A Highly Salt-Dependent Enthalpy Change for *Escherichia coli* SSB Protein−Nucleic Acid Binding Due to Ion−Protein Interactions†

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ABSTRACT: We have examined the linkage between salt concentration and temperature for the equilibrium binding of the tetrameric *Escherichia coli* single-stranded binding (SSB) protein to three single-stranded nucleic acids, poly(U), $dA(pA)_{69}$, and $dT(pT)_{69}$, by van't Hoff analysis and isothermal titration calorimetry (ITC). For SSB binding to poly(U) in its $(SSB)_{65}$ mode, the equilibrium association constant, K_{obs}, decreases with increasing salt concentration at all temperatures examined, and binding is enthalpy-driven; however, the value of $\partial \log K_{obs}/\partial \log [NaCl]$ is highly temperature-dependent, varying from -9.3 ± 0.3 at 10 °C to -5.1 ± 0.4 at 37 °C. This indicates that ΔH_{obs} for SSB−poly(U) binding is strongly dependent on [NaCl]; based on van't Hoff analyses, ΔH_{obs} varies from -57 ± 3 kcal/mol at 0.18 M NaCl to -34 ± 3 kcal/mol at 0.42 M NaCl ($\partial \Delta H_{obs}/\partial \log [NaCl] = 60 \pm 5$ kcal/mol). However, $\partial \Delta H_{obs}/\partial \log [NaF]$ is independent of temperature (25−37 °C), indicating that the effect of [NaCl] on ΔH_{obs} is due primarily to Cl^-. Similar effects were also observed for SSB binding to $dA(pA)_{69}$. We also measured ΔH_{obs} and its dependence on [NaCl] for SSB binding to $dT(pT)_{69}$ by ITC and find $\Delta H_{obs} = -144 \pm 4$ kcal/mol (0.175 M NaCl, pH 8.1, 25 °C) and $\partial \Delta H_{obs}/\partial \log [NaCl] = 46 \pm 2$ kcal/mol (0.175−2.0 M NaCl). These large effects of [NaCl] on ΔH_{obs} appear to result, at least partly, from the release of preferentially bound Cl^- from SSB protein upon binding nucleic acid, with the release of Cl^- being linked to a process with $\Delta H >> 0$. Effects of salt concentration on ΔH_{obs} are not observed for processes in which only monovalent cations are released from the nucleic acid, presumably since Na^+ or K^+ are bound to linear nucleic acids as delocalized, fully hydrated cations. Such salt effects on ΔH_{obs} may serve as a signature for differential ion−protein binding. These results underscore the need to examine the linkage of [salt] to ΔH_{obs}, as well as $\Delta G°_{obs}$ and $\Delta S°_{obs}$, in order to understand the bases for stability and specificity of protein−nucleic acid interactions.

The equilibrium affinities and kinetic rate constants for proteins and oligocations binding to linear nucleic acids are generally sensitive functions of salt concentration and type (Record et al., 1978, 1991; Lohman, 1986; Lohman & Mascotti, 1992a). For simple oligocations (e.g., oligolysines, oligoarginines, polyamines), an increase in salt concentration decreases the observed equilibrium association constant, K_{obs}, for binding to linear nucleic acids. This sensitivity of K_{obs} to salt concentration is due to the polyelectrolyte nature of the nucleic acid, and the consequent accumulation of counterions (e.g., Na^+, K^+) in the local vicinity of the nucleic acid. Binding of an oligocation to a nucleic acid partially neutralizes the phosphate charges on the nucleic acid resulting in a concomitant release of counterions from the nucleic acid (Record et al., 1976, 1978, 1991; Lohman & Mascotti, 1992a). Studies of the temperature dependence of K_{obs} for simple oligocations [e.g., oligolysines (Mascotti & Lohman, 1992, 1993), polyamines (Braunlin et al., 1982)] binding to linear nucleic acids indicate that the effects of salt concentration on K_{obs} ($\Delta G°_{obs}$) are entirely entropic; i.e., $\Delta S°_{obs}$ increases with decreasing [salt], whereas ΔH_{obs} is independent of salt concentration and generally small in magnitude (Mascotti & Lohman, 1992, 1993; Lohman & Mascotti, 1992a). The increase in $\Delta S°_{obs}$ upon oligocation−nucleic acid binding is due primarily to counterion release (a free energy of dilution) which provides the major thermodynamic driving force for the binding of these simple oligocations to linear nucleic acids at low salt (counterion) concentrations (Record et al., 1976, 1978; Anderson & Record, 1995).

Many studies of proteins binding to linear nucleic acids *in vitro* also indicate that K_{obs} generally decreases with increasing salt concentration (de Haseth et al., 1977; Revzin & von Hippel, 1977) and the increase in $\Delta S°_{obs}$ due to the release of cations from the nucleic acid contributes substantially to the favorable free energy of binding. However, the effects of changes in salt concentration on protein−nucleic acid equilibria are often more complex than for simple oligocations since direct binding of ions to the protein can result in ion uptake by or release from the protein in addition to cation release from the nucleic acid (Overman et al., 1988; Kowalczykowski et al., 1981; Newport et al., 1981; Bujalowski & Lohman, 1989b; Ha et al., 1992). Such effects

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are clearly evident from studies of the equilibrium binding of the *E. coli* SSB tetramer to single-stranded nucleic acids (Overman et al., 1988; Bujalowski et al., 1988; Overman & Lohman, 1994).

We have shown previously (Ferrari & Lohman, 1994) that K_{obs} for *E. coli* SSB tetramer binding to $dC(pC)_X$ and $dT(pT)_X$ ($X = 55$ or 69) is highly temperature-dependent, with values of ΔH_{obs} ranging from −70 to −100 kcal/mol tetramer at high [NaBr]. In fact, these values are much larger than have been reported for any protein−nucleic acid interaction, including proteins that bind with high affinity to specific DNA sequences. To explore the origins of this extremely large exothermic ΔH_{obs}, and to attempt to understand in more detail the origins of the effects of salt concentration on K_{obs} ($\Delta G°_{obs}$) for protein−nucleic acid interactions, we have examined the thermodynamic linkage between temperature and monovalent salt concentration and type (NaCl and NaF) for SSB tetramer binding to three different single-stranded nucleic acids: an RNA polymer [poly(U)] and two oligodeoxynucleotides, $dT(pdT)_{69}$ and $dA(pdA)_{69}$, using both van't Hoff and direct calorimetric approaches. An understanding of the origins of these linkages is essential for a molecular understanding of the stability and specificity of protein−nucleic acid interactions.

