Calorimetric Studies of \textit{E. coli} SSB Protein–Single-stranded DNA Interactions. Effects of Monovalent Salts on Binding Enthalpy

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Isothermal titration calorimetry (ITC) was used to examine the effects of monovalent salts (NaCl, NaBr, NaF and ChCl) on the binding enthalpy ($\Delta H_{\text{obs}}$) for \textit{E. coli} SSB tetramer binding to the single-stranded oligodeoxymidylates, dT(pT)$_{69}$ and dT(pT)$_{34}$ over a wide range of salt concentrations from 10 mM to 2.0 M (25°C, pH 8.1), and when possible, the binding free energy and entropy ($\Delta G_{\text{obs}}$, $\Delta S_{\text{obs}}$). At low monovalent salt concentrations (<0.1 M), the total $\Delta H_{\text{obs}}$ for saturating all sites on the SSB tetramer with ssDNA shows little dependence on salt concentration, but is extremely large and exothermic ($\Delta H_{\text{obs}} \approx -150(\pm5)$ kcal/mol). This is much larger than any $\Delta H_{\text{obs}}$ previously reported for a protein–nucleic acid interaction. However, at salt concentrations above 0.1 M, $\Delta H_{\text{obs}}$ is quite sensitive to NaCl and NaBr concentration, becoming less negative with increasing salt concentration ($\Delta H_{\text{obs}} \approx -70(\pm1)$–kcal/mol in 2 M NaBr). These salt effects on $\Delta H_{\text{obs}}$ were mainly a function of anion type and concentration, with the largest effects observed in NaBr, and then NaCl, with little effect of [NaF]. These large effects of salt on $\Delta H_{\text{obs}}$ appear to be coupled to a net release of weakly bound anions (Br$^-$ and Cl$^-$) from the SSB protein upon DNA binding. However, at lower salt concentrations (<0.1 M), specific cation effects on $\Delta H_{\text{obs}}$ also are observed. Under conditions where we can determine $\Delta G_{\text{obs}}$, $\Delta S_{\text{obs}}$, and $\Delta H_{\text{obs}}$ (25°C, pH 8.1, 0.17 to 2 M NaBr), SSB binding to dT(pT)$_{69}$ is enthalpically driven with a large unfavorable entropic contribution, both of which are dependent upon [NaBr]. These studies show that weak anion bonding to a protein can result in large effects of salt concentration on $\Delta H_{\text{obs}}$ (as well as $\Delta G_{\text{obs}}$ and $\Delta S_{\text{obs}}$) for a protein–ssDNA interaction. The possibility of such effects needs to be considered in any interpretation of the thermodynamics of this and other protein–nucleic acid interactions.

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Introduction

Thermodynamic studies are required to provide the energetic information to understand the origins of stability and specificity of protein–nucleic acid interactions. However, the stability of these and other macromolecular interactions are generally a strong function of solution conditions (temperature, pH, salt concentration and type). Furthermore, the effects of each of these variables are generally linked, so that an understanding of the effects of one variable requires an understanding of the effects of the others. In fact, much of the power of thermodynamic studies of macromolecular equilibria lies in the information obtained from analysis of these linkage effects, which provides the means by which one can expose the complexities of such interacting systems.

It is well established that the stabilities and specificities of protein–nucleic acid interactions display significant electrostatic effects and thus are highly sensitive to the bulk salt concentration (for
reviews see Record et al., 1978, 1991; Anderson & Record, 1995; Lohman et al., 1978; Lohman & Mascotti, 1992; Hard & Lundback, 1996). A major source of these salt effects is the polyelectrolyte nature of nucleic acids, which are highly charged polyanions. The high charge density of the linear sugar-phosphate backbone results in a sequestering of low molecular weight cations (e.g. Na⁺, K⁺, Mg²⁺) in the vicinity of the nucleic acid (Manning, 1969; Record et al., 1976; Record, 1975). Any perturbation of the nucleic acid linear charge density, for example, due to binding of a protein, will generally result in a reduction in the amount of cation binding to the nucleic acid. As a direct consequence of this cation release, increases in cation concentration will lower the equilibrium binding constant for most protein–nucleic acid interactions (Record et al., 1976, 1978; Lohman & Mascotti, 1992).

Equilibrium binding studies of simple positively charged oligopeptides to linear nucleic acids have shown that the release of counterions from the nucleic acid provides a major favorable entropic contribution to the binding free energy (a drastic entropy of dilution; Record et al., 1976; Lohman et al., 1980; Braunlin et al., 1982; Mascotti & Lohman, 1990, 1992, 1993, 1997; Zhang et al., 1996). In fact, the ΔH_{obs} for binding of these simple oligopeptides to linear nucleic acids appears to be independent of salt concentration (Lohman et al., 1980; Mascotti & Lohman, 1992, 1993, 1997), consistent with the effects of salt on ΔG_{obs} being entirely entropic in nature.

Cation release from the nucleic acid also is a major contributor to the stability of protein–nucleic acid interactions (Record et al., 1976, 1978, 1991; Lohman & Mascotti, 1992). However, the effects of salt concentration on these systems can also be more complex than on the simple model peptides due to the fact that the protein itself is also capable of binding ions (both anions and cations). Clear evidence for both ion release from and/or uptake by the protein upon DNA binding has been reported (Overman et al., 1988; Overman & Lohman, 1994; Bujalowski et al., 1988; Bujalowski & Lohman, 1989a; Leirimo et al., 1987; Ha et al., 1989; Kowalczykowski et al., 1981; Lohman et al., 1996). Furthermore, many of these effects appear to be due to weak preferential binding of anions, which exert their influence according to their position in the Hofmeister series (Baldwin, 1996; Collins, 1997).

