.2)	8.1 (±0.3) × 10^4	370 (±130)
poly(U)	NaF	0.66 (±0.01)	−4.8 (±1.5)	3.1 (±0.4)	1.9 (±0.9) × 10^5[a]	440 (±120)
poly(U)	potassium glutamate	0.65 (±0.02)[c]	−5.7 (±0.4)	3.1 (±0.1)	5.0 (±0.9) × 10^5[a]	380 (±160)
poly(U)	NaBr	0.54–0.57 (±0.01)[d]	−6.7 (±0.6)	−1.1 (±0.5)	90 (±50)[a]	380 (±190)
poly(dT)	NaBr	0.78–0.88[d]	−5.7 (±0.7)	6.7 (±0.3)	~2 × 10^9[a]	130 (±70)

[a] Extrapolated. [b] A salt-independent average value of $\omega_{T/O}$ = 413 was used to determine K_{obsd} from the salt-back titrations. [c] Q_{max} is dependent on the potassium glutamate concentration over the range from 0.40 to 0.65 M. [d] Fluorescence quenching is not proportional to the fraction of bound SSB protein.

in the $(SSB)_{65}$ binding mode in buffer T (pH 8.1) at 25 °C, and Table II compares the values of Q_{max} determined for the SSB–poly(U) interaction in NaCl, NaF, $NaCH_3COO$, KCl, NaBr, and KGlu. Except in solutions of NaBr and KGlu, we find that Q_{max} is independent of salt concentration, for a given homopolynucleotide and salt type. We note that although one can determine a unique value for Q_{max} in the limit of low SSB binding density for the SSB–poly(U) experiments in NaBr, one must evaluate the binding parameters for this interaction using the general method of analysis (Bujalowski & Lohman, 1987a), since we have shown that Q_{obsd} is not directly proportional to the fraction of bound SSB (L_B/L_T) in the presence of NaBr. There is a definite hierarchy of affinities of SSB protein for homopolynucleotides, and the value of Q_{max} seems to be correlated with this affinity; however, the molecular basis for this correlation is not well understood. It is clear that Q_{max} is not simply related to the affinity of the SSB–polynucleotide complex, since, in the majority of cases, Q_{max} does not decrease with increasing salt concentration, whereas K_{obsd} clearly does decrease.

Salt Dependence of the $(SSB)_{65}$ Equilibrium Complex Resides in the Intrinsic Binding Constant Rather Than the Cooperativity Parameter. A series of titrations of SSB protein with poly(U) performed at different NaCl concentrations (0.20–0.275 M) and a single SSB concentration is shown in Figure 2 along with theoretical binding isotherms generated by using the tetramer/octamer model (Bujalowski & Lohman, 1987b), as described under Materials and Methods. Recall that under these conditions only the $(SSB)_{65}$ complex is formed (Lohman & Overman, 1985). We have directly measured the site size of the SSB–poly(U) interaction in 0.20 M NaCl, using a high SSB concentration [5.2 × 10^{-6} M (tetramer); 392 μg/mL] to ensure stoichiometric binding, and obtained a site size of 66 ± 2 nucleotides per tetramer (data not shown). From quantitative analyses of the data in Figure 2, we note that the affinity of SSB protein for poly(U) decreases as the NaCl concentration increases. A constant cooperativity parameter of $\omega_{T/O}$ = 420 provides a good fit for the titrations at all NaCl concentrations, indicating that $\omega_{T/O}$ is independent of NaCl concentration in the range of 0.20 M ≤ [NaCl] ≤ 0.4 M. This was checked at each NaCl concentration, using titrations that covered the full range of fractional saturation of SSB protein (data not shown). However, the value of the intrinsic association equilibrium constant decreases steeply with increasing NaCl concentration. The steep dependence of the stability of the $(SSB)_{65}$ complex on [NaCl] is also apparent from a salt-back titration of the SSB–poly(U) complex shown in Figure 3. Dissociation of the SSB–poly(U) complex occurs over the range from approximately 0.2 to 0.4 M NaCl, with a midpoint of 0.28 M NaCl. Complete dissociation of poly(U) is obtained upon increasing the [NaCl], indicating reversibility of complex formation.

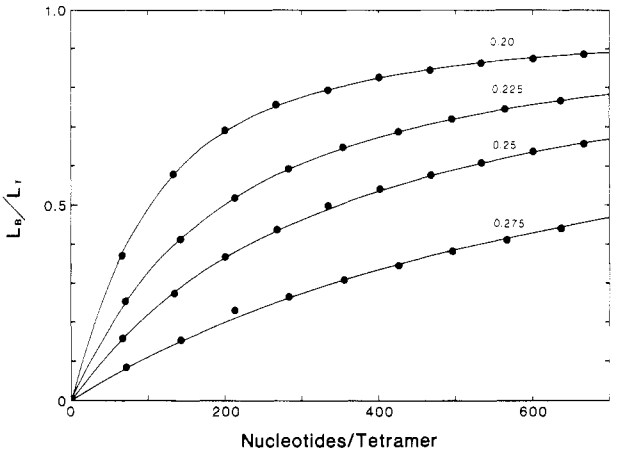

FIGURE 2: "Reverse" titrations of SSB protein with poly(U) at four NaCl concentrations (buffer T, pH 8.1, 25 °C) indicate that the binding constant is salt dependent but the cooperativity parameter is not. L_B/L_T (=Q_{obsd}/Q_{max}) is plotted versus the mole ratio of total nucleotides per total SSB tetramer. The NaCl concentrations are indicated in the figure. The initial SSB protein concentration was 0.10 μM tetramer. The smooth curves are the best theoretical fits to the data generated by using the tetramer/octamer model (Bujalowski & Lohman, 1987b), as described under Materials and Methods, with $\omega_{T/O}$ = 420, n = 65 nucleotides/tetramer, and Q_{max} = 0.59. The intrinsic binding constants used to generate the theoretical curves are 5.7 × 10^4, 2.5 × 10^4, 1.4 × 10^4, and 6.1 × 10^3 M^{-1} for the curves at 0.20, 0.225, 0.25, and 0.275 M NaCl, respectively.

On the basis of the observation that the cooperativity of the

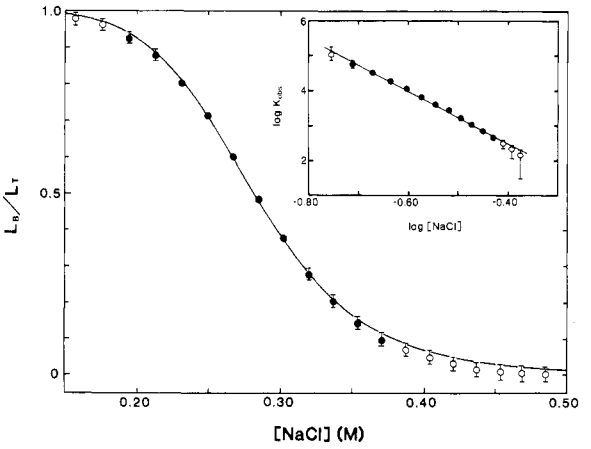

FIGURE 3: "Salt-back" titration of an SSB protein–poly(U) complex can be used to determine the salt dependence of the binding constant (K_{obsd}). Salt-back titration of an SSB protein–poly(U) complex preformed in 0.10 M NaCl (buffer T, pH 8.1, 25 °C); initial [SSB] = 0.15 μM, initial [poly(U)] = 117 μM nucleotide. The fraction of bound SSB protein, L_B/L_T, is plotted versus NaCl concentration. Error bars result from the accuracy in determining the fluorescence quenching (Q_{max} = 0.59 ± 0.01). Closed symbols indicate the region in which $0.10 \leq L_B/L_T \leq 0.90$. The smooth curve is the theoretical prediction generated by using the tetramer/octamer model (Bujalowski & Lohman, 1987b) with $\omega_{T/O}$ = 420 and the salt dependence of K_{obsd} (slope and y intercept) obtained from the inset. The inset shows the log–log plot of the binding constant calculated from the data in the salt-back titration as described in the text. Error bars were determined from the error in the measurement of fluorescence quenching in the salt-back titration and the error in Q_{max}. Closed and open symbols correspond to those points indicated in the salt-back titration. The equation for the linear least-squares line in the inset is log K_{obsd} = −7.2 log [NaCl] − 0.66.