MATERIALS AND METHODS

Reagents and Buffers. All chemicals were reagent grade. All solutions were prepared with distilled and deionized (Milli-Q) water. Buffer T is 10 mM Tris [tris(hydroxymethyl)aminomethane], pH 8.1, 0.1 mM Na_3EDTA (ethylenediaminetetraacetic acid); buffer H is 10 mM Hepes, pH 8.1, 0.1 mM Na_3EDTA. The pH of the buffers did not vary by more than ±0.1 over the salt concentration range examined.

E. coli SSB Protein and Nucleic Acids. SSB protein was purified as described (Lohman & Overman, 1985) with the addition of a double-stranded DNA column to remove a small exonuclease contaminant. The SSB concentration was determined spectrophotometrically in buffer T + 0.20 M NaCl using an extinction coefficient of $\epsilon_{280} = 1.13 \times 10^5$ M^{-1} (tetramer) cm^{-1} (Lohman & Overman, 1985). As shown previously (Lohman & Overman, 1985; Bujalowski & Lohman, 1991a,b; Ferrari & Lohman, 1994; Overman & Lohman, 1994), greater than 95% of the protein is tetrameric even at concentrations as low as 0.5 nM (tetramer) (pH 8.1, 0.2 M NaCl, 37 °C), and the SSB tetramer is stable under all conditions and at all protein concentrations used in these studies. $dT(pT)_{69}$ and $dA(pA)_{69}$ were synthesized and purified as described (Ferrari et al., 1994). The DNA was ≥98% pure as judged by analysis by denaturing polyacrylamide gel electrophoresis and autoradiography of a sample that was labeled at the 5′ end with ^{32}P using polynucleotide kinase. Oligodeoxynucleotide concentrations were determined by UV absorbance in buffer T (pH 8.1), 0.1 M NaCl, using the following extinction coefficients (per nucleotide): $dT(pT)_{69}$, $\epsilon_{260} = 8.1 \times 10^3$ M^{-1} cm^{-1}; $dA(pA)_{69}$, $\epsilon_{257} = 1.0 \times 10^4$ M^{-1} cm^{-1}. Poly(U) (Boehringer) had $s_{20,w} = 9.5$ S, corresponding to an average length of 1100 ± 200 nucleotides. A fractionated sample of poly(U) having $s_{20,w} = 14$ S gave identical results in binding assays. Poly(U) concentration was determined spectrophotometrically using $\epsilon_{260} = 9.2 \times 10^3$ M^{-1} (nucleotide) cm^{-1} (buffer T + 0.10 M NaCl). DNA and SSB samples were dialyzed extensively against the same buffer for each experiment.

Equilibrium Binding Constants by Fluorescence Titrations. Equilibrium titrations of SSB protein with poly(U) ("reverse" titrations), monitoring the quenching of the SSB tryptophan fluorescence ($\lambda_{ex} = 282$ or 296 nm; $\lambda_{em} = 347$ nm), were performed in an SLM 8000 spectrofluorometer as described (Overman et al., 1988; Overman & Lohman, 1994). The temperature of each experiment was controlled to ±0.1 °C, and all measurements were corrected for dilution, photobleaching, and inner filter effects as described (Overman et al., 1988; Lohman & Mascotti, 1992b).

Equilibrium constants, K_{obs}, for SSB tetramer binding to $dA(pA)_{69}$ to form 1:1 complexes were determined from titrations monitoring SSB tryptophan fluorescence quenching as described (Ferrari & Lohman, 1994). Binding isotherms for SSB binding to poly(U) were constructed using a model-independent binding density function analysis (Bujalowski & Lohman, 1987b; Lohman & Bujalowski, 1991) as described (Overman et al., 1988; Lohman & Mascotti, 1992b) under conditions such that binding occurs exclusively in the $(SSB)_{65}$ polynucleotide binding mode. In this binding mode, a "limited" cooperativity model (Bujalowski & Lohman, 1987a) was used to analyze the equilibrium binding isotherms to determine the equilibrium constant, K_{obs}, for SSB tetramer binding to an isolated site and the "limited" cooperativity parameter, $\omega_{T/O}$. Nonlinear least-squares analysis of the equilibrium isotherms, with the site size constrained to $n = 65$ nucleotides per SSB tetramer, was used to determine the best-fit values of K_{obs} and $\omega_{T/O}$ as described (Overman et al., 1988; Overman & Lohman, 1994). For SSB binding to poly(U) in the $(SSB)_{65}$ binding mode in buffers containing NaCl and NaF salts (pH 8.1), the observed fluorescence quenching, Q_{obs}, is directly proportional to the fraction of bound SSB tetramers (i.e., $L_B/L_T = Q_{obs}/Q_{max}$), and Q_{max}, n, $\omega_{T/O}$, and the free SSB protein fluorescence are independent of salt concentration (Lohman & Mascotti, 1992b; Overman et al., 1988; Overman & Lohman, 1994). Therefore, the dependence of K_{obs} on salt concentration was obtained with high precision from "salt-back" titrations (Overman et al., 1988; Lohman & Mascotti, 1992b; Overman & Lohman, 1994).

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) of SSB tetramer binding to $dT(pT)_{69}$ was performed with an OMEGA microcalorimeter (Microcal, Inc., Northhampton, MA) under conditions such that a 1:1 complex is formed in which $dT(pT)_{69}$ occupies all four SSB subunits. Under all solution conditions used in the ITC experiments, SSB−$dT(pT)_{69}$ binding is stoichiometric; thus, only values of ΔH_{obs} could be determined. The reaction cell (1.37 cm^3) was filled with one species [either $dT(pT)_{69}$ or SSB tetramer at concentrations of 1−2 μM] and titrated with 10 μL aliquots of the other species [stock concentrations of 16−19 μM SSB tetramer or 32−35 μM $dT(pT)_{69}$]. Samples were degassed prior to use. The measured values of ΔH_{obs} were independent of sample concentration and order of addition.