We have been studying the thermodynamics of single-stranded (ss) DNA binding to the Escherichia coli SSB protein (for reviews see Lohman & Bujalowski, 1990; Lohman & Ferrari, 1994), which functions in DNA replication, recombination and repair (Meyer & Laine, 1990). This protein, which is a stable homotetramer under a wide range of solution conditions, can bind to ss-polynucleotides in several different binding modes, referred to as (SSB)_{35}, (SSB)_{56}, and (SSB)_{65} binding modes all four subunits of the SSB tetramer interact with ssDNA, whereas in the (SSB)_{35} binding mode only two subunits interact with ssDNA. The relative stabilities of these different polynucleotide binding modes are dependent on salt concentration and type (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988) and the inter-tetramer cooperativities of these binding modes also differ dramatically (Lohman et al., 1986b; Bujalowski & Lohman, 1987; Ferrari et al., 1994; Griffith et al., 1984). The ability of the SSB protein to form these different binding modes is due at least in part to its tetrameric nature and to a salt-dependent negative cooperativity for ssDNA binding within individual SSB tetramers (Lohman & Bujalowski, 1988, 1994; Bujalowski & Lohman, 1989a,b). This negative cooperativity becomes more prominent at low salt concentrations, thus decreasing the apparent affinity of DNA for the third and fourth subunits of the SSB tetramer, and stabilizing the (SSB)_{35} mode. A recent crystal structure of the chymotryptic DNA binding core of the SSB tetramer, SSBc, shows that it is predominantly a β-strand protein with D₂ symmetry (Raghunathan et al., 1997).

The equilibrium association constant, K_{diss}, for E. coli SSB tetramer binding to ss-nucleic acids decreases dramatically with increasing salt concentration resulting from a net release of both cations and anions upon formation of the ss-nucleic acid complex (Overman et al., 1988; Bujalowski & Lohman, 1989a,b; Overman & Lohman, 1994). However, we have recently shown that the salt dependence of K_{diss} (ΔG_{diss}) is not entirely entropic in nature (Lohman et al., 1996), behavior which differs from that observed for simple oligosilenes and oligoarginines binding to ss-nucleic acids (Mascotti & Lohman, 1992, 1993, 1997). In fact, a very large effect of [NaCl] on the observed binding enthalpy, ΔH_{obs}, was measured for SSB tetramer binding to the ss-RNA, poly(U), by van’t Hoff analysis, whereas little effect of [NaF] was observed. This effect was also demonstrated calorimetrically; the value of ΔH_{obs} for SSB binding to dT(pT)₆₉ ranged from −145(±5) kcal/mol at 0.17 M NaCl to −95(±5) kcal/mol at 2 M NaCl (25.0°C, pH 8.1). However, the limited range of [NaCl] examined made it difficult to determine the origin of these effects, although several possibilities were considered (Lohman et al., 1996). The current studies reported here were designed to test these different possibilities by examining the effects of different monovalent salts over a much wider range of salt concentrations. Our results indicate that these effects are mainly due to the net release of weakly bound anions from the SSB protein upon formation of the SSB–nucleic acid complex. Although such studies have not yet been performed for other protein–nucleic acid interactions, it is likely that similar effects will be observed.
Results

Calorimetric determination of the effects of monovalent salt concentration on $\Delta H_{\text{obs}}$ for SSB binding to $\text{dT(pT)}_{69}$

As shown previously (Lohman et al., 1996), at the protein concentrations required for our ITC experiments (>0.5 μM SSB tetramer), $\text{dT(pT)}_{69}$ binding to the SSB tetramer is stoichiometric at all concentrations of NaCl studied (0.17 to 2.0 M; buffer H, pH 8.1, 25°C). Thus, ITC experiments performed in buffers containing NaCl as the only monovalent salt can only provide an accurate estimate of $\Delta H_{\text{obs}}$. Our previous studies also showed that the value of $\Delta H_{\text{obs}}$ for the SSB-$\text{dT(pT)}_{69}$ interaction is extremely large and highly dependent upon [NaCl] (examined from 0.17 to 2 M NaCl); $\Delta H_{\text{obs}}$ ranges from $-145(\pm5)$ kcal/mol at 0.17 M NaCl to $-95(\pm5)$ kcal/mol at 2 M NaCl, displaying an approximately linear dependence on log[NaCl] ($\Delta H_{\text{obs}}$ (kcal/mol) = (46.3 ± 1.8)log [NaCl] — (109.8 ± 0.9)) (Lohman et al., 1996).

To further investigate the origins of this salt effect, we performed ITC experiments with the SSB-$\text{dT(pT)}_{69}$ system as a function of both [NaBr] and [NaF] under the same solution conditions (buffer H, pH 8.1, 25°C). Figure 1 shows typical results from an ITC titration of SSB tetramer (1.4 μM) with $\text{dT(pT)}_{69}$ (28.3 μM) at 1.3 M NaBr (25°C, pH 8.1), plotted in the form of normalized heats versus the ratio, $[\text{dT(pT)}_{69}]_{\text{tot}}/[\text{SSB}]_{\text{tot}}$. These are conditions under which 1:1 complexes of $\text{dT(pT)}_{69}$ per SSB tetramer are formed, as can be observed by the non-linear least squares fit of the data to equation (1), which yields $n = 1.04$ with $K_{\text{abs}} = 9.5(\pm0.6) \times 10^7$ M$^{-1}$, $\Delta H_{\text{obs}} = -85.0(\pm0.4)$ kcal/mol.

Table 1 and Figure 2 show the values of $\Delta H_{\text{obs}}$ measured for SSB tetramer binding to $\text{dT(pT)}_{69}$ as a function of [NaCl], [NaBr] and [NaF] (25°C, pH 8.1). Under all salt conditions, $\Delta H_{\text{obs}}$ is highly exothermic, but is also a sensitive function of the type of monovalent salt. At a constant salt concentration, $\Delta H_{\text{obs}}$ becomes less negative, proceeding from NaF to NaCl to NaBr. However, the dependence of $\Delta H_{\text{obs}}$ on salt concentration also differs for these three salts, being largest in NaBr, less in NaCl and nearly independent of NaF concentration, although we could only measure this over a narrow range of [NaF] (0.2 to 0.6 M) due to the limited solubility of NaF and SSB in NaF. Although the uncertainties associated with the data in NaF are larger than in NaCl or NaBr due to the lower SSB concentrations used (see Materials and Methods), these results clearly indicate that $\Delta H_{\text{obs}}$ is very sensitive to the type of monovalent anion. We also note that the values of $\Delta H_{\text{obs}}$ estimated previously by van’t Hoff analysis (Ferrari & Lohman, 1994) for SSB binding to $\text{dT(pT)}_{69}$ in 2.0 M NaBr ($-82(\pm17)$ kcal/mol) and 2.4 M NaBr (69(±4) kcal/mol; pH 8.1, 25°C) agree well with those measured here by ITC.