SSB protein–ss nucleic acid interaction is independent of NaCl concentration over the range from 0.2 to 0.4 M NaCl (see Table I) and that Q_{max} is independent of [NaCl] over this same range, we have determined values of K_{obsd} as a function of [NaCl] from salt-back titrations using the procedures outlined under Materials and Methods, and these are plotted in the inset to Figure 3. Over the [NaCl] range examined (0.2 M ≤ [NaCl] ≤ 0.4 M), the log–log plot of K_{obsd} vs [NaCl] is linear with $\partial \log K_{obsd}/\partial \log$ [NaCl] = −7.4 ± 0.5 at 25.0 °C, pH 8.1. The smooth curve in Figure 3 is a theoretical prediction of the salt-back titration based on the measured salt dependence of K_{obsd} obtained from the "log–log" plot in the inset to Figure 3, using the tetramer/octamer model with $\omega_{T/O}$ = 400 to describe the interaction (Bujalowski & Lohman, 1987b; see eq 9–11).

Identical salt dependences were obtained from salt-back titrations, independent of protein concentration or extent of initial fractional saturation of nucleic acid (as long as nucleic acid was in excess). Note that the error in the determination of K_{obsd} from a salt-back titration increases significantly in the regions where the protein is either less than 10% or greater than 90% covered with nucleic acid. The major sources of error originate from the determination of the maximum extent of fluorescence quenching, Q_{max}. For this reason, only the data in the mid range of the salt-back titration [$0.1 \leq (L_B/L_T) \leq 0.9$] were used to estimate the salt dependence of K_{obsd}.

For conditions in which the relationship $L_B/L_T = Q_{obsd}/Q_{max}$ has been shown to hold, we have generally used reverse titrations performed at a series of salt concentrations to obtain the value of the cooperativity parameter and to determine whether it is independent of [NaCl] in this region. If these constraints hold, then a salt-back titration can be used to obtain a more accurate determination of the salt dependence of K_{obsd}. In Figure 4, values of K_{obsd} determined by reverse titrations

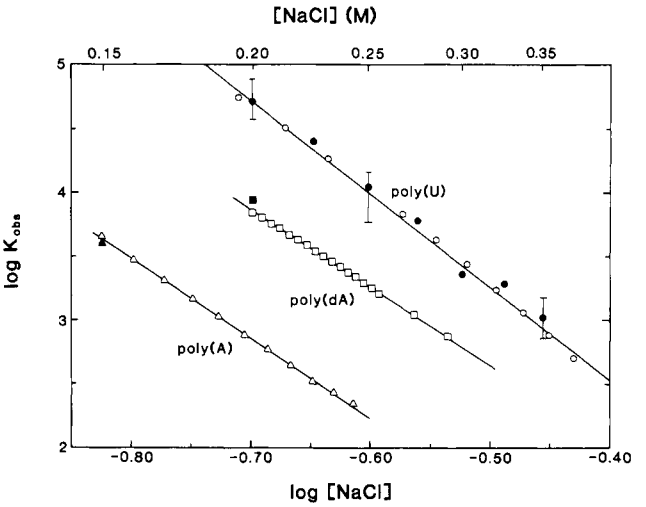

FIGURE 4: Dependence of the intrinsic binding constant on [NaCl] (log–log plot) for the interaction of SSB protein with poly(U), poly(dA), and poly(A). The binding constants were calculated by using the procedures given under Materials and Methods with $\omega_{T/O}$ = 413. Open symbols represent data from salt-back titrations, and closed symbols are from reverse titrations performed at constant NaCl concentrations. Error bars on reverse titration data show the range of values obtained from multiple determinations. Linear least-squares lines are shown for salt-back titrations and the equation for each line is given in Table I. The polynucleotides are (●, ○) poly(U), (■, □) poly(dA), and (▲, △) poly(A) (25 °C, pH 8.1).

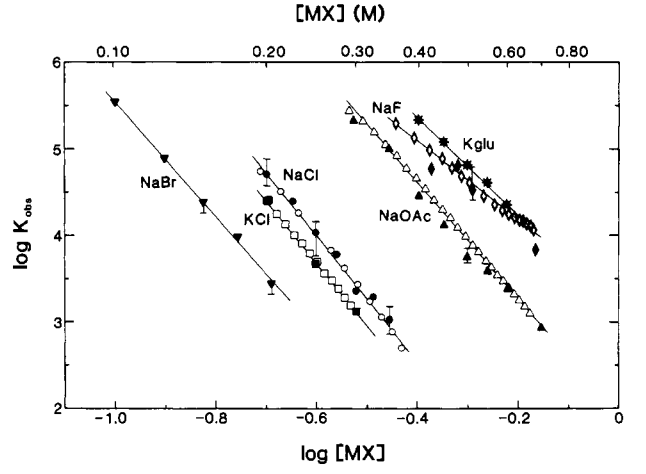

FIGURE 5: Dependence of the intrinsic binding constant on salt concentration (log–log plot) for the interaction of SSB protein with poly(U) in salts of different types. The binding constants were calculated by using the procedures given under Materials and Methods with $\omega_{T/O}$ = 413. Open symbols represent data from salt-back titrations, and closed symbols are from reverse titrations performed at constant salt concentrations. The linear least-squares lines shown are based on salt-back titrations for KCl, NaCl, $NaCH_3COO$, and NaF and on the reverse titration data for NaBr and potassium glutamate. The equation corresponding to each line is given in Table II. The salts are (▼) NaBr, (■, □) KCl, (●, ○) NaCl, (▲, △) $NaCH_3COO$, (♦, ◇) NaF, and (★) potassium glutamate (25 °C, pH 8.1).

at a number of salt concentrations are directly compared to values of K_{obsd} determined by the salt-back titration method for poly(U), poly(dA), and poly(A) as a function of [NaCl]. Figure 5 shows similar comparisons for the SSB–poly(U) interaction in a series of different salts. The agreement between the two methods is very good, indicating that the system is at equilibrium under the conditions of our experiments and that both methods result in valid binding constants. Table II gives a further comparison of these two methods. Since SSB protein–ssDNA interactions can show severe nonequilibrium effects at low salt (Lohman et al., 1986a), it was important

to be certain that the measurements at these higher [NaCl] are at equilibrium.

Specificity of the Interaction of SSB Protein with Single-Stranded Nucleic Acids in NaCl Buffers. *E. coli* SSB protein exhibits a dramatic base and sugar specificity in its interaction with ss homopolynucleotides; however, most previous comparisons of relative binding parameters have been made under low-salt conditions (Molineux et al., 1975), where a mixture of binding modes as well as nonequilibrium phenomena can occur, or with oligonucleotides (Krauss et al., 1981). In this section, we report the equilibrium binding specificity of SSB protein to a numer of ss homopolynucleotides, in the $(SSB)_{65}$ binding mode, as a function of [NaCl]. Quantitative estimates of K_{obsd} and $\omega_{T/O}$ are only reported for SSB binding to poly(U), poly(dA), and poly(A) in buffers containing NaCl. Poly(C) binds very weakly to SSB in [NaCl] $\geq$ 60 mM, although we have measured binding parameters at lower [NaCl]. Poly(dT) binds too tightly to SSB to accurately measure K_{obsd} and $\omega_{T/O}$ in buffers containing only NaCl, even in 5 M NaCl (Lohman & Overman, 1985); however, the equilibrium constant for the SSB–ss nucleic acid interaction is weakened sufficiently in high concentrations of NaBr so that an accurate measurement of the salt dependence of K_{obsd} can be made in this salt for poly(dT) (see below).