The heat of reaction was obtained by integration of the peak obtained after each injection of titrant, using the software provided by the manufacturer. Control experiments were performed in which the same aliquots of titrant were injected into buffer and the heat of dilution was subtracted from the total heat to obtain the heat of binding. Values of

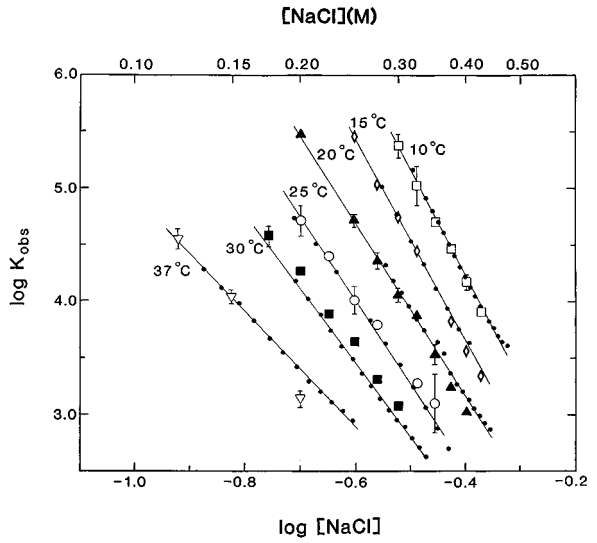

FIGURE 1: [NaCl] dependence of the equilibrium binding constant, K_{obs}, for SSB tetramer binding to poly(U) is temperature-dependent. Values of K_{obs} are for SSB tetramer−poly(U) binding in the $(SSB)_{65}$ polynucleotide mode (pH 8.1). The small circles represent determinations from salt-back titrations, and the large symbols are determinations from reverse titrations performed at constant salt concentrations. Error bars represent the range of values obtained from multiple determinations. The linear least-squares lines shown are based on the data from the salt-back titrations. The linear least-squares lines describing each line are given in Table 1. The temperatures are (□) 10 °C, (◇) 15 °C, (▲) 20 °C, (○) 25 °C, (■) 30 °C, and (▽) 37 °C.

ΔH_{obs} were calculated using two approaches. Since binding was stoichiometric under all solution conditions used, the ΔH_{obs} was obtained directly as the heat released from each injection divided by the moles of titrant injected (after correction for the heat of dilution) (differential approach). The values of ΔH_{obs} reported are averages of 5−8 injections. The reported standard deviations in ΔH_{obs} were calculated as $[\mathrm{var}(\Delta H_b) + \mathrm{var}(\Delta H_d)]^{1/2}$ where $\mathrm{var}(\Delta H_b)$ and $\mathrm{var}(\Delta H_d)$ are the variances in the heats obtained from addition of titrant to the sample and addition of titrant to buffer (heat of dilution), respectively. The value of ΔH_{obs} was also determined by summing the individual heats (corrected for the heat of dilution) and dividing by the total moles of titrant at saturation (after correction for the volume displaced during the titration) (integral approach). For 13 titrations, the values of ΔH_{obs} calculated using these 2 approaches agreed to within ∼2% for 10 and ∼6% for 3.

We also measured ΔH_{obs} for the binding of SSB to $dT(pT)_{69}$ in several buffers (Pipes, HEPES, Tricine, Tris, and phosphate) possessing different ionization enthalpies in order to determine whether ΔH_{obs} has contributions from protonation of the buffer due to uptake or release of protons accompanying protein−DNA binding. If protonation of SSB occurs upon binding $dT(pT)_{69}$, then ΔH_{obs} will be composed of two terms ($\Delta H_{obs} = \Delta H_{react} + \Delta\nu_H \Delta H_{ion}$), where ΔH_{react} is the enthalpy change for the SSB−$dT(pT)_{69}$ binding reaction and $\Delta\nu_H \Delta H_{ion}$ reflects any contributions from ionization of the buffer. We observed only a slight dependence of ΔH_{obs} on ΔH_{ion}, yielding $\Delta\nu_H = 1.6$ (±1.1), consistent with our studies of SSB binding to poly(U) indicating a net uptake of a fraction of a proton near pH 8.1 (Overman & Lohman, 1994). Due to the large ΔH_{obs} for the SSB−$dT(pT)_{69}$ reaction, corrections due to buffer ionization are negligible within our experimental error and thus were not applied.

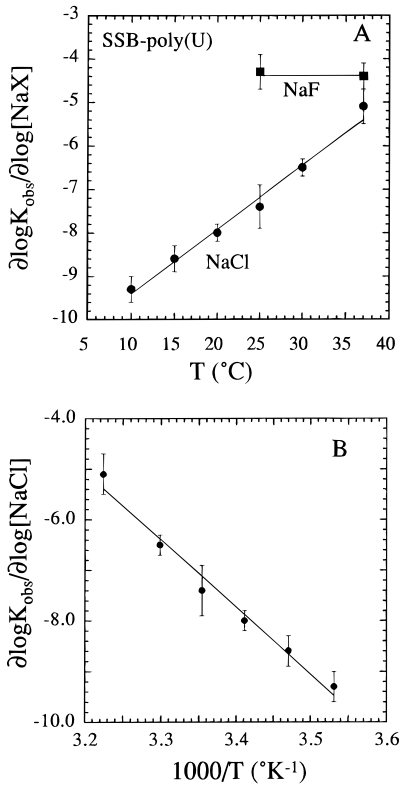

FIGURE 2: Temperature dependence of $\partial \log K_{obs}/\partial \log [NaX]$ for SSB tetramer−poly(U) binding in the $(SSB)_{65}$ polynucleotide mode (pH 8.1). (A) $\partial \log K_{obs}/\partial \log [NaX]$, determined by linear regression of the salt dependence of K_{obs}, is plotted as a function of temperature for NaCl (■) and NaF (●). (B) The data in NaCl from panel A are replotted vs $1/T$. The linear least-squares line shown has a slope of -1.33×10^4 K.