The lack of a dependence of $\Delta H_{\text{obs}}$ on [NaF] for the SSB-$\text{dT(pT)}_{69}$ interaction is consistent with our previous van’t Hoff studies of SSB tetramer binding to poly(U) in the (SSB)$_{65}$ binding mode (Lohman et al., 1996). For this system, the [NaF] dependence of $K_{\text{obs}}$ (log $K_{\text{obs}}$/log[NaF] (0.4 to 0.9 M NaF), is not influenced by temperature (between 25°C and 37°C; Lohman et al., 1996). Since ($\Delta S_{\text{obs}}/\Delta T$)$_{\text{obs}}$ was ~$(1/2.3R)\Delta H_{\text{obs}}$/log [NaF], where $\Delta S_{\text{obs}} = (\log K_{\text{obs}}$/log[NaF]), this indicates that $\Delta H_{\text{obs}}$ (~58(±5) kcal/mol under these conditions) is independent of [NaF].

In Figure 2, the values of $\Delta H_{\text{obs}}$ for SSB-$\text{dT(pT)}_{69}$ binding in all three salts (NaBr, NaCl and NaF) appear to converge at low salt concentrations to the value determined in [NaF]. This behavior is
similar to that observed previously for the SSB-poly(U) interaction, with $\Delta H_{\text{obs}}$ determined at low [NaCl] approaching the value estimated in 0.20 M NaCl (Lohman et al., 1996). Unfortunately, we are unable to determine values of $\Delta H_{\text{obs}}$ for the SSB-dT(pT)34 interaction at salt concentrations below 0.2 M due to the fact that different modes of SSB binding begin to become populated.

**Table 1.** Effect of salt concentration and type on $\Delta H_{\text{obs}}$ for SSB binding to dT(pT)69 and dT(pT)34

<table>
<thead>
<tr>
<th>[Salt] (M)</th>
<th>dT(pT)34 (kcal/mol)</th>
<th>dT(pT)69 (kcal/mol)</th>
<th>NaBr</th>
<th>NaCl</th>
<th>CholineCl</th>
<th>NaF</th>
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<td>0.010</td>
<td>-141.6(±8.0)</td>
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<td>-146.4(±12.1)</td>
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<td>-149.6(±1.1)</td>
<td></td>
<td></td>
<td>-159.5(±5.4)</td>
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<td>-155.9(±1.6)</td>
<td></td>
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<td>-150.8(±0.9)</td>
<td></td>
<td></td>
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<td>-116.0(±2.1)</td>
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<td>-94.2(±1.0)</td>
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</table>

*Conditions: buffer H, pH 8.1, 25°C; for SSB binding to dT(pT)34 $\Delta H_{\text{obs}} = \Delta H_{\text{obs,2}} = \Delta H_{\text{obs,1}} + \Delta H_{\text{obs,2}}$.

* Taken from Lohman et al. (1996).

**Figure 2.** Dependence on salt concentration and type of the binding enthalpy ($\Delta H_{\text{obs}}$) for SSB tetramer binding to dT(pT)69. Binding enthalpies (see also Table 1) were measured by ITC in buffer H (pH 8.1), 25°C. (▲) NaCl; (▲) NaBr; (■) NaF.

**Calorimetric studies of dT(pT)34 binding to the SSB tetramer**

In order to measure $\Delta H_{\text{obs}}$ over a wider range of salt concentrations, including salt concentrations <0.2 M, we examined the binding of dT(pT)34 to the SSB tetramer. Each SSB tetramer can bind two molecules of dT(pT)34, although with negative cooperativity (Lohman & Bujalowski, 1988; Bujalowski & Lohman, 1989a,b; Ferrari et al., 1994). We can readily determine the stoichiometry and average degree of DNA binding per tetramer and thus can examine the binding of one or two molecules of dT(pT)34 per SSB tetramer over any range of salt concentrations.

We performed ITC experiments in the following salts: NaCl (0.01 to 1.0 M); NaBr (0.04 to 1.0 M); NaF (0.01 to 0.06 M), LiCl (0.2 M) and choline chloride (ChCl; 0.02 to 1.0 M), where choline is a monovalent tetra-alkyl ammonium derivative ((CH3)3N(CH2)2OH). Figure 3 shows the results of a typical ITC titration of SSB tetramer (2.0 µM) with dT(pT)34 (34.0 µM) in 60 mM NaCl (25.2°C, pH 8.1). Under these conditions, the first molecule of dT(pT)34 binds to the SSB tetramer with sufficiently high affinity such that only the enthalpy change can be determined accurately (Bains & Freire, 1991). From the fit of the data to equation (3), we determined $\Delta H_{\text{obs,1}} = -67.1(±0.5)$ kcal/mol for the binding of the first molecule of dT(pT)34 ([dT(pT)34tot]/[SSB]tot < 1). The further decrease of the normalized heat at ratios of [dT(pT)34tot]/[SSB]tot > 1, indicates that binding of the second dT(pT)34 molecule is more exothermic ($\Delta H_{\text{obs,2}} = -82.3(±0.6)$ kcal/mol) than binding of the first molecule. However, due to the high negative cooperativity, the affinity of dT(pT)34 for the
second site is much lower than for the first and is within a range that can be determined accurately ($k_2 = 1.49(\pm 0.11) \times 10^8$ M$^{-1}$).

Figure 4 shows the results of a series of ITC titrations of SSB with dT(pT)$_{34}$ (buffer H, pH 8.1, 25°C), clearly indicating a dramatic effect of NaCl concentration. We analyzed these isotherms by non-linear least squares fitting of the data to equation (3) to obtain the step-wise microscopic binding constants, $k_1$ and $k_2$, and the binding enthalpies, $\Delta H_1$ and $\Delta H_2$, for the binding of the first and second molecules of dT(pT)$_{34}$, respectively. In general, we find that $k_1$ is always higher than $k_2$, reflecting the known negative cooperativity (Lohman & Bujalowski, 1988; Bujalowski & Lohman, 1989a,b); however, due to the very high affinity of dT(pT)$_{34}$ for the first site, $k_1$ can only be determined accurately at [NaCl] $\geq$ 1 M. On the other hand $k_2$ can be determined accurately over the entire salt concentration range studied and the values determined here are in good agreement with those determined previously based on titrations monitoring SSB Trp fluorescence quenching (Bujalowski & Lohman, 1989b).