Figure 4 shows the dependence of K_{obsd} on NaCl concentration for the interaction of SSB with poly(U), poly(A), and poly(dA) in the $(SSB)_{65}$ binding mode. Under these conditions, K_{obsd} decreases in the order poly(U) > poly(dA) > poly(A), whereas the cooperativity parameter is the same within experimental error for all three nucleic acids ($\omega_{T/O}$ = 400 ± 100). The equilibrium constant for SSB binding to these homopolynucleotides, in the $(SSB)_{65}$ mode, is highly salt dependent; the values of $\partial \log K_{obsd}/\partial \log$ [NaCl], determined from the salt-back titration data shown in Figure 4, are −7.4 ± 0.5 for poly(U), −6.1 ± 0.6 for poly(dA), and −6.2 ± 0.3 for poly(A) (see Table I). The dramatic decrease in K_{obsd} with increasing [NaCl] for all three polynucleotides indicates that there is a significant release of ions (Na^+ and/or Cl^-) upon formation of the $(SSB)_{65}$ complex, suggesting contributions to the binding free energy due to electrostatic interactions (Record et al., 1976; Lohman et al., 1980). Although the data in Figure 4 indicate a steep [NaCl] dependence of K_{obsd} for all three polynucleotides, there is a quantitative difference between the slopes for poly(A) and poly(dA) binding and the slope for poly(U) binding, indicating slight polynucleotide-dependent differences in the extent of ion release.

It is not possible to obtain equilibrium binding constants for formation of the $(SSB)_{65}$ complex with poly(C) or poly(dT) in NaCl at 25 °C (pH 8.1). A meaningful measure of an equilibrium binding constant for the SSB protein–ssM13 DNA interaction also cannot be made due to the presence of competing intramolecular base-paired structures in the DNA; an apparent midpoint of ~1.4 M NaCl is obtained at 25.0 °C, pH 8.1 (data not shown). Although we have not directly measured K_{obsd} and $\omega_{T/O}$ for the interaction of SSB protein with poly(dC) and poly(I), we have qualitatively ranked their relative affinities using salt-back titrations. As a result, we can rank the homopolynucleotides in order of equilibrium binding affinity, *in the* $(SSB)_{65}$ *binding mode*, as follows (in 0.2 M NaCl, pH 8.1, 25.0 °C): K_{obsd}[poly(dT)] > K_{obsd}[poly(dC)] ≫ K_{obsd}(ssM13 DNA) > K_{obsd}[poly(I)] > K_{obsd}[poly(U)] = $8K_{obsd}$[poly(dA)] = $87K_{obsd}$[poly(A)] ≫ K_{obsd}[poly(C)]. We have also obtained quantitative measurements of K_{obsd} and $\omega_{T/O}$ for both poly(U) and poly(dT) in buffers containing NaBr, and these are discussed below.

Table I shows the values of K_{obsd} and $\omega_{T/O}$ for a number of solution conditions, obtained by fitting the experimental isotherms to the tetramer/octamer model (Bujalowski & Lohman, 1987b) for the three polynucleotides examined. The cooperativity parameter obtained by using this model is 380 ± 100 for poly(U), 450 ± 250 for poly(A), and 350 ± 250 for poly(dA). The larger error limits for poly(A) and poly(dA) result from attempting to determine the cooperativity under conditions where the binding affinity is much weaker for these polynucleotides than for poly(U). Values of $\omega_{T/O}$ for SSB binding to poly(A) and poly(dA) are the same as for poly(U), within experimental error, under conditions which promote tighter binding for these polynucleotides, such as in buffers containing NaF (L. B. Overman, unpublished experiments). In NaCl, the most accurate estimate of $\omega_{T/O}$ for the SSB–poly(A) interaction was made in 0.15 M NaCl so that the potential presence of other binding modes, in particular the $(SSB)_{56}$ mode, may complicate the analysis of these data. However, in this case, the experiments were performed at low binding densities, where the $(SSB)_{65}$ complex predominates (W. Bujalowski, unpublished results).

Preferential Anion and Cation Effects on the Equilibrium Binding of SSB Protein in the $(SSB)_{65}$ *Mode.* The NaCl concentration dependences of K_{obsd} shown in Figure 4 indicate that a net release of ions accompanies the formation of the $(SSB)_{65}$ complex, for all three homopolynucleotides examined (Record et al., 1976, 1978). Although the values of $\partial \log K_{obsd}/\partial \log$ [NaCl] differ slightly for the individual nucleic acids, these data indicate that there are approximately 7 ± 1 ions released in each case. For a protein–nucleic acid interaction, the salt dependence of the equilibrium binding constant may have contributions from preferential interaction of both cations and anions, although there is no electroneutrality constraint on the relative contributions of cation vs anion release.[1] In order to interpret the salt dependences shown in Figure 4 and Table I in more detail, we have determined the relative contributions of cation and anion release to the slopes in Figure 4 by measuring the salt dependence of K_{obsd} for the SSB–poly(U) interaction in a number of salts which differ only in the anion type.

We have measured K_{obsd} and $\omega_{T/O}$, for formation of the $(SSB)_{65}$ complex with poly(U), as a function of the concentration of the following salts: NaCl, KCl, NaBr, $NaCH_3COO$, NaF, and potassium glutamate (pH 8.1, 25 °C), and the results are displayed in Figure 5 and Table II. A constant value of the cooperativity parameter ($\omega_{T/O}$ = 410 ± 120) provides a good fit to all of the isotherms; hence, $\omega_{T/O}$ seems to be independent of anion type and concentration. Salt-back titration data are not presented for the SSB–poly(U) interaction in KGlu, since Q_{max} was found to be slightly salt-dependent in this salt. As a result, Q_{max} was determined at each KGlu concentration (data not shown). The binding isotherms for the SSB–poly(U) interaction in NaBr were determined at each NaBr concentration using the analysis of Bujalowski and Lohman (1987a), since we have found that Q_{obsd} is a nonlinear function of L_B/L_T under these conditions and also that Q_{max} and the free SSB protein fluorescence are dependent upon [NaBr].

It is clear from Figure 5 that the anion type and concentration have a major effect on both the magnitude and salt dependence of K_{obsd}. At 0.35 M Na^+ (pH 8.1, 25 °C), the value of K_{obsd} in the presence of the different anions is as follows: K_{obsd}(Glu) = $5.6K_{obsd}$(F) = $11K_{obsd}$(CH_3COO) = (1.1×10^3)K_{obsd}(Cl) = (1.1×10^4)K_{obsd}(Br). Over the limited range of salt concentrations investigated, the log–log plots of

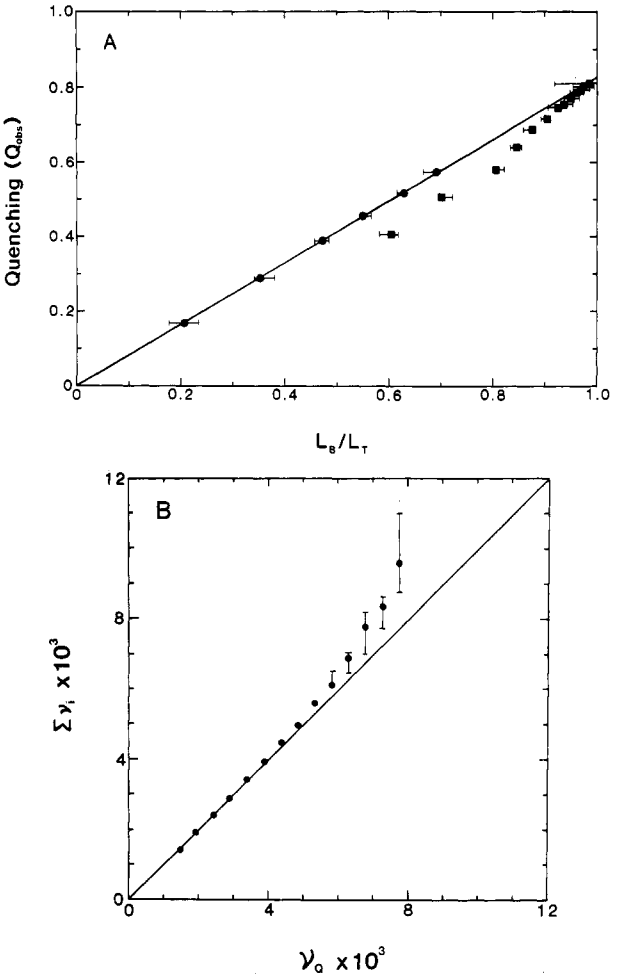

FIGURE 6: Demonstration that Q_{obsd}/Q_{max} is *not* equal to L_B/L_T for SSB protein binding to poly(dT) in 2.0 M NaBr (buffer T, pH 8.1, 25 °C). (A) Data from five reverse titrations were analyzed by using the method of Bujalowski and Lohman (1987a) to determine the fraction of bound SSB, L_B/L_T, as a function of the degree of quenching (Q_{obsd}). Data from only two of the titrations are shown in which the SSB tetramer concentrations are (●) 0.036 μM and (■) 0.56 μM. The line shows the expected relationship if $Q_{obsd}/Q_{max} = L_B/L_T$ as is the case in NaCl (see Figure 1). (B) The apparent average binding density, ν_Q, calculated by assuming $L_B/L_T = Q_{obsd}/Q_{max}$, with Q_{max} = 0.825, deviates from the true average binding density, $\sum\nu_i$, determined by the general method of analysis. The line shows the expected relationship if $Q_{obsd}/Q_{max} = L_B/L_T$.