RESULTS

A Large, Exothermic ΔH_{obs} Accompanies SSB Binding to Poly(U). Equilibrium isotherms for SSB tetramer binding to poly(U) in its $(SSB)_{65}$ mode were obtained by monitoring quenching of the SSB tryptophan fluorescence. These isotherms were analyzed using a "limited" cooperativity model (Bujalowski & Lohman, 1987a) to obtain the equilibrium association constant, K_{obs}, for the binding of an SSB tetramer to an isolated site on poly(U) and the nearest-neighbor cooperativity parameter, $\omega_{T/O}$, as previously described (Bujalowski & Lohman, 1987b; Overman et al., 1988). The linkage between temperature and [NaCl] was examined by performing a series of reverse titrations as well as salt-back titrations at different temperatures (10, 15, 20, 25, 30, and 37 °C). The dependence of log K_{obs} on log [NaCl] at each temperature is shown in Figure 1. At each temperature, K_{obs} decreases with increasing [NaCl], i.e., $\partial \log K_{obs}/\partial \log [NaCl] < 0$, indicating that SSB−poly(U) complex formation is accompanied by a net release of ions (Na^+ and Cl^-) as previously shown (Overman et al., 1988; Overman & Lohman, 1994). Furthermore, at constant [NaCl], K_{obs} decreases with increasing temperature, indicating $\Delta H_{obs} < 0$ for complex formation. However, as shown in Figure 2A, $\partial \log K_{obs}/\partial \log [NaCl]$ increases (becoming less negative) as the temperature increases; hence, the net ion release accompanying SSB−poly(U) binding decreases with increasing temperature. Over the salt concentration ranges

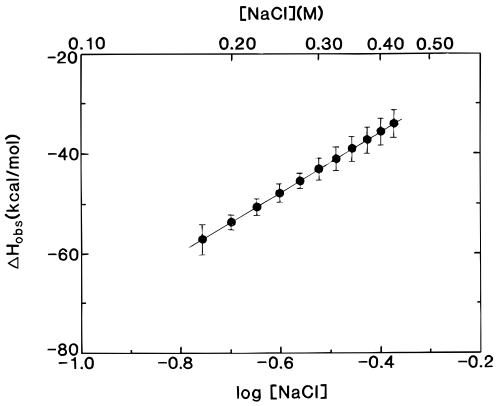

FIGURE 3: van't Hoff enthalpy change (ΔH_{obs}) for SSB tetramer binding to poly(U) in its $(SSB)_{65}$ mode increases with increasing [NaCl]. ΔH_{obs} values, determined from van't Hoff analysis of the data in Figure 1, are plotted *vs* log [NaCl] (pH 8.1). Error bars represent the standard error of the estimate. The line shown is the linear least-squares representation of the data: $\Delta H_{obs} = 60$ log [MX] − 12 (kcal/mol).

investigated, ∂ log K_{obs}/∂ log [MX] has values of -9.3 ± 0.3 at 10 °C, -8.6 ± 0.3 at 15 °C, -8.0 ± 0.2 at 20 °C, -7.4 ± 0.5 at 25 °C, -6.5 ± 0.2 at 30 °C, and -5.1 ± 0.4 at 37 °C.

Since ∂ log K_{obs}/∂ log [NaCl] ($\equiv S_a K_{obs}$) is temperature-dependent, it necessarily follows from the reciprocity relationship in eq 1 that ΔH_{obs} for this interaction must be dependent on [NaCl].

$$(\partial S_a K_{obs}/\partial T^{-1})_{[NaCl]} = -(1/2.3R)(\partial \Delta H_{obs}/\partial \log [NaCl])_T \quad (1)$$

Figure 2B shows a plot of $S_a K_{obs}$ as a function of $1/T$ indicating that $(\partial S_a K_{obs}/\partial T^{-1})_{[NaCl]} < 0$ over the entire temperature range. A linear least-squares line through the data has a slope equal to -1.33×10^4 K. Therefore, it follows from eq 1 that $(\partial \Delta H_{obs}/\partial \ln [NaCl])_T > 0$ over this same range of conditions, and we calculate $(\partial \Delta H_{obs}/\partial \log [NaCl])_T = 61$ kcal/mol. Although a linear least-squares line does describe the data in Figure 3 within experimental error, there is some indication of curvature in the direction that the dependence of ΔH_{obs} on [NaCl] may be slightly greater at higher temperature.

The data in Figure 1 were used to construct a series of van't Hoff plots at different NaCl concentrations (data not shown). Within experimental error, these van't Hoff plots are linear over the temperature range examined, consistent with our previous observations for SSB binding to oligodeoxythymidylates and oligodeoxycytidylates, although van't Hoff plots for SSB binding to oligodeoxyadenylates show definite curvature with an associated apparent $\Delta C_p < 0$ (Ferrari & Lohman, 1994). Linear regression analysis of these van't Hoff plots yielded estimates of ΔH_{obs} and $\Delta S°_{obs}$ for the SSB−poly(U) interaction. As shown in Figure 3, the van't Hoff ΔH_{obs} is negative and very large at each [NaCl]. In fact, the value of $\Delta H_{obs} = -57 \pm 3$ kcal/mol at 0.18 M NaCl (pH 8.1) is considerably larger than any ΔH_{obs} yet reported for a protein−nucleic acid interaction. However, ΔH_{obs} is also a strong function of [NaCl] (pH 8.1), increasing (becoming less negative) from -57 ± 3 kcal/mol at 0.18 M NaCl to -34 ± 3 kcal/mol at 0.42 M NaCl. The linear least-squares line describing the dependence of the van't Hoff ΔH_{obs} on [NaCl] in Figure 3 is given in eq 2. The value of $\partial \Delta H_{obs}/\partial$ log [NaCl] $= 60 \pm 5$ kcal/mol agrees very well with the prediction based on eq 1 and the data in Figure 2B.

$$\Delta H_{obs} \text{ (kcal/mol)} = 60 \log [NaCl] - 12 \quad (2)$$

The entropy change associated with SSB−poly(U) binding is also [NaCl]-dependent and is well described as $\Delta S°_{obs} = 170$ log[NaCl] − 40 (eu) over the same [NaCl] range shown in Figure 3. Hence, $\Delta G°_{obs}$ for SSB tetramer binding to poly(U) in its $(SSB)_{65}$ mode is dominated by a large favorable ΔH_{obs} over the entire range of [NaCl] and temperatures studied. However, ΔH_{obs} becomes less favorable and $\Delta S°_{obs}$ becomes less unfavorable with increasing [NaCl]. As mentioned above, although Figure 3 indicates that ΔH_{obs} is well described as a linear function of log [NaCl] over the [NaCl] examined, the slight curvature in the plot in Figure 2B in conjunction with eq 1, predicts that $\partial \Delta H_{obs}/\partial$ log [NaCl] should display some curvature. However, this trend is not apparent in Figure 3.