Figure 5 shows that a plot of log $k_1$ versus log [salt] has a convex shape, with a maximum value of $k_2$ near $\sim$0.10 M for NaBr, which shifts to higher salt concentrations in NaCl. At salt concentrations <0.1 M, $k_2$ is very nearly independent of anion type (with the exception of the data in choline chloride) and increases with increasing salt concentration. However, in the higher salt region ($\geq$0.1 M), $k_2$ decreases with increasing salt concent-
anion type, with $k_2$ decreasing approximately tenfold, although the position of the maximum does not change. Thus, the presence of the more bulky cation, choline, reduces the affinity of $dT(pT)_{34}$ to the second site and magnifies the negative cooperativity at low salt concentration. This is consistent with the proposed role of cations in reducing the negative cooperativity between sites (Bujalowski & Lohman, 1989b) and indicates that Na$^+$ is more effective at relieving the negative cooperativity than the bulkier choline cation.

Effect of salt concentration and type on $\Delta H_{\text{tot}}$ for SSB tetramer binding to $dT(pT)_{34}$

Before discussing the individual behavior of $\Delta H_{\text{obs,1}}$ and $\Delta H_{\text{obs,2}}$, we first consider the total $\Delta H_{\text{obs}}$ for saturating the SSB tetramer with two molecules of $dT(pT)_{34}$, i.e. $\Delta H_{\text{obs,tot}} = (\Delta H_1 + \Delta H_2)$ and compare this to $\Delta H_{\text{obs}}^{\text{tot}}$ for binding $dT(pT)_{69}$. The values of $\Delta H_{\text{obs}}^{\text{tot}}$ and $\Delta H_{\text{obs,tot}}$ are presented in Figure 6 and in Table 1. Direct comparisons of $\Delta H_{\text{obs}}^{\text{tot}}$ and $\Delta H_{\text{obs,tot}}$ can be made under identical solution conditions in NaCl, ChCl and NaBr, from 0.1 to 1.0 M. For all of these conditions, $\Delta H_{\text{obs}}^{\text{tot}}$ and $\Delta H_{\text{obs,tot}}$ are identical within our experimental uncertainty, i.e. the enthalpic contribution for binding one molecule of $dT(pT)_{69}$ is equivalent to that measured for binding two molecules of $dT(pT)_{34}$. This is consistent with the fact that one molecule of $dT(pT)_{69}$ interacts with all four SSB subunits whereas two molecules of $dT(pT)_{34}$ are required to interact with all four SSB subunits (Bujalowski & Lohman, 1989a,b).

At monovalent salt concentrations less than 0.1 M, we observe that $\Delta H_{\text{obs,tot}}$ converges for all three salts and becomes nearly independent of salt concentration and there is a clear dependence of $k_2$ on anion type, with $k_{2,F} > k_{2,Cl} > k_{2,Br}$ (see also Table 2). This behavior of $k_2$ has been demonstrated previously based on isotherms determined by tryptophan fluorescence quenching (Bujalowski & Lohman, 1989b).

The data in Figure 5 also indicate that cation type influences $k_2$. Upon substitution of choline chloride ((CH$_3$)$_2$N (CH$_2$)$_2$OH)Cl) for NaCl, $k_2$ decreases approximately tenfold, although the position of the maximum does not change. Thus, the presence of the more bulky cation, choline, reduces the affinity of $dT(pT)_{34}$ to the second site and magnifies the negative cooperativity at low salt concentration. This is consistent with the proposed role of cations in reducing the negative cooperativity between sites (Bujalowski & Lohman, 1989b) and indicates that Na$^+$ is more effective at relieving the negative cooperativity than the bulkier choline cation.