K_{obsd} vs [MX] appear linear with slopes of −5.7 ± 0.4 in KGlu, −4.3 ± 0.4 in NaF, −6.5 ± 0.3 in $NaCH_3COO$, and −6.7 ± 0.6 in NaBr, as compared to −7.4 ± 0.5 in NaCl and −7.1 ± 0.1 in KCl. Although a straight line describes the data very well over the limited experimental salt concentration range covered for each salt, the log–log plots are not expected to be linear over a wider range of salt concentrations since there is clearly an anion contribution to the salt dependence.[2]

Salt Dependence of K_{obsd} for the SSB Protein–Poly(dT) Interaction. In order to accurately measure the equilibrium binding constant for the SSB protein–poly(dT) interaction, as well as its salt dependence, it has been necessary to study the interaction in salts other than NaCl, since the affinity is too high even in 5 M NaCl (25 °C, pH 8.1). On the basis of our observations that the affinity of SSB protein for poly(U)

[2] Since anion binding to the protein should be describable by a mass action phenomenon, rather than a condensation effect (Manning, 1969), the log–log plots of K_{obsd} vs [MX] are not expected to remain linear over a wide range of salt concentrations, although this nonlinearity is difficult to detect over a limited salt concentration range.

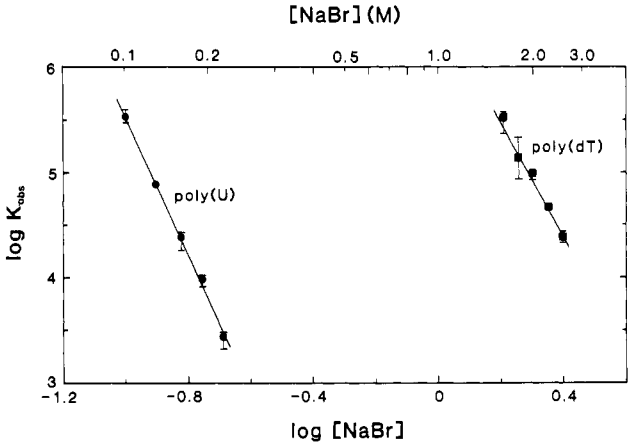

FIGURE 7: Dependence of the intrinsic binding constant on NaBr concentration (log–log plot) for the interaction of SSB protein with poly(dT). The binding constants were estimated by obtaining the best fit of the tetramer/octamer model (Bujalowski & Lohman, 1987b) to the experimental isotherm determined by using the general method of analysis (Bujalowski & Lohman, 1987a) with $\omega_{T/O}$ = 126 for the poly(dT) data (■). Error bars indicate the range of values estimated from Scatchard plots. The data from Figure 5 for the SSB–poly(U) interaction (●) are included for comparison ($\omega_{T/O}$ = 413). Linear least-squares lines are shown, and the equation for each line is given in Table II (25 °C, pH 8.1).

is dramatically decreased in buffers containing either $MgCl_2$ or NaBr, we performed salt-back titrations of SSB–poly(dT) complexes in order to determine whether dissociation of the complex occurs in these salts. We observed the onset of dissociation of a preformed SSB protein–poly(dT) complex at ~1.8 M $MgCl_2$; however, precipitation occurred at approximately 2.2 M $MgCl_2$; hence, the experiment could not be completed. However, we found that complete dissociation of the SSB protein–poly(dT) complex could be achieved in concentrations of NaBr > 3 M, without precipitation. These two observations indicate that the SSB–poly(dT) complex is salt dependent; however, it is necessary to lower the equilibrium binding constant, $K\omega$, below ~10^8 M^{-1} in order to detect dissociation in the fluorescence experiments described here.

The general method of analysis (Bujalowski & Lohman, 1987a) was performed at 2.0 M NaBr, in order to test whether $L_B/L_T = Q_{obsd}/Q_{max}$ for the SSB–poly(dT) interaction in NaBr. This was of particular concern, since we observed that the intrinsic fluorescence of the SSB protein is dependent upon [NaBr], although it is not dependent upon the concentrations of NaCl, KCl, NaF, or $NaCH_3COO$ (Lohman & Overman, 1985). Just as we had observed for the SSB–poly(U) interaction in NaBr solutions, the results of this analysis indicated that Q_{obsd} is *not* directly proportional to the fraction of bound SSB protein, L_B/L_T, over the entire binding density range. In Figure 6A, a plot of Q_{obsd} vs L_B/L_T is shown which clearly demonstrates the nonlinear relationship between these two quantities for the SSB–poly(dT) interaction in NaBr. Another demonstration of this nonlinearity is shown in Figure 6B, where we have plotted the actual binding density, $\sum\nu_i$, determined from a binding density analysis of five titration curves vs the apparent binding density, ν_Q, calculated by using the assumption that $L_B/L_T = Q_{obsd}/Q_{max}$ (with Q_{max} = 0.825). It is clear that $L_B/L_T \neq Q_{obsd}/Q_{max}$, over the entire range of binding densities in NaBr. As a result, it is not possible to obtain an accurate binding isotherm from a titration at a single SSB protein concentration in NaBr nor is it possible to determine the NaBr concentration dependence of K_{obsd} and $\omega_{T/O}$ by a salt-back titration for the SSB–poly(dT) interaction. Hence, the general method of analysis (Bujalowski & Lohman,

1987a) had to be performed at each of five NaBr concentrations, from 1.8 to 2.5 M.

The NaBr concentration dependence of K_{obsd} for the SSB protein–poly(dT) interaction is shown in Figure 7, from which we calculate $\partial \log K_{obsd}/\partial \log [NaBr] = -5.7 \pm 0.7$. The SSB–poly(U) data in NaBr are also directly compared with the poly(dT) data in Figure 7. It is clear that the SSB protein–poly(dT) interaction is highly salt dependent, with a log–log slope slightly lower than that found for the interaction with poly(U) (-6.7 ± 0.6). However, a significantly lower value for the cooperativity parameter was obtained for the SSB–poly(dT) interaction in this range of NaBr concentrations, with $\omega_{T/O} = 130 \pm 70$. We do not know whether this lower value of $\omega_{T/O}$ reflects some inherent property of poly(dT) or an effect of the high NaBr concentrations used in these experiments (1.8–2.5 M). The SSB–poly(U) interaction at lower NaBr concentrations shows the same higher value of $\omega_{T/O} = 400 \pm 100$, which we have found for most of the other solution conditions that have been examined. Upon extrapolating both sets of data in Figure 7 to a common NaBr concentration (1.0 M), we estimate that the affinity of SSB protein is a factor of $\sim 10^8$ greater for poly(dT) than for poly(U). We also note that since the NaBr concentrations required for the poly(dT) experiments are in the molar range, the salt dependence for the interaction with poly(dT) may be lower due to contributions from the release of bound water, which may partially offset the salt dependence due to the release of ions (Tanford, 1969; Record et al., 1978). In addition, since anion release accompanies complex formation, the log–log slope is not expected to remain constant over such a wide range of NaBr concentrations (0.1–2.5 M);[2] hence, the values of K_{obsd} obtained from the linear extrapolations of poly(U) and poly(dT) to 1 M NaBr should be viewed only as estimates.