The "unlimited" cooperativity parameter, $\omega_{T/O}$, obtained from analysis of the SSB tetramer−poly(U) equilibrium binding isotherms is also dependent on temperature, increasing from ∼170 ± 130 at 10 °C to ∼1000 ± 400 at 37 °C (see Table 1), although it is independent of salt concentration (Overman et al., 1988). This suggests that the nearest-neighbor cooperative interactions involved in formation of SSB "octamers" are entropically driven.

We have previously shown that SSB−poly(U) and SSB−ss nucleic acid interactions, in general, are influenced by anion type (Overman et al., 1988; Overman & Lohman, 1994). To determine the relative contributions of anions *vs* cations to the salt dependence of ΔH_{obs}, we measured the dependence of K_{obs} on [NaF] at 25 and 37 °C (pH 8.1). These measurements were restricted to ≥25 °C and ≤37 °C for two reasons. The low-temperature limit was due to the inability to perform experiments at a sufficiently high [NaF] to reduce K_{obs} to a measurable range (due to the large $\Delta H_{obs} = -58 \pm 5$ kcal/mol and the low solubility of NaF). The high-temperature limit resulted from the necessity of maintaining the [NaF] ≥ 0.2 M in order to ensure that SSB bound in its $(SSB)_{65}$ binding mode (Bujalowski et al., 1988). At both 25 and 37 °C, plots of log K_{obs} *vs* log [NaF] are linear with identical values of ∂ log K_{obs}/∂ log [NaF] $= -4.3 \pm 0.4$ (see Figure 2A and Table 2). In contrast, recall that the value of ∂ log K_{obs}/∂ log [NaCl] changes from -5.1 ± 0.4 at 37 °C to -7.4 ± 0.5 at 25 °C. The negligible effect of temperature on ∂ log K_{obs}/∂ log [NaF] indicates that the dependence of ΔH_{obs} on [NaCl] at pH 8.1 is due at least in part to differential Cl^- binding to SSB protein. Although ∂ log K_{obs}/∂ log [NaF] is not sensitive to temperature, the value of K_{obs} in NaF does increase with decreasing temperature; based on experiments at 25 and 37 °C, we estimate $\Delta H_{obs} = -58 \pm 5$ kcal/mol, independent of [NaF] (pH 8.1).

[NaCl] Dependence of K_{obs} for SSB−dA(pA)$_{69}$ Binding Is Also Temperature-Dependent. Quantitative equilibrium studies of SSB tetramer binding to polynucleotides such as poly(U) are complicated by the fact that in the $(SSB)_{65}$ polynucleotide mode, binding occurs with a "limited" intertetramer positive cooperativity ($\omega_{T/O}$); thus, the energetics of cooperativity must be resolved from the intrinsic binding constant, K_{obs}. To eliminate these complexities, we examined the binding of SSB to $dA(pA)_{69}$ under conditions such that a

Table 1: Effect of Temperature on K_{obs} for SSB Tetramer−Poly(U) Binding in the $(SSB)_{65}$ Binding Mode in NaCl (pH 8.1)

temp (°C)	Q_{max}	$\partial \log K_{obs}/\partial \log$ [NaCl]	$\log K_{obs}$[a] (1.0 M NaCl)	K_{obs} (M^{-1}) (0.35 M NaCl)	$\omega_{T/O}$
10	0.69−0.71 (±0.01)[b]	−9.3 (±0.3)	0.5 (±0.1)	5.5 (±0.5) x 10^4	170 (±130)
15	0.66 (±0.01)	−8.6 (±0.3)	0.1 (±0.1)	1.0 (±0.1) × 10^4	170 (±70)
20	0.63 (±0.01)	−8.0 (±0.2)	0.1 (±0.1)	5.6 (±0.1) × 10^3	290 (±150)
25	0.59 (±0.01)	−7.4 (±0.5)	−0.4 (±0.2)	9.4 (±0.6) × 10^2	380 (±100)
30	0.54 (±0.01)	−6.5 (±0.2)	−0.5 (±0.1)	2.9 (±0.1) × 10^2	330 (±130)
37	0.51 (±0.01)	−5.1 (±0.4)	−0.2 (±0.2)	1.3 (±0.1) × 10^2	1000 (±400)

[a] Extrapolated. [b] Q_{max} is dependent on the NaCl concentration at this temperature.

Table 2: Effect of Temperature on K_{obs} for SSB Tetramer−Poly(U) Binding in the $(SSB)_{65}$ Binding Mode in NaF (pH 8.1)

temp (°C)	Q_{max}	$\partial \log K_{obs}/\partial \log$ [NaF]	$\log K_{obs}$[a]	K_{obs} (M^{-1})	$\omega_{T/O}$[b]
					170
25	0.66 (±0.01)	−4.3 (±0.4)	3.3 (±0.3)	1.8 (±0.5) × 10^5	410
37	0.56 (±0.01)	−4.4 (±0.3)	1.8 (±0.3)	6.4 (±2.5) × 10^3	1000

[a] Extrapolated. [b] From NaCl data.

1:1 complex is formed in which the DNA interacts with all four SSB subunits (Ferrari et al., 1994; Ferrari & Lohman, 1994). K_{obs} was determined from isotherms monitored by the quenching of the SSB tryptophan fluorescence (Ferrari & Lohman, 1994). At each temperature (from 5 to 37 °C), the values of $\partial \log K_{obs}/\partial \log$ [NaCl] are negative, indicating a net release of ions upon formation of the SSB−$dA(pA)_{69}$ complex. Furthermore, $\partial \log K_{obs}/\partial \log$ [NaCl] increases with increasing temperature from -5.7 ± 0.1 at 5 °C to -4.3 ± 0.2 at 37 °C. Between 25 and 37 °C, the value of $\partial S_a K_{obs}/\partial(1/T) = (-7.1 \pm 1) \times 10^3$ K, from which we estimate $\partial \Delta H_{obs}/\partial \log$ [NaCl] = 32 ± 4 kcal/mol, according to eq 1, although this becomes smaller at lower temperatures.