Table 2. Equilibrium constants for $dT(pT)_{34}$ binding to SSB tetramer

<table>
<thead>
<tr>
<th>[Salt] (M)</th>
<th>NaF $k_2$ (M$^{-1}$)</th>
<th>NaCl $k_2$ (M$^{-1}$)</th>
<th>NaBr $k_2$ (M$^{-1}$)</th>
<th>CholineCl $k_2$ (M$^{-1}$)</th>
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<tr>
<td>0.010</td>
<td>$(3.85 \pm 1.20) \times 10^8$</td>
<td>$(4.75 \pm 0.87) \times 10^8$</td>
<td>$(8.01 \pm 0.53) \times 10^8$</td>
<td>$(4.40 \pm 0.40) \times 10^8$</td>
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<tr>
<td>0.014</td>
<td>$(1.76 \pm 0.11) \times 10^9$</td>
<td>$(1.82 \pm 0.17) \times 10^9$</td>
<td>$(1.04 \pm 0.11) \times 10^9$</td>
<td>$(1.65 \pm 0.08) \times 10^9$</td>
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<td>0.020</td>
<td>$(7.85 \pm 0.48) \times 10^8$</td>
<td>$(8.01 \pm 0.53) \times 10^8$</td>
<td>$(1.49 \pm 0.40) \times 10^8$</td>
<td>$(8.56 \pm 1.00) \times 10^8$</td>
</tr>
<tr>
<td>0.040</td>
<td>$(6.90 \pm 0.70) \times 10^7$</td>
<td>$(6.75 \pm 1.50) \times 10^7$</td>
<td>$(1.97 \pm 0.40) \times 10^7$</td>
<td>$(3.83 \pm 2.00) \times 10^7$</td>
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<tr>
<td>0.060</td>
<td>$(8.00 \pm 3.50) \times 10^6$</td>
<td>$(6.75 \pm 1.50) \times 10^7$</td>
<td>$(1.97 \pm 0.40) \times 10^7$</td>
<td>$(3.83 \pm 2.00) \times 10^7$</td>
</tr>
<tr>
<td>0.10</td>
<td>$(2.08 \pm 0.57) \times 10^8$</td>
<td>$(1.39 \pm 0.34) \times 10^8$</td>
<td>$(3.34 \pm 0.28) \times 10^7$</td>
<td>$(3.83 \pm 2.00) \times 10^7$</td>
</tr>
<tr>
<td>0.14</td>
<td>$(3.88 \pm 0.56) \times 10^6$</td>
<td>$(1.13 \pm 0.75) \times 10^7$</td>
<td>$(3.28 \pm 0.28) \times 10^7$</td>
<td>$(3.83 \pm 2.00) \times 10^7$</td>
</tr>
<tr>
<td>0.20</td>
<td>$(4.02 \pm 0.82) \times 10^6$</td>
<td>$(5.44 \pm 1.70) \times 10^7$</td>
<td>$(3.28 \pm 0.28) \times 10^7$</td>
<td>$(3.83 \pm 2.00) \times 10^7$</td>
</tr>
<tr>
<td>0.30</td>
<td>$(2.54 \pm 0.48) \times 10^6$</td>
<td>$(2.19 \pm 0.14) \times 10^7$</td>
<td>$(3.83 \pm 2.00) \times 10^7$</td>
<td>$(3.83 \pm 2.00) \times 10^7$</td>
</tr>
<tr>
<td>0.40</td>
<td>$(9.01 \pm 0.92) \times 10^7$</td>
<td>$(2.71 \pm 0.22) \times 10^8$</td>
<td>$(3.35 \pm 0.13) \times 10^9$</td>
<td>$(4.75 \pm 0.34) \times 10^7$</td>
</tr>
<tr>
<td>0.50</td>
<td>$(4.90 \pm 0.37) \times 10^7$</td>
<td>$(1.64 \pm 0.07) \times 10^7$</td>
<td>$(2.78 \pm 1.10) \times 10^6$</td>
<td>$(3.45 \pm 0.21) \times 10^6$</td>
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<tr>
<td>0.60</td>
<td>$(8.88 \pm 0.63) \times 10^6$</td>
<td>$(4.73 \pm 0.25) \times 10^6$</td>
<td>$(1.10 \pm 0.08) \times 10^6$</td>
<td>$(1.27 \pm 0.03) \times 10^6$</td>
</tr>
<tr>
<td>0.80</td>
<td>$(1.21 \pm 0.10) \times 10^7$</td>
<td>$(2.48 \pm 0.19) \times 10^7$</td>
<td>$(1.78 \pm 0.17) \times 10^7$</td>
<td>$(5.61 \pm 0.39) \times 10^7$</td>
</tr>
</tbody>
</table>

Conditions: buffer H, pH 8.1, 25°C.
Figure 6. Salt dependence of $\Delta H_{obs}$ for SSB tetramer binding to t(t(pT))$_{69}$ and t(t(pT))$_{34}$. The total calorigraphic $\Delta H_{obs}$ for saturating all four SSB subunits with one molecule of dT(t(pT))$_{69}$ (filled symbols) or two molecules of dT(t(pT))$_{34}$ (open symbols, total $\Delta H_{tot,obs} = \Delta H_{obs,1} + \Delta H_{obs,2}$) in buffer H (pH 8.1), 25°C; (●, ○) NaCl, (▲, △) NaBr, (■, □) NaF, (○) ChCl; (●) estimate of $\Delta H_{obs}$ from van’t Hoff analysis of fluorescence titrations for SSB-dT(pT)$_{69}$ binding in 2.0 and 2.4 M NaBr (Ferrari & Lohman, 1994). ITC data from one titration in 0.2 M LiCl (+) for SSB binding to t(t(pT))$_{69}$ ($\Delta H_{obs} = \Delta H_{obs,1} + \Delta H_{obs,2} = -69.9(\pm 0.3) \text{ kcal/mol} + -74.4(\pm 0.3) \text{ kcal/mol} = -144.3(\pm 0.4) \text{ kcal/mol}$).

concentration, with $\Delta H_{obs,tot} = -150(\pm 5) \text{ kcal/mol}$. In fact, $\Delta H_{obs,tot}$ is essentially independent of [NaF] from 10 mM to 0.6 M. In NaCl and NaBr $\Delta H_{obs,tot}$ reaches $-150(\pm 5) \text{ kcal/mol}$ below 0.10 M salt concentrations. Therefore, the most dramatic effects of salt concentration and anion type occur at salt concentrations $> 0.10 \text{ M}$, suggesting that these effects result from relatively weak interactions of anions with the SSB protein.

Individual values of $\Delta H_{obs,1}^{35}$ and $\Delta H_{obs,2}^{35}$ for binding the first and second molecules of dT(t(pT))$_{69}$, respectively, to the SSB tetramer can also be resolved at all salt concentrations investigated (see Table 3), although the values of $\Delta H_{obs,2}^{35}$ have larger uncertainties at both high and low salt concentrations, where binding to the second site is weakest. As shown in Figure 7, the dependences of $\Delta H_{obs,1}^{35}$ and $\Delta H_{obs,2}^{35}$ on both salt concentration and type are quite different. In general $\Delta H_{obs,1}^{35}$ increases with increasing salt concentration, whereas $\Delta H_{obs,2}^{35}$ decreases and then increases, showing a minimum at $\approx 60 \text{ mM}$ in both NaCl and ChCl. We cannot say whether a similar minimum in $\Delta H_{obs,2}^{35}$ occurs in NaBr, since experiments were not performed at [NaBr] $< 0.4 \text{ M}$. In all salts, the absolute magnitude of $\Delta H_{obs,1}^{35}$ is always less than $\Delta H_{obs,2}^{35}$, up to approximately 0.5 M, whereas above this salt concentration the situation is reversed and $|\Delta H_{obs,1}^{35}| > |\Delta H_{obs,2}^{35}|$. We also note that $\Delta H_{obs,1}^{35}$ and $\Delta H_{obs,2}^{35}$ appear to converge to the same value (≈ 70(± 5) kcal/mol) in the limit of low salt.

From the data in Figure 7, we see that the influence of anions on both $\Delta H_{obs,1}^{35}$ and $\Delta H_{obs,2}^{35}$ increases with increasing salt concentration, whereas cation-specific effects are apparent mainly at lower salt concentrations. As observed for the SSB-dT(t(pT))$_{69}$ interaction the absolute values of $\Delta H_{obs,1}^{35}$ and $\Delta H_{obs,2}^{35}$ decrease more steeply as a function of [NaBr], suggesting that the associated release of Br$^-$ from the protein upon DNA binding results in a larger positive contribution to the overall binding enthalpy than does the release of Cl$^-$.