Specific Effects of Sodium vs Potassium Ion on the $(SSB)_{65}$ Equilibria. In addition to the differential effects of anions which are apparent in Figure 5, a slight but significant difference in K_{obsd} for formation of the $(SSB)_{65}$ complex is observed in the presence of NaCl vs KCl. The salt dependences of K_{obsd} are identical in these two monovalent salts, indicating that identical numbers of ions are released upon forming the $(SSB)_{65}$ complex. However, the binding constants are shifted by approximately a factor of 2 such that $K_{obsd}(NaCl) = 2K_{obsd}(KCl)$. This difference is not likely to be due to any significant difference in the affinities of these cations for poly(U). From NMR studies, it has been shown that the affinities for duplex DNA of a series of monovalent cations, including Na^+ and K^+, are quite similar (Anderson et al., 1978; Bleam et al., 1980). In addition, we have observed no effect of the replacement of K^+ for Na^+ on the equilibrium binding constant of poly(A) for the tripeptide Lys-Trp-Lys (Wei and T. M. Lohman, unpublished experiments). Therefore, if Na^+ and K^+ were only being displaced from poly(U) upon binding of SSB protein, we would not expect a significant shift in K_{obsd}. Since the transitions from the $(SSB)_{35}$ to the $(SSB)_{56}$ complex, as well as from the $(SSB)_{56}$ to the $(SSB)_{65}$ complex, both result in the *uptake* of cations (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988), this differential effect of Na^+ vs K^+ on K_{obsd} is likely to reflect a difference in affinity of these two cations for the $(SSB)_{65}$ complex, rather than for poly(U) (see Discussion).

Effect of Glycerol on the Stability of the $(SSB)_{65}$ Complex. Since some investigators have included glycerol in the buffers used to study the *E. coli* SSB protein, we have examined the effect of glycerol on the equilibrium binding of SSB to poly(U), although we do not routinely include glycerol in our buffers. A direct comparison of equilibrium binding isotherms for the SSB protein–poly(U) interaction, in buffer T + 0.2 M NaCl + 10% glycerol, yielded the identical salt dependence and cooperativity parameter as in the absence of glycerol. However, a 6-fold *decrease* in the magnitude of K_{obsd} was observed in the presence of 10% glycerol (data not shown). Thus, glycerol has a significant effect on SSB protein–poly(U) interactions and cannot be regarded simply as a passive agent that only stabilizes the protein. This effect of glycerol (as well as other solution variables) on SSB protein–nucleic acid interactions must be considered when comparisons of binding constants are made from different laboratories.

In aqueous solutions, glycerol has been shown to increase the preferential hydration of proteins (Lee & Timasheff, 1977; Gekko & Timasheff, 1981a,b). The fact that we observe a decrease in K_{obsd} in the presence of glycerol suggests that preferential hydration increases upon formation of the SSB protein–poly(U) complex, resulting in an apparent release of glycerol, in a thermodynamic sense. This does not necessarily mean that preferential hydration increases upon complex formation in the absence of glycerol. Lee and Timasheff (1977) observed the opposite effect of glycerol on formation of a protein complex, since glycerol enhances the self-assembly of tubulin to form microtubules, suggesting that preferential hydration decreases upon microtubule formation in the presence of glycerol.

Discussion

Relationship between the Fractional Fluorescence Quenching of the SSB Protein and the Fraction of Bound SSB Protein. In buffers containing NaCl, KCl, $NaCH_3COO$, NaF, and potassium glutamate, we and Bujalowski and Lohman (1987a) have shown that the fractional quenching of the SSB fluorescence is a linear function of the fraction of bound SSB protein; i.e., $Q_{obsd}/Q_{max} = L_B/L_T$. However, in buffers containing NaBr, the fractional fluorescence quenching is *not* a linear function of the fraction of bound SSB protein, as shown in Figure 6. The fluorescence of the free and nucleic acid bound SSB protein is quenched by NaBr, and the data in Figure 6A indicate that the extent of quenching of the bound SSB protein is dependent on the binding density, such that the degree of quenching decreases at higher binding densities. This suggests that at higher binding densities the tryptophans of the nucleic acid bound SSB protein are less accessible to the bromide ion. In the $(SSB)_{65}$ binding mode, the protein seems to exist as an equilibrium mixture of tetramers and octamers (dimers of tetramers), and octamer formation is favored at higher binding densities (Bujalowski & Lohman, 1987b); therefore, the lower accessibility of Br^- may be correlated with the increase in the extent of octamer formation. Regardless of the molecular interpretation, these data clearly demonstrate that it is crucial to check the relationship between the fractional change in the optical signal being monitored and the fraction of bound ligand, rather than assume that this relationship is linear.

Intrinsic Equilibrium Binding Constant for Formation of the $(SSB)_{65}$ Complex Is Highly Salt Dependent in Contrast to Its Equilibrium Cooperativity. The equilibrium constant for the interaction of SSB protein with homopolynucleotides, in the $(SSB)_{65}$ binding mode, decreases steeply with increasing salt concentration, reflecting a net release of ions upon formation of the complex. For the interaction with poly(U), we measure $\partial \log K_{obsd}/\partial \log [NaCl] = -7.4 \pm 0.5$, whereas for poly(A) and poly(dA), we find a slightly smaller value of -6.2 ± 0.6. Since these salt dependences have contributions from a net release of both cations and anions, we do not yet have

sufficient information to determine the origin of the slightly larger value for poly(U). The value of ψ, the thermodynamic extent of counterion association per nucleotide (see eq 4 and 5), has been estimated to be slightly smaller for poly(U) than for poly(A) (0.68 vs 0.78) (Record et al., 1976); hence, there must be other contributions to the difference in the salt dependences for these polynucleotides (possibly differences in cation and anion uptake as discussed below). At a minimum, our results indicate that electrostatic interactions between the SSB protein and ss nucleic acid, with the concomitant release of previously bound ions into the bulk solution (Record et al., 1976, 1978), play an important role in determining the stability of the complex.

We have also measured the dependence of K_{obsd} on NaBr concentration for the interaction of the SSB protein with both poly(dT) and poly(U) (25 °C, pH 8.1) and found that $\partial \log K_{obsd}/\partial \log [NaBr] = -5.7 \pm 0.7$ and -6.7 ± 0.6, respectively. This clearly shows that the SSB–poly(dT) interaction is quite salt dependent, contrary to some previous reports. In order to detect the salt dependence of the SSB protein–poly(dT) interaction, it was necessary to use buffers containing NaBr in order to lower the affinity of the SSB protein for poly(dT) into a range such that it can be measured using fluorescence titrations. Previous attempts to measure the equilibrium binding constant and cooperativity parameter for the SSB–poly(dT) interaction in NaCl yielded only minimum estimates of $K_{obsd} \geq 10^8$ M^{-1}. Furthermore, the values of K_{obsd} appeared to be independent of NaCl concentration, since the same minimum estimates were obtained at all NaCl concentrations (Williams et al., 1983). This apparent salt independence results from the fact that the true binding constants in NaCl buffers are much greater than 10^8 M^{-1} and hence lie outside the "window" of the fluorescence titration method.

In contrast to K_{obsd}, the cooperativity parameter, $\omega_{T/O}$, seems to be independent of salt concentration and type as well as polynucleotide base and sugar composition. A value of $\omega_{T/O} = 410 \pm 120$ was obtained from analyses of the equilibrium binding isotherms using a model in which the SSB cooperativity is limited to the formation of octamers of SSB (dimers of tetramers) rather than the formation of SSB clusters of unlimited length (Bujalowski & Lohman, 1987b). Therefore, $\omega_{T/O}$ represents the equilibrium constant for formation of a nucleic acid bound SSB octamer from two nucleic acid bound SSB tetramers. This value of $\omega_{T/O} = 410 \pm 120$ corresponds to a free energy change of -3.6 ± 0.15 kcal/mol of SSB octamer formed at 25 °C. The equilibrium distribution of nucleic acid bound SSB tetramers and octamers predicted from the tetramer/octamer model, using $\omega_{T/O} = 410 \pm 120$, indicates that a binding density dependent mixture of SSB tetramers and octamers should form on the nucleic acid (Bujalowski & Lohman, 1987b); i.e., cooperativity is not high enough to completely form octamers. In a previous study, Lohman et al. (1986a) used a model for cooperative binding that assumes the formation of unlimited clusters of SSB tetramers on the nucleic acid (McGhee & von Hippel, 1974) and obtained a value of 50 ± 10 for the cooperativity parameter. There is adequate evidence indicating that in the $(SSB)_{65}$ binding mode, cooperativity is limited to the formation of octamers (Chrysogelos & Griffith, 1982; Bujalowski & Lohman, 1987b); hence, this latter value of 50 ± 10 is likely to have little physical significance.