Calorimetric Determination of the [NaCl] Dependence of ΔH_{obs} for SSB−dT(pT)$_{69}$ Binding. We also determined the ΔH_{obs} for SSB tetramer binding to $dT(pT)_{69}$ at a series of [NaCl] from 0.17 M to 2.0 M (pH 8.1, 25.0 °C) using isothermal titration calorimetry (ITC). We used $dT(pT)_{69}$ for these ITC experiments since under these conditions the SSB tetramer forms a 1:1 complex with $dT(pT)_{69}$ with sufficiently high affinity so that binding is stoichiometric at all [NaCl], even 5 M (Bujalowski & Lohman, 1989a,b). Therefore, determination of ΔH_{obs} and its dependence on [NaCl] are simplified, although estimates of K_{obs} ($\Delta G°_{obs}$) or $\Delta S°_{obs}$ could not be obtained.

The results of a calorimetric titration of SSB (1.8 μM tetramer) with $dT(pT)_{69}$ (35 μM) in 0.17 M NaCl (pH 8.1, 25 °C) are shown in Figure 4. Figure 4A shows that each injection of $dT(pT)_{69}$ is accompanied by a large exothermic heat of reaction, and as shown in Figure 4B, the total heat increases linearly with [$dT(pT)_{69}$] until saturation is reached at a stoichiometry of 1 SSB tetramer per $dT(pT)_{69}$. The calorimetric enthalpy determined from this titration is $\Delta H_{obs} = -146.0 \pm 1.5$ kcal/mol ($dT(pT)_{69}$) calculated either by the differential or by the integral approach (see Materials and Methods). This extremely large value of ΔH_{obs} is 2.5-fold larger than that estimated from van't Hoff analysis of SSB binding to poly(U) at the same [NaCl]. A similar experiment carried out by titrating a solution of $dT(pT)_{69}$ (1 μM) with SSB (16 μM) yielded $\Delta H_{obs} = -148.6 \pm 2.2$ kcal/mol by the differential approach and −140.1 kcal/mol by the integral approach.

ITC measurements of ΔH_{obs} for SSB−$dT(pT)_{69}$ binding were performed at 0.17, 0.25, 0.40, 1.0, and 2.0 M NaCl (25 °C, pH 8.1). At each [NaCl], titrations of SSB protein with $dT(pT)_{69}$ as well as $dT(pT)_{69}$ with SSB protein were both performed, and stoichiometries of 1 ± 0.05 were obtained. As shown in Figure 5, the calorimetric ΔH_{obs} increases significantly with increasing [NaCl]. The linear least-squares line describing the data in Figure 5 is given in eq 3.

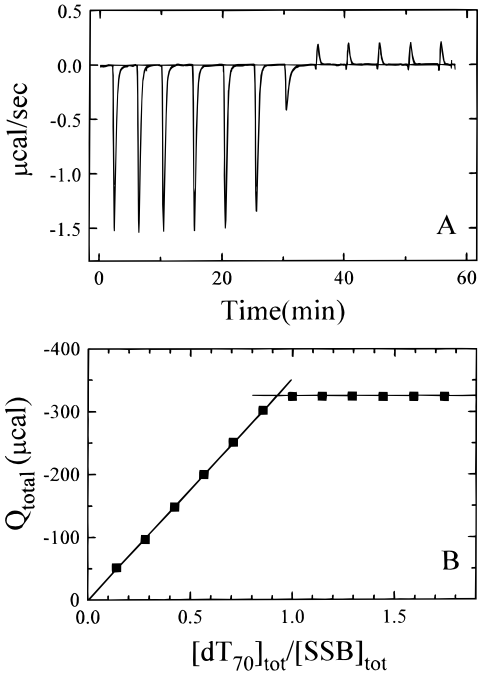

FIGURE 4: Determination of ΔH_{obs} for SSB tetramer binding to $dT(pT)_{69}$ by isothermal titration calorimetry. (A) Measured heats of reaction upon titration of a solution of SSB protein (1.84 μM tetramer) in buffer H (pH 8.1) + 0.17 M NaCl (25 °C) with 10 μL aliquots of $dT(pT)_{69}$ (35 μM). (B) Plot of total heat (Q_{total}) (μcal) as a function of the molar ratio of $dT(pT)_{69}$ to SSB tetramer. A value of $\Delta H_{obs} = -146 \pm 1$ kcal/mol was determined from these data.

$$\Delta H_{obs}\ (\text{kcal/mol}) = (46.3 \pm 1.8) \log [\text{NaCl}] - (109.8 \pm 0.9) \quad (3)$$

This increase in ΔH_{obs} with increasing [NaCl] is expected based on the linkage relationship in eq 1 and the observations that $\partial \log K_{obs}/\partial \log$ [NaCl] decreases (becomes more negative) with decreasing temperature for SSB binding to poly(U) and $dA(pA)_{69}$. The value of $\partial \Delta H_{obs}/\partial \log$ [NaCl] = 46.3 ± 1.8 kcal/mol determined calorimetrically for SSB−$dT(pT)_{69}$ binding is somewhat less than the value of 60 ± 5 determined for SSB−poly(U) binding [in the $(SSB)_{65}$ mode] by van't Hoff analysis (see eq 2 and Figure 3), but is greater than that predicted from the observed temperature dependence of $S_a K_{obs}$ for SSB−$dA(pA)_{69}$ binding in the range from 25 to 37 °C. These differences may reflect effects of nucleic

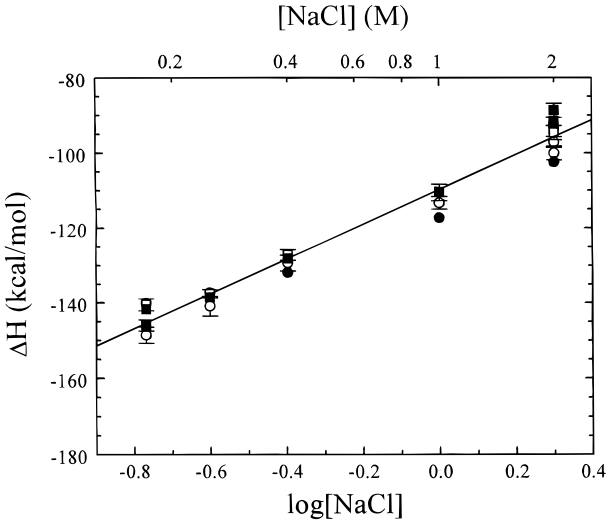

FIGURE 5: Calorimetric ΔH_{obs} for SSB tetramer binding to dT-$(pT)_{69}$ increases with increasing [NaCl]. Values of ΔH_{obs}, plotted as a function of log [NaCl], were determined by isothermal titration calorimetry (25 °C, buffer H, pH 8.1), by the differential approach (open symbols) or the integral approach as shown in Figure 4 (filled symbols). (●, ○) Experiments performed by titrating $dT(pT)_{69}$ with SSB protein; (■, □) experiments performed by titrating SSB protein with $dT(pT)_{69}$. The line shown is the linear least-squares line describing the data: $\Delta H_{obs} = 46.3 \log [NaCl] - 109.8$.

acid base composition or length (poly(U) *vs* $(dX)_{70}$) on the ion binding properties of the SSB protein (Lohman & Bujalowski, 1994).