<table>
<thead>
<tr>
<th>Salt [M]</th>
<th>$\Delta H_{1}$ (NaF)</th>
<th>$\Delta H_{2}$ (NaCl)</th>
<th>$\Delta H_{1}$ (NaBr)</th>
<th>$\Delta H_{2}$ (CholineCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.010</td>
<td>-74.0(±1.4)</td>
<td>-72.4(±12)</td>
<td>-69.9(±0.9)</td>
<td>-71.7(±7.9)</td>
</tr>
<tr>
<td>0.014</td>
<td>-74.3(±0.5)</td>
<td>-85.2(±2.6)</td>
<td>-75.1(±0.6)</td>
<td>-68.8(±2.2)</td>
</tr>
<tr>
<td>0.020</td>
<td>-75.2(±0.5)</td>
<td>-71.9(±1.3)</td>
<td>-72.1(±0.6)</td>
<td>-74.3(±1.3)</td>
</tr>
<tr>
<td>0.028</td>
<td>-74.4(±0.6)</td>
<td>-77.1(±1.2)</td>
<td>-71.1(±0.5)</td>
<td>-78.7(±0.9)</td>
</tr>
<tr>
<td>0.040</td>
<td>-76.1(±1.5)</td>
<td>-87.8(±1.8)</td>
<td>-69.5(±0.7)</td>
<td>-81.5(±0.9)</td>
</tr>
<tr>
<td>0.060</td>
<td>-79.0(±2.0)</td>
<td>-81.0(±2.6)</td>
<td>-70.2(±2.3)</td>
<td>-83.9(±2.9)</td>
</tr>
<tr>
<td>0.10</td>
<td>-78.5(±2.5)</td>
<td>-74.0(±3.7)</td>
<td>-67.1(±0.5)</td>
<td>-82.3(±0.6)</td>
</tr>
<tr>
<td>0.14</td>
<td>-77.5(±2.9)</td>
<td>-75.1(±4.4)</td>
<td>-67.1(±0.5)</td>
<td>-82.3(±0.6)</td>
</tr>
<tr>
<td>0.20</td>
<td>-66.2(±1.7)</td>
<td>-82.7(±2.4)</td>
<td>-62.6(±1.1)</td>
<td>-74.6(±1.3)</td>
</tr>
<tr>
<td>0.30</td>
<td>-65.3(±0.7)</td>
<td>-76.9(±0.8)</td>
<td>-60.8(±0.5)</td>
<td>-67.0(±0.8)</td>
</tr>
<tr>
<td>0.40</td>
<td>-63.3(±1.1)</td>
<td>-70.2(±1.2)</td>
<td>-60.1(±0.5)</td>
<td>-66.7(±0.8)</td>
</tr>
<tr>
<td>0.50</td>
<td>-62.1(±1.2)</td>
<td>-64.0(±1.3)</td>
<td>-56.4(±0.3)</td>
<td>-61.5(±0.8)</td>
</tr>
<tr>
<td>0.60</td>
<td>-54.3(±0.2)</td>
<td>-55.0(±0.8)</td>
<td>-64.8(±0.4)</td>
<td>-59.4(±1.1)</td>
</tr>
<tr>
<td>0.80</td>
<td>-52.1(±0.4)</td>
<td>-45.1(±1.2)</td>
<td>-61.2(±0.2)</td>
<td>-57.5(±0.6)</td>
</tr>
<tr>
<td>1.0</td>
<td>-58.4(±0.6)</td>
<td>-53.7(±0.9)</td>
<td>-52.4(±0.8)</td>
<td>-45.8(±3.1)</td>
</tr>
</tbody>
</table>

Conditions: buffer H, pH 8.1, 25°C.
Anion Effects on SSB Protein–DNA Binding Enthalpy

The effects of cations (Figure 7(b)) seem to be more complex; the value of $\Delta H_{\text{obs},1}$ is larger in magnitude in CHCl than in NaCl, whereas $\Delta H_{\text{obs},2}$ is slightly lower. However, this difference becomes insignificant at salt concentrations close to 1 M.

ITC measurements of dT(pT)$_{34}$ binding to SSB were also performed in 0.2 M LiCl. Although binding affinities were too high to measure, we could measure $\Delta H_{\text{obs},1} = -144.3(\pm 0.4)$ kcal/mol, with $\Delta H_{\text{obs},1} = -69.9(\pm 0.3)$ kcal/mol and $\Delta H_{\text{obs},2} = -74.4(\pm 0.3)$ kcal/mol. These values differ only slightly from those measured at 0.2 M NaCl and choline chloride, indicating no major effect of cation type.

Thermodynamics of SSB binding to dT(pT)$_{69}$ in NaBr

The affinity of the SSB-dT(pT)$_{69}$ interaction is lower in the presence of NaBr than at the equivalent [NaCl], so that in the range from 1.0 to 2.0 M NaBr (25.0°C, pH 8.1), $K_{\text{obs}}$ and thus $\Delta G_{\text{obs}}$ and $\Delta S_{\text{obs}}$ as well as $\Delta H_{\text{obs}}$ can be determined accurately by ITC. These values are given in Table 4 and also plotted in Figure 8. Over the range of [NaBr] investigated, both $T \Delta S_{\text{obs}}$ and $\Delta H_{\text{obs}}$ are negative and increase (becoming less negative) with increasing [NaBr]. Therefore, binding is enthalpically driven with a large and unfavorable entropic contribution. Since $\Delta H_{\text{obs}}$ and $\Delta S_{\text{obs}}$ are of the same sign and have a similar dependence on [NaBr], the free energy change ($\Delta G_{\text{obs}}$) is smaller in magnitude (of the order $-10$ kcal/mol) and also displays a much smaller dependence on [NaBr]. However, the value of $\partial \log K_{\text{obs}}/\partial \log [\text{NaBr}] = -7.4(\pm 0.3)$ is still quite large and indicates that a net release of seven to eight ions accompanies complex formation.