The one exception to the apparent invariance of the cooperativity parameter to changes in salt conditions is for the SSB protein–poly(dT) interaction in NaBr, where $\omega_{T/O} = 130 \pm 70$. This deviation from 410 ± 120 may reflect an effect that is specific to poly(dT), or it may be due to the high NaBr concentrations which were necessary to lower the value of K_{obsd} so that it can be measured by using fluorescence titrations. Since we have not observed any other polynucleotide-specific effects on the value of $\omega_{T/O}$, it is more likely that $\omega_{T/O}$ is slightly salt dependent. However, the change in $\omega_{T/O}$ may be so slight that is is not apparent over a narrow range of salt concentrations; rather, it may only be observable when experiments are performed at extremely high salt concentrations, such as in the poly(dT) studies in 1.8–2.5 M NaBr.

We find quite good agreement between the values of K_{obsd} and $\omega_{T/O}$ determined from titrations at a constant salt concentration and those determined from a salt-back titration, in the case of data in NaCl, NaF, $NaCH_3COO$, and KCl (salt-back titrations cannot be interpreted in NaBr and potassium glutamate). This indicates that the experiments are at equilibrium. Furthermore, we do not detect any inconsistency in the estimates of the cooperativity parameter, determined by the two methods, as was observed in the case of the bacteriophage M13 and Ike gene 5 proteins (Bulsink et al., 1985; de Jong et al., 1987).

Salt Dependence of K_{obsd} for $(SSB)_{65}$ Formation Has Contributions from Cation and Anion Release as Well as Cation and Anion Uptake. The salt dependence of K_{obsd}, for *E. coli* SSB protein binding to ss nucleic acids in the $(SSB)_{65}$ binding mode, is more complicated than the simple case of oligocation binding described in eq 5. From the experiments reported in this paper, we can conclude that the salt dependence has contributions from a net release of both cations and anions upon formation of the complex. We can obtain a rough estimate of the relative contributions of anions vs cations to the salt dependence of K_{obsd} by comparing the values of $\partial \log K_{obsd}/\partial \log [MX]$ for the experiments performed with a common cation, e.g., Na^+. From the data in Table II, we see that there are approximately three more Cl^- ions released than F^- ions and roughly two more acetate ions than F^- ions. In general, it has been found that fluoride ions interact only weakly with proteins (von Hippel & Schleich, 1969). We also have independent evidence which suggests that the fluoride anion interacts only weakly, if at all, with the SSB protein (L. B. Overman, unpublished experiments); hence, to a first approximation, the salt dependence in NaF should only reflect preferential interactions of Na^+; i.e., the slope of -4 in NaF indicates that four Na^+ ions are released upon complex formation. With this as a reference point, the rest of the salt dependences in Table II indicate that in sodium acetate there are approximately four Na^+ ions and approximately two acetate ions released per bound SSB tetramer, whereas in NaCl there are approximately four Na^+ ions and approximately three Cl^- ions released. Similarly, in NaBr, there are approximately four Na^+ ions and approximately three Br^- ions released. We emphasize that these are only estimates, since the comparisons of the salt dependences are made over different salt concentration ranges. These interpretations are further complicated, since the salt dependences have a hidden contribution due to cation uptake, which is probably not constant over the range of Na^+ concentrations for which we are making these comparisons. Nevertheless, the qualitative result is that the extent of anion release increases in the order $F^- < CH_3COO^- < Cl^- \sim Br^-$. Experiments performed with buffers containing mixtures of these anions suggest that the anions bind competitively to the SSB protein (L. B. Overman, unpublished experiments). The fact that the slopes in NaCl, $NaCH_3COO$, and NaBr are nearly equal, yet $K_{obsd}(NaBr) < K_{obsd}(NaCH_3COO) < K_{obsd}(NaCl)$, suggests that in these cases

the anion binding sites on the protein are fully saturated by each anion in the salt concentration range of the experiments yet it is harder to displace Br^- than Cl^- than CH_3COO^- from these sites upon formation of the $(SSB)_{65}$ complex. The data in NaF suggest that this anion does not fully saturate the anion binding sites on the protein; hence, less fluoride is released, yielding a lower value of $|\partial \log K_{obsd}/\partial \log [MX]|$ in buffers containing fluoride.

Although we have shown that there is a *net* release of cations and anions, we also have independent evidence indicating that an *uptake* of cations and anions occurs upon formation of the $(SSB)_{65}$ binding mode from the lower site size binding modes (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988). As a result, the observed salt dependence, which indicates a *net* release of cations and anions upon formation of the $(SSB)_{65}$ complex from free SSB and nucleic acid, must have hidden contributions from cation and anion uptake (see eq 4). The slight differential effect of Na^+ vs K^+, on the magnitude of K_{obsd} (see Figure 5), may be a reflection of this cation uptake component. Therefore, at least two terms, of opposite sign, contribute to the cation component of the salt dependence ($z\psi - b = 4$ in eq 4). Hence, there must be $4 + b$ condensed cations released from the nucleic acid due to the formation of z ionic interactions between an SSB tetramer and the nucleic acid, since the uptake of b cations will cancel the release of b condensed cations from the nucleic acid. As a result, even though we can estimate the cation contribution to the salt dependence of SSB protein binding, we can only calculate a minimum estimate for the number of ionic interactions, z, formed in the $(SSB)_{65}$ complex with poly(U). Since $\psi = 0.68$ for poly(U) (Record et al., 1976), the minimum estimate for z is $4/0.68 = 5.9$ per SSB tetramer; however, z must be significantly greater, since there is a substantial uptake of cations ($b \geq 6$ for Na^+) which accompanies complex formation (Bujalowski et al., 1988). On the basis of these findings, we estimate $z \geq 15$ ionic interactions per SSB tetramer. Clearly, we need more quantitative information concerning cation uptake before we can accurately determine the number of ionic interactions, z, formed in the $(SSB)_{65}$ complex from an analysis of the salt dependence.

Although the cations that are released upon formation of the $(SSB)_{65}$ complex must originate primarily from the nucleic acid (Record et al., 1976), we have shown that an *uptake* of ions accompanies the transitions from the lower to the higher site size binding modes (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988). Since the higher SSB site sizes seem to reflect additional wrapping of the nucleic acid around the SSB protein and/or aggregation of bound SSB tetramers to form octamers, it is likely that cation uptake may be required for the additional wrapping. In addition, cation and anion uptake may also be required for the formation of bound SSB octamers.

The relative ability of anions to lower the binding constant for the SSB protein–poly(U) interaction in the $(SSB)_{65}$ binding mode is $Br^- > Cl^- > CH_3CO_2^- > F^- \sim$ glutamate. This hierarchy follows the so-called Hofmeister or lyotropic series (von Hippel & Schleich, 1969), although glutamate has not been examined in this context. We favor an interpretation that the anion effects observed in these studies reflect the preferential interaction of anions with the protein vs protein–nucleic acid complex, rather than more general effects of salts on water structure (chaotropic effects), for the following reasons. Studies of the interaction of the oligopeptide pentalysine with duplex DNA show no differential effect of chloride vs acetate on the equilibrium binding constant for this interaction (Lohman et al., 1980), suggesting that the effects we see for the SSB protein–ss nucleic acid interaction are due to specific binding of anions to the SSB protein. In addition, preliminary studies of the effects of pH on K_{obsd} for $(SSB)_{65}$ formation also indicate that the extent of anion binding and release is coupled to the protonation of sites on the SSB protein (L. B. Overman, unpublished data).

There have been other reports of differential effects of anions on protein–nucleic acid interactions including bacteriophage T4 gene 32 protein–poly(A) (Kowalczykowski et al., 1981; Lohman, 1984), *E. coli lac* repressor–DNA (de Haseth et al., 1977; Barkely et al., 1981), ribosomal protein S8–16S rRNA (Mougel et al., 1986), *E. coli* RNA polymerase, and type II restriction endonucleases (Leirmo et al., 1987). In each case, the anions that were examined follow the same hierarchy as we observe for the SSB protein, with the exception of *lac* repressor where fluoride and acetate have comparable effects on the magnitude of K_{obsd}. The effects of anions on the T4 gene 32 protein–poly(A) interaction most closely parallel the effects we see for the *E. coli* SSB protein–poly(U) interaction, in that both a change in the magnitude of K_{obsd} and also a change in the salt dependence are observed. In both cases $|\partial \log K_{obsd}/\partial \log [NaF]| < |\partial \log K_{obsd}/\partial \log [NaCH_3CO_2]| < |\partial \log K_{obsd}/\partial \log [NaCl]|$. However, there is no indication of a differential anion effect on the salt dependences for the *lac* repressor or ribosomal S8 proteins. This difference in $\partial \log K_{obsd}/\partial \log [MX]$ for different anions provides further evidence that in the case of *E. coli* SSB protein, the major role of anions results from their direct interactions with the protein and the DNA–SSB protein complex.