DISCUSSION

Equilibrium constants, K_{obs} ($\Delta G°_{obs}$), for nearly all protein−nucleic acid interactions are very sensitive to changes in [salt] and type (Record et al., 1978, 1991; Lohman & Mascotti, 1992a). Much of this effect is generally due to the interactions of cations with the nucleic acid and the increase in entropy ($\Delta S°_{obs} > 0$) resulting from the thermodynamic release of counterions (e.g., Na^+, Mg^{2+}) from the nucleic acid polyanion upon complex formation. This has been demonstrated in studies of nucleic acids binding to simple oligocations such as positively charged oligolysines and oligoarginines. For such simple oligocations, the [salt]-dependent component of $\Delta G°_{obs}$ is entirely entropic in origin with ΔH_{obs} being independent of [salt] (Mascotti & Lohman, 1992, 1993; Lohman & Mascotti, 1992a). However, in this report, we show that the [NaCl]-dependence of K_{obs} for *E. coli* SSB tetramer binding to three different single-stranded nucleic acids is strongly temperature-dependent, reflecting a [NaCl]-dependent ΔH_{obs}.

From van't Hoff analyses and the linkage relationship in eq 1, we find $\partial \Delta H_{obs}/\partial \log [NaCl] = 60 \pm 5$ kcal/mol for SSB binding to poly(U), with ΔH_{obs} increasing from -57 ± 3 kcal/mol at 0.18 M NaCl to -34 ± 3 kcal/mol at 0.42 M NaCl. This linkage does not appear to be due to cation release from the nucleic acid since ΔH_{obs} is independent of salt concentration for nonspecific binding of oligolysines and oligoarginines to poly(U), at least in the range from 0.10 to 0.30 M (Mascotti & Lohman, 1992, 1993). Therefore, the [NaCl] dependence of ΔH_{obs} must result from differential ion binding to the protein. The fact that $\partial \Delta H_{obs}/\partial \log [NaCl]$ is significantly larger than $\partial \Delta H_{obs}/\partial \log [NaF]$ suggests that the effect is due, at least in part, to differential Cl^- binding. Consistent with this view, we also note that the value of $\Delta H_{obs} = -58 \pm 5$ kcal/mol determined in NaF (pH 8.1) for SSB binding to poly(U) is nearly the same as the value determined at the lowest [NaCl] investigated ($\Delta H_{obs} = -57 \pm 3$ kcal/mol at 0.18 M NaCl). The [NaCl] dependence of K_{obs} for SSB−$dA(pA)_{69}$ binding also displays the same qualitative dependence on temperature. Direct calorimetric measurements of ΔH_{obs} for SSB binding to $dT(pT)_{69}$ also show that ΔH_{obs} increases with [NaCl] ($\partial \Delta H_{obs}/\partial \log [NaCl] = 46 \pm 2$ kcal/mol (pH 8.1, 25.0 °C)).

Of additional interest is the fact that the values of ΔH_{obs} are very large and considerably more exothermic than have been reported for other protein−nucleic acid interactions. The value of $\Delta H_{obs} = -144 \pm 4$ kcal/mol for SSB binding to $dT(pT)_{69}$ (0.17 M NaCl, pH 8.1, 25 °C) is nearly a factor of 5 larger than the largest value reported for any other protein−nucleic acid interaction, including sequence-specific binding proteins (de Haseth et al., 1977; Ha et al., 1989; Jin et al., 1993; Ladbury et al., 1994; Merabet & Ackers, 1995). These values are consistent with our previous estimates of ΔH_{obs} from van't Hoff analyses of SSB binding to $dT(pT)_{69}$ and $dC(pC)_{69}$ measured in buffer containing NaBr (Ferrari & Lohman, 1994).

In the studies reported here, we have been careful to determine that the SSB tetramer does not undergo disassembly or additional assembly processes over the range of protein concentrations and solution conditions examined, and we have used homopolynucleotides, such as poly(U), $dT(pT)_{69}$, and $dA(pA)_{69}$, so that all binding sites are equivalent and no intramolecular base pairing can occur. We have also restricted our studies of the SSB−poly(U) interaction to solution conditions that we know support only the $(SSB)_{65}$ binding mode in which all four subunits interact with nucleic acid, in order to avoid complications of multiple binding modes that may possess different values of ΔH_{obs}. Furthermore, our analyses of fluorescence titrations were performed using model-independent methods (Bujalowski & Lohman, 1987b; Lohman & Bujalowski, 1991; Lohman & Mascotti, 1992b) which show that the quenching of SSB fluorescence is directly proportional to the extent of SSB binding to poly(U) in the $(SSB)_{65}$ mode (Overman et al., 1988; Overman & Lohman, 1994). Therefore, we are confident that the [NaCl]-dependent ΔH_{obs} is an intrinsic property of the SSB tetramer−poly(U) interaction. The results with the simpler oligodeoxynucleotides, $dA(pA)_{69}$ and $dT(pT)_{69}$, support this conclusion.

The observation that there is less net Cl^- released as the temperature increases suggests that some of the Cl^- that is released must bind to the protein with $\Delta H < 0$. Therefore, as the temperature increases, less Cl^- is bound to the protein, and thus less Cl^- is released upon protein binding to the nucleic acid. As the $[Cl^-]$ is increased, more Cl^- binds to the protein, and thus more Cl^- ions are released upon formation of the SSB−nucleic acid complex. Since the release of these Cl^- ions is accompanied by $\Delta H > 0$, the net ΔH_{obs} for formation of the SSB−nucleic acid complex will increase with increasing [NaCl] as observed.