NaCl concentration effects on the circular dichroism spectra of complexes of SSB with dT(pT)$_{69}$

The large effects of salt concentration and type on $\Delta H_{\text{obs}}$ for SSB binding to dT(pT)$_{34}$ and dT(pT)$_{69}$ could have contributions from a salt-induced conformational transition within the SSB tetramer. To investigate this possibility we examined the [NaCl] dependencies of the circular dichroism (CD) spectra of SSB protein alone and in a 1:1 complex with dT(pT)$_{69}$. Previous studies have used CD to probe SSB-DNA complexes (Anderson & Coleman, 1975), although the emphasis was on the spectral properties of the nucleic acid. CD spectra of SSB protein (buffer H, pH 8.1, 25°C) at 0.01, 0.2 and 1 M NaCl are shown in Figure 9(a). The spectra have two negative bands, one in the near UV with a minimum at 280 nm and another in the far UV with a minimum at 208 nm. These spectra agree well with those reported by Anderson & Coleman (1975), after recalculation of the ellipticity per mole of SSB monomer. The spectra presented in Figure 9(a) show very little effect of [NaCl], with only a slight increase in the absolute value of the molar ellipticity at 280 nm with decreasing [NaCl].

We next examined the effect of [NaCl] on the CD spectrum of SSB in a 1:1 complex with dT(pT)$_{69}$. However, we restricted our studies to [NaCl] $\geq 10$ mM, where 1:1 complexes are formed exclusively (Bujalowski & Lohman, 1989b). The CD spectra of SSB protein alone, dT(pT)$_{69}$ alone, and their 1:1 complexes are shown in Figure 9(b). The molar ellipticity of the free protein is small in comparison with that of free dT(pT)$_{69}$ in the near UV region. Previous studies of proteins bound to ssDNA have shown that the spectral contribution of the DNA generally dominates the CD spectrum of the complex in the near UV region (Scheerhagen et al., 1986; Powell & Gray, 1993; van Amerongen et al., 1987). Therefore the difference between the CD spectra of free DNA and of protein-DNA complex should be dominated by any changes in DNA conformation occurring upon complex formation. In fact, Figure 9(b) shows that the spectrum of the SSB-dT(pT)$_{69}$ complex differs from that of free
Fluorescence and ITC data

The CD spectrum of the SSB-dT(pT)_{69} complex is unaffected by [NaCl]. Therefore, CD experiments show no evidence for significant effects of [NaCl] on the conformation of free SSB protein or dT(pT)_{69} within the SSB complex. However, it is more difficult to comment on changes to the SSB protein bound to DNA.

**Discussion**

Studies of the stability and specificity of protein–nucleic acid interactions have shown that a dominant effect of salt concentration is due to the polyelectrolyte nature of the nucleic acid. The fact that the nucleic acid is a highly negatively charged linear polymer results in delocalized binding of cations to the nucleic acid. These cations are then released into solution upon binding of a protein or other ligand. This polyelectrolyte effect is exclusively due to cation–DNA interactions and has been shown to be entirely entropic in nature for simple positively charged oligopeptides (Mascotti & Lohman, 1992, 1993, 1997; Lohman & Mascotti, 1996). Although such effects have not been explored for other protein–nucleic acid systems, it seems unlikely that such effects are unique to SSB protein.

Our previous van’t Hoff studies demonstrating a linkage between salt and temperature effects and indicating that \( \Delta H_{\text{obs}} \) is salt-dependent were per-

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**Table 4. Effect of NaBr concentration on thermodynamic parameters for SSB-dT(pT)_{69} binding**

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>[NaBr] (M)</th>
<th>( K_{\text{obs}} ) (M^{-1})</th>
<th>( \frac{\Delta \log K_{\text{obs}}}{\Delta \log [\text{NaBr}]} )</th>
<th>( n )</th>
<th>( \Delta H_{\text{obs}} ) (kcal/mol)</th>
<th>( \Delta G_{\text{obs}} ) (kcal/mol)</th>
<th>( \Delta S_{\text{obs}} ) (cal/mol K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITC data</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.0</td>
<td>0.17</td>
<td>8.5(±1.5) \times 10^{8}</td>
<td>0.99</td>
<td>124(±1.0)</td>
<td>-130.3(±1.2)</td>
<td>-124.5(±1.0)</td>
<td>-107.0(±1.0)</td>
</tr>
<tr>
<td>25.4</td>
<td>0.25</td>
<td>9.5(±0.6) \times 10^{7}</td>
<td>1.02</td>
<td>128(±1.5)</td>
<td>-95.2(±0.5)</td>
<td>-85.0(±1.5)</td>
<td>-80.8(±0.4)</td>
</tr>
<tr>
<td>25.2</td>
<td>0.6</td>
<td>7.4(±0.5) \times 10^{6}</td>
<td>1.03</td>
<td>130(±1.0)</td>
<td>-83.8(±1.1)</td>
<td>-10.3(±0.0)</td>
<td>-9.1(±0.0)</td>
</tr>
<tr>
<td>25.0</td>
<td>1.0</td>
<td>5.6(±0.4) \times 10^{5}</td>
<td>0.98</td>
<td>126(±1.5)</td>
<td>-71.0(±0.9)</td>
<td>-248.5(±1.4)</td>
<td>-204.7(±0.0)</td>
</tr>
<tr>
<td>Fluorescence data</td>
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<td>-11.1</td>
<td>-10.9</td>
</tr>
<tr>
<td>25.0</td>
<td>1.3</td>
<td>1.3(±0.6) \times 10^{4}</td>
<td>1.00</td>
<td>10.9</td>
<td>-9.0</td>
<td>-9.0</td>
<td></td>
</tr>
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</tr>
<tr>
<td>25.0</td>
<td>2.0</td>
<td>4.0(±0.3) \times 10^{2}</td>
<td>1.00</td>
<td>8.9</td>
<td>8.9</td>
<td>8.9</td>
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</tr>
<tr>
<td>25.0</td>
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<td>2.0(±0.0) \times 10^{1}</td>
<td>-7.1(±0.5)</td>
<td>8.9</td>
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</tr>
<tr>
<td>25.0</td>
<td>2.0</td>
<td>1.3(±0.01) \times 10^{0}</td>
<td>-7.1(±0.5)</td>
<td>8.9</td>
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<tr>
<td>25.0</td>
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<td>-7.1(±0.5)</td>
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<tr>
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<td>2.4(±0.01) \times 10^{-1}</td>
<td>-7.1(±0.5)</td>
<td>8.9</td>
<td>8.9</td>
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</tr>
</tbody>
</table>