We examined the effect of glutamate on the $(SSB)_{65}$ equilibria since glutamate is one of the dominant free anions found in *E. coli* and the concentration of glutamate increases substantially (to ~0.2 M) when *E. coli* is grown in high osmotic media [Munro et al., 1972; Measures, 1975; reviewed by Record et al. (1985)]. In light of this, it is interesting that the observed binding constant for formation of the $(SSB)_{65}$ complex is approximately 10^3-fold larger in the presence of glutamate than in chloride (0.35 M anion, 25 °C, pH 8.1). To a first approximation, this effect seems to be due to the *lack* of affinity of glutamate for the SSB protein, resulting in a higher SSB–nucleic acid affinity since glutamate anions would not have to be displaced from SSB upon binding to the nucleic acid. However, the fact that $-(\partial \log K_{obsd}/\partial \log [MX])$ is larger for glutamate than for fluoride (5.7 vs 4.8, respectively), whereas K_{obsd} is slightly larger in glutamate than in fluoride (see Figure 5), is not totally consistent with this explanation. Similar qualitative observations of the effects of glutamate on *E. coli* RNA polymerase–promoter interactions have been made by Leirmo et al. (1987); however, in that case, the effect of glutamate is mainly on K_{obsd}, rather than on $\partial \log K_{obsd}/\partial \log [MX]$. The observation of these and other dramatic effects of salt on the thermodynamics of protein–nucleic acid interactions indicates that comparisons of in vitro with in vivo studies must be made cautiously.

Polynucleotide Binding Specificity of E. coli SSB Protein in the $(SSB)_{65}$ Binding Mode Differs from That of the T4 Gene 32 and M13 Gene 5 Proteins. The affinity of *E. coli* SSB protein in the $(SSB)_{65}$ binding mode adheres to the following hierarchy (in 10 mM Tris-HCl and 0.20 M NaCl, 25 °C, pH 8.1): $K_{obsd}[poly(dT)] > K_{obsd}[poly(dC)] \gg K_{obsd}$(M13 ss DNA) $> K_{obsd}[poly(I)] > K_{obsd}[poly(U)] = 8K_{obsd}[poly(dA)] = 87K_{obsd}[poly(A)] \gg K_{obsd}[poly(C)]$. The affinity of SSB protein is clearly greater for the polydeoxyribonucleotide than for the ribo homologue in the two cases

that we have examined (adenine and cytosine). In the case of the polyadenylates, the [NaCl] dependences of K_{obsd} are identical for both the ribo and deoxyribo forms, even though the binding constants differ by a factor of ~11. This suggests that the difference is due primarily to a nonelectrostatic component of the free energy of binding (Record et al., 1976), although more studies are necessary to confirm this suggestion.

There also seems to be a correlation between the maximum degree of quenching, Q_{max}, and the polynucleotide specificity of the $(SSB)_{65}$ complex, based on the homopolynucleotides that we have examined (see Table I). As the relative affinity increases, so does the value of Q_{max}, ranging from 0.39 ± 0.01 for poly(A) to 0.87 ± 0.02 for poly(dT). Since the mechanism of tryptophan quenching is not understood for any of the helix-destabilizing proteins, we have no molecular interpretation for this observation. However, recent studies suggest that one or more tryptophan residues of *E. coli* SSB protein are in close contact with the bases in a complex with poly(5-HgU) (Cha & Maki, 1984).

The hierarchy of SSB–polynucleotide affinities observed here is different than that reported by Molineux et al. (1975). This discrepancy is likely due to the fact that the previous estimates were obtained in buffers with no added salt (20 mM Tris, pH 7.4). These are conditions in which nonequilibrium behavior can occur (Lohman et al., 1986a) and where binding is also too tight to accurately measure binding constants, even on a relative scale, unless competition experiments are performed. Furthermore, the mode of binding of the *E. coli* SSB protein to ss nucleic acids is different under low-salt conditions, where the $(SSB)_{35}$ and $(SSB)_{56}$ binding modes dominate, than under the conditions which favor the $(SSB)_{65}$ binding mode as examined in this work (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988).

The majority of ss nucleic acid binding proteins display a hierarchy of binding affinities for ss homopolynucleotides of different base and sugar composition. It is interesting to compare the hierarchy of specificities which we observe for the *E. coli* SSB protein with those of two other helix-destabilizing proteins. For the homopolynucleotides that we have studied here, the bacteriophage T4 gene 32 protein hierarchy is K_{obsd}[poly(dT)] ≫ K_{obsd}[poly(dC)] = $8K_{obsd}$[poly(dA)] = $40K_{obsd}$[poly(U)] = $50K_{obsd}$[poly(A)] = $5000K_{obsd}$[poly(C)], in 10 mM HEPES, pH 7.7, 0.1 mM Na_2EDTA, and 0.20 M NaCl, 25 °C (Newport et al., 1981), whereas the M13 gene 5 protein hierarchy is K_{obsd}[poly(dT)] ≫ K_{obsd}[poly(U)] = $15K_{obsd}$[poly(A)] = $20K_{obsd}$[poly(C)] = $22K_{obsd}$[poly(dA)], in 10 mM sodium cacodylate, pH 6.9, and 0.23 M NaCl, 37 °C (Bulsink et al., 1985). Several similarities among the three systems are evident. Each protein shows the highest affinity for poly(dT) as compared to the other homopolynucleotides, a higher affinity for the polydeoxyribonucleotide than for the ribonucleotide homologue, and a salt-independent cooperativity parameter. This latter point also holds for *E. coli* SSB protein, when bound in the $(SSB)_{65}$ mode; however, recall that the molecular bases for cooperative binding are extremely different for each of the three proteins (Bujalowski & Lohman, 1987b). The qualitative specificity rankings are very similar, with the exception of the relative placement of poly(dA), which varies for each of the three proteins. These properties suggest a common component to the binding specificity for each of these proteins, although there are clearly characteristics that are unique to each protein.

It is interesting that de Jong et al. (1987) have observed a differential effect of Na^+ vs K^+ on the interaction of the phage Ike gene 5 protein with poly(A); however, the effect was exactly the opposite of what we observe for the *E. coli* SSB protein–poly(U) interaction (see Figure 5), although the magnitude of the effect is approximately a factor of 2 in both cases. As discussed above, it is unlikely that this reflects differential interactions of Na^+ vs K^+ with the polynucleotide but rather must indicate some interaction of the cations with both proteins.

Previous Studies of the Salt Dependence of E. coli SSB Protein–ss Nucleic Acid Binding. From previous studies (Lohman et al., 1986a), and those reported here, we conclude that the intrinsic equilibrium constant for the interaction of *E. coli* SSB protein with ss nucleic acids to form the $(SSB)_{65}$ complex is highly salt dependent, suggesting the involvement of ionic interactions between the protein and the nucleic acid with the concomitant release of ions into solution. Contrary to these conclusions, there have been a number of reports that SSB–ss polynucleotide interactions are only slightly salt dependent and that there is only a very small electrostatic component to the binding free energy (Ruyechan & Wetmur, 1976; Williams et al., 1983; Clore et al., 1986; Chase & Williams, 1986). The conclusion that SSB–ss polynucleotide interactions are not highly salt dependent seems to be based mainly on two observations: (1) SSB–ss *oligo*nucleotide binding is salt independent in the low [NaCl] range from 4 to 200 mM NaCl, although the oligonucleotide binding constant dramatically decreases above 200 mM NaCl or 10 mM $MgCl_2$ (Ruyechan & Wetmur, 1976); and (2) the failure to observe a salt-dependent binding constant for the SSB protein–poly(dT) interaction.