There are several possible origins of the increase in ΔH_{obs} with increasing [NaCl], any or all of which may contribute: (1) site binding of Cl^- ions to the protein that is associated with $\Delta H << 0$ (Scatchard & Yap, 1964; Diebler et al., 1969); (2) site binding of Cl^- ions to the protein that is coupled to protonation of the protein, with an accompanying $\Delta H < 0$ for protonation; (3) Cl^- binding coupled to a protein

conformational change with $\Delta H < 0$; or (4) preferential interaction or accumulation of Cl^- in the vicinity of the protein that is both anion-specific and highly temperature-dependent. Our previous studies suggest that some of the Cl^- that is bound to the SSB protein and thus released upon SSB binding to poly(U) requires protonation of the protein (Overman & Lohman, 1994). Therefore, deprotonation of these sites would accompany SSB binding to ss DNA. Since protonation of some amino acids occurs with a large $\Delta H < 0$ (Shiao & Sturtevant, 1976), the linkage of de-protonation to Cl^- release should contribute a $\Delta H > 0$ to ΔH_{obs} and thus result in $\partial \Delta H_{obs}/\partial \log [NaCl] > 0$ as observed.

The large dependence of ΔH_{obs} on [NaCl] may have contributions from site binding of ions to the protein, with its resulting large negative ΔH (Scatchard & Yap, 1964; Diebler et al., 1969). In fact, site binding of a single Na^+ to human α-thrombin is accompanied by $\Delta H_{obs} = -25 \pm 2$ kcal/mol, although conformational changes in thrombin that accompany Na^+ binding may also contribute to this large ΔH (Wells & Di Cera, 1992). However, the binding of ions that do not undergo dehydration is generally accompanied by a small ΔH and thus should not contribute substantially to ΔH_{obs}. For example, ΔH_{obs} for oligolysine binding to poly(U) is relatively small (−2 kcal/mol) and insensitive to $[K^+]$ even though release of K^+ from poly(U) accompanies oligolysine binding (Mascotti & Lohman, 1992; Lohman & Mascotti, 1992a). This is likely due to the fact that monovalent cations such as Na^+ and K^+ bind to linear nucleic acids in a delocalized manner, retaining their waters of hydration (Manning, 1978; Anderson et al., 1978; Braunlin, 1995).

Although some site binding of Cl^- to SSB protein cannot be discounted, the fact that the effect of [NaCl] on ΔH_{obs} does not reach a saturation point, but continues even up to 2 M NaCl, suggests contributions from weaker preferential interactions of Cl^- relative to water. Unfortunately, there is no available information on the temperature dependence of such weak Hofmeister effects (Record et al., 1978; von Hippel & Schleich, 1969) to compare with our results.

The thermodynamics of SSB−ss nucleic acid binding are of interest not only for the large [NaCl] dependence of ΔH_{obs} but also for the extremely large negative ΔH_{obs}. Although a full understanding must await further studies, a partial explanation may be due to the fact that 2−3 groups on SSB require protonation in order for SSB to bind poly(U) (Overman & Lohman, 1994). These protonation events are distinct from those discussed above that are linked to Cl^- binding. In fact, they are not linked to effects of [NaCl] and thus should not contribute to the [NaCl] dependence of ΔH_{obs}; however, they should contribute a $\Delta H < 0$ to ΔH_{obs}. A similar requirement for protonation of two groups on lac repressor upon binding to nonspecific DNA contributes to the negative ΔH_{obs} (−11 kcal/mol) observed at pH >7.5 (de Haseth et al., 1977).

Although no other reports have described similar effects for protein−nucleic acid interactions, this simply reflects the fact that influences of [salt] on ΔH_{obs} have not previously been examined for protein−nucleic acid interactions. However, such effects should be observed for other protein−DNA interactions accompanied by differential binding of ions to the protein or ion binding that is linked to protonation or conformational changes with an associated ΔH. Based on recent examples of site binding of cations to complex RNA molecules (Wang et al., 1993), it is also likely that the ΔH_{obs} for some RNA conformational transitions or RNA−protein interactions may be [salt]-dependent.

A few reports of [salt]-dependent values of ΔH_{obs} for ligand−DNA interactions have appeared. The ΔH_{obs} for daunomycin binding to calf thymus DNA increases (becoming less negative) with increasing [NaCl], in the range from 50 mM to 0.2 M NaCl, but is constant at -11 ± 2 kcal/mol from 0.2 to 1 M NaCl (Chaires, 1985). Similar observations have been reported for the binding of calf thymus DNA to daunomycin, adriamycin (Barcelo et al., 1988) and ethidium (LePecq & Paoletti, 1967). However, the basis for these effects may not be related to the salt effects we observe for SSB−nucleic acid interactions, but rather to the sequence heterogeneity of calf thymus DNA and the fact that these ligands bind with some base sequence specificity (Chaires et al., 1987) and thus different binding sites may be selectively populated at different salt concentrations. Salt-dependent daunomycin aggregation may also complicate these results. A salt-dependent ΔH_{obs} is also observed for the binding of 3′-CMP to ribonuclease A (Bolen et al., 1971; Eftink et al., 1983). Although this was interpreted as a simple "ionic strength" effect on the pK_a's of the two active site histidines in RNase A that are involved in binding, direct ion binding to the protein is also possible.

The observations reported here for *E. coli* SSB protein have important implications for studies of this and other protein−nucleic acid interactions. They underscore that attempts to understand the molecular origins of the stability and specificity of these interactions or to correlate thermodynamic quantities with structural changes must be made cautiously in the absence of systematic studies of the effects of solution conditions. However, we emphasize that ΔH_{obs} is independent of salt concentration for simple systems [e.g., oligolysines binding to poly(U)], where release of hydrated cations from the nucleic acid is the predominant salt effect on $\Delta G°_{obs}$ (Mascotti & Lohman, 1992, 1993; Lohman & Mascotti, 1992a). Therefore, the existence of a salt-dependent ΔH_{obs} provides an indication that salt-dependent processes other than those originating from release of hydrated cations from the nucleic acid must be linked to the binding equilibrium. The effects of [salt] and type on ΔH_{obs} and $\Delta S°_{obs}$ as well as $\Delta G°_{obs}$ need to be examined for other protein−nucleic acid systems, and macromolecular interactions in general, in order to assess the generality of the observations reported here.

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