**Figure 8.** Dependence on NaBr concentration of the thermodynamic parameters for the binding of dT(pT)_{69} to the SSB tetramer. Thermodynamic parameters (\( \Delta H_{\text{obs}} \), \( \Delta G_{\text{obs}} \), \( \Delta S_{\text{obs}} \)) were determined by isothermal titration calorimetry (buffer H, pH 8.1, 25°C).
formed for SSB binding to poly(U) in its (SSB)$_{65}$ binding mode (Lohman et al., 1996). Upon binding to ss-polynucleotides in this mode, the SSB tetramer can form cooperative inter-tetramer interactions of the “limited” type (Bujalowski & Lohman, 1987; Overman et al., 1988). As a result, the energetic contributions from these cooperative interactions had to be resolved from the intrinsic protein–nucleic acid binding. Furthermore, in order to insure that the effects of salt were on the intrinsic protein–nucleic acid interaction, we had to restrict our studies to salt concentrations >0.2 M, since other SSB-polynucleotide binding modes can become populated at lower salt concentrations (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988). However, an effect of [NaCl] on $\Delta H_{\text{obs}}$ was also confirmed by direct isothermal titration calorimetric (ITC) studies using dT(pT)$_{69}$. All of the studies reported here used either dT(pT)$_{69}$ or dT(pT)$_{34}$ in order to eliminate these complexities. In addition, $\Delta H_{\text{obs}}$ was measured directly using ITC over a wider range of salt concentrations (10 mM to 2.0 M) and types (NaCl, NaBr, NaF, LiCl and choline chloride).

It is also important to emphasize that we have also determined that the SSB tetramer is the stable species present under all of the solution conditions examined in this study and thus no assembly/disassembly processes are linked to DNA binding. Our use of homo-oligodeoxynucleotides, containing only thymidine also insures that no intramolecular base-pairing occurs within the ssDNA. For these and others reasons discussed previously (Lohman et al., 1996) we are confident that the salt-dependent $\Delta H_{\text{obs}}$ reported here reflects an intrinsic property of the SSB–ssDNA interaction.

The major results of the current study are summarized in Figure 6, which shows the dependence of $\Delta H_{\text{obs}}$ on the concentrations of NaBr, NaCl and NaF for the equilibrium binding of one molecule of dT(pT)$_{69}$ or two molecules of dT(pT)$_{34}$ per SSB tetramer. At salt concentrations below...
0.10 M (25.0°C, pH 8.1), a very large $\Delta H_{\text{obs}}$ ($\approx -150(\pm 5)$ kcal/mol) is measured for saturating all subunits of the SSB tetramer with oligodeoxythymidylates, which is nearly independent of salt type and concentration. The possible origins of this extremely large $\Delta H_{\text{obs}}$ are discussed below. Within our experimental uncertainty, this value of $\Delta H_{\text{obs}}$ is essentially unaffected by [NaF] up to 0.60 M, the highest [NaF] examined. However, upon raising either the [NaCl] or [NaBr] above 0.10 M, $\Delta H_{\text{obs}}$ increases (becoming less negative) continuously up to the highest salt concentrations examined (2.0 M). Furthermore, the salt dependence of $\Delta H_{\text{obs}}$ is steeper in [NaBr] than in [NaCl]. The lowest value of $\Delta H_{\text{obs}} = -70(\pm 1)$ kcal/mol is measured in the presence of 2.0 M NaBr, whereas in 2.0 M NaCl, $\Delta H_{\text{obs}} = -94(\pm 1)$ kcal/mol. Thus, the major effect of salt concentration on $\Delta H_{\text{obs}}$ occurs above 0.1 M, does not plateau at high salt concentration and is primarily an effect of anions, following the Hofmeister series (von Hippel & Schleich, 1969; Collins, 1995, 1997; Baldwin, 1996). This suggests that the effects are due to weak preferential interactions of anions with the SSB protein and that release of anions from the protein makes a positive enthalpic contribution to the overall $\Delta H_{\text{obs}}$ (Lohman et al., 1996). However, we have also obtained evidence for cation-specific effects on $\Delta H_{\text{obs}}$ at lower salt concentrations.

The values of $\Delta H_{\text{obs}}$ measured at fixed salt concentration are dependent only on the anion type. In the presence of three chloride salts (0.2 M LiCl, NaCl and choline chloride) we measure the same $\Delta H_{\text{obs}} = -142(\pm 2)$ kcal/mol, even though all three cations (Li+, Na+, choline) display very different properties in aqueous solution. According to Collins (Collins, 1995, 1997; Collins & Washabaugh, 1985), Li+ is a strong kosmotrope (water structure maker), Na+ is only a marginal kosmotrope and choline ($\text{CH}_3\text{CH}_2\text{N}^+\text{(CH}_3\text{CH}_2\text{OH})\text{O}^-$) is a strong chaotrope, comparable to $\text{K}^+$ and $\text{NH}_4^+$. The absence of any difference in $\Delta H_{\text{obs}}$ for SSB-ssDNA binding among these cations suggests that the weak preferential binding of these cations to SSB does not occur. However, in order to confirm these conclusions, additional experiments need to be performed at higher salt concentrations. These results also provide further indication that the effects of salt on $\Delta H_{\text{obs}}$ do not reflect cation binding to the DNA.

Based on our previous results (Lohman et al., 1996), we considered the following possibilities to explain the effect of [NaCl] on $\Delta H_{\text{obs}}$ for the SSB–nucleic acid interaction. (1) Site binding of anions (Cl– and Br–) to the protein, occurring with $\Delta H < 0$. (2) Site binding of anions to the protein that requires protonation of sites on the protein, with an accompanying $\Delta H > 0$ for protonation. (3) Anion binding that is coupled to a protein conformational change with $\Delta H < 0$, or (4) weak, preferential binding of anions to the protein, occurring with $\Delta H < 0$, and resulting in