It may be that the binding affinity of *E. coli* SSB protein to ss oligonucleotides is less salt dependent below 200 mM NaCl; however, one should not conclude from those studies that the binding of SSB to ss *poly*nucleotides is also salt independent, since we do not know whether SSB binds to oligo- and polynucleotides in a similar manner. Furthermore, one must be careful about drawing conclusions about electrostatic effects on protein–*poly*nucleotide binding from studies of protein–*oligo*nucleotide binding. Counterions interact less well with oligonucleotides than with polynucleotides; hence, even if the number of ionic interactions are equivalent in a protein–oligo- vs protein–polynucleotide complex, the number of counterions released upon formation of the protein–oligonucleotide complex (and hence the observed salt dependence) will be *less* than for the protein–polynucleotide complex (Record & Lohman, 1978). The apparent insensitivity of SSB protein–oligonucleotide binding to changes in NaCl concentrations below 0.2 M (Ruyechan & Wetmur, 1976) may be related to the observations that a mixture of several SSB–polynucleotide binding modes can exist below this NaCl concentration. The salt concentration for which Ruyechan and Wetmur (1976) observed the dramatic decrease in the binding constant for oligonucleotides (>0.2 M NaCl or >10 mM $MgCl_2$) correlates well with the conditions in which we observe a complete conversion of SSB protein–poly(dT) complexes to the $(SSB)_{65}$ mode by both NaCl and $MgCl_2$ (Lohman & Overman, 1985; Bujalowski & Lohman, 1986). However, we do not have quantitative information about the salt dependences of the equilibrium constants for SSB binding in the other lower site size binding modes to comment further on this.

Unfortunately, the same conclusion that *E. coli* SSB protein–ss polynucleotide complexes are not very salt dependent and hence form few ionic interactions has been reached based on attempts to estimate equilibrium binding constants from titrations of SSB protein with poly(dT) (Molineux et al., 1975; Williams et al., 1983) in buffers containing only NaCl or KCl.

As we have shown previously, the binding of SSB protein to poly(dT) is stoichiometric, even in 5 M NaCl (Lohman & Overman, 1985; Bujalowski & Lohman, 1986); hence, one can only obtain a lower limit for the binding constant of this complex in a buffer containing only NaCl. Since the binding of SSB protein to poly(dT) remains stoichiometric up to 5 M NaCl ($K_{obsd} \geq 5 \times 10^8$ M^{-1}), previous attempts to measure K_{obsd} for this interaction have obtained the same lower limit binding constant ($\sim 5 \times 10^8$ M^{-1}) at all NaCl concentrations, even though the actual value of K_{obsd} is larger than this and does decrease with increasing salt concentration. This has led to the erroneous conclusion that the binding constant is salt independent (Williams et al., 1983; Molineux et al., 1975; Chase & Williams, 1986). As we have shown here, all SSB protein–ss polynucleotide complexes display a steep decrease in affinity with increasing salt concentration, indicating the existence of a significant number of electrostatic interactions in the complex. One must exceed 1.8 M $MgCl_2$ or 1.8 M NaBr in order to weaken the SSB protein–poly(dT) complex sufficiently to measure K_{obsd} by monitoring quenching of the SSB fluorescence (at 25 °C, pH 8.1). Under these conditions, the SSB protein–poly(dT) binding constant does decrease significantly with increasing $MgCl_2$ or NaBr concentration ($\partial \log K_{obsd}/\partial \log [NaBr] = -5.7 \pm 0.7$). Therefore, the equilibrium interaction of SSB protein with all ss nucleic acids that we have investigated is highly salt dependent and must involve significant electrostatic interactions, at least in the $(SSB)_{65}$ binding mode.

In order to quantitatively analyze the NaBr concentration dependence of K_{obsd} for poly(U) vs poly(dT), we have calculated $\partial \log K_{obsd}/\partial \log a_{\pm}$, where $a_{\pm}$ is the mean ion activity for NaBr, since each of these sets of data was obtained in quite different NaBr concentration ranges. We find that $\partial \log K_{obsd}/\partial \log a_{\pm} = -7.1$ and -4.6 for poly(U) and poly(dT), respectively. Therefore, although both interactions are clearly quite salt dependent, the quantitative values differ significantly. This difference may reflect real thermodynamic differences in the interactions of ions and water with these two polynucleotides. On the other hand, the difference may also reflect the release of a constant number of water molecules upon formation of any SSB protein–ss polynucleotide complex. Even if the ion release terms are identical for the interaction of SSB protein with poly(U) and poly(dT), the last term in eq 4 can become significant at salt concentrations in the molar range if there is significant water release accompanying the interaction. If the different values of $\partial \log K_{obsd}/\partial \log a_{\pm}$ for poly(U) vs poly(dT) are due solely to water release, then eq 4 indicates that the difference in slopes could be explained if $\sim 30 \pm 3$ water molecules per tetramer are released upon formation of the complex. Further studies are required to resolve these possibilities.

Further Thermodynamic Considerations. The thermodynamic stability of most protein–nucleic acid complexes, including the *E. coli* SSB protein–ss nucleic interaction studied here, is tightly linked to several solution variables (temperature, pH, cation and anion type, and concentration), and the net binding free energy is therefore dependent upon a balance of a number of free energy contributions, each of which is sensitive to the solution conditions. Therefore, systematic investigations of the effects of changes in all of these solution conditions for each polynucleotide may be necessary to fully understand the basis for the polynucleotide specificities which have been observed for the *E. coli* SSB protein as well as for the T4 gene 32 and M13 gene 5 proteins. The extreme sensitivity to solution conditions of the observed binding free energy of protein–nucleic acid complexes also indicates that an understanding of the molecular bases for the stability of these complexes will only come through investigations of the thermodynamics of these interactions in solution and that reliable predictions of stability *cannot* be made solely from considerations of the crystal structures of the macromolecules.

In this context, we point out that the salt dependences, as well as the values of $\Delta H°$, differ for the interactions of *E. coli* SSB protein with many of the different homopolynucleotides (L. B. Overman and T. M. Lohman, unpublished results); hence, the apparent polynucleotide binding hierarchies will clearly be dependent upon the conditions used for the comparisons. This is also true for the M13 gene 5 protein (Bulsink et al., 1985). Hence, comparisons of K_{obsd} made under a single set of solution conditions are *not adequate* to determine the molecular basis of polynucleotide specificity, since the thermodynamics of the reaction determine the equilibrium specificity. As a result, even qualitative specificity rankings will change with solution conditions. This is a general problem that must be recognized whenever attempts are made to correlate specificities for the interaction of a protein with a series of nucleic acids possessing structural differences. Until a thorough thermodynamic analysis of the interaction of the *E. coli* SSB protein with each of the homopolynucleotides is obtained, caution must be used in drawing conclusions concerning the molecular basis for specificity. It has recently been observed that upon introducing a single base pair substitution in the *lac* operator site, the electrostatic and nonelectrostatic contributions to the net free energy of complex formation with *lac* repressor protein can be drastically altered, including ion release (Mossing & Record, 1986). This indicates that the total free energy contribution to binding cannot always be viewed as a simple sum of free energy contributions from the individual interactions that contribute to the overall binding. This is likely to be a general conclusion for most protein–nucleic acid interactions and emphasizes the need to thermodynamically dissect a binding interaction when comparisons are made between modified (mutant) proteins or nucleic acids.

Finally, we emphasize that the salt effects reported in this paper are not due to simple ionic strength (screening) effects but rather reflect the direct, preferential binding of ions to the various macromolecular species. Consequently, *meaningful quantitative comparisons of protein–nucleic acid interactions cannot be made on the basis of ionic strength*; i.e., particular attention must be paid to the buffer composition when such comparisons are made. Specific ion effects are important for all protein–nucleic acid interactions, as well as macromolecular equilibria in general, and therefore cannot be ignored in any quantitative analysis.

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Registry No. Poly(A), 24937-83-5; poly(U), 27416-86-0; poly(dA), 25191-20-2; poly(dT), 25086-81-1; glutamate, 56-86-0; Na, 7440-23-5; K, 7440-09-7; Cl^-, 16887-00-6; Br^-, 24959-67-9; AcOH, 64-19-7; F^-, 16984-48-8; $HOCH_2CH(OH)CH_2OH$, 56-81-5.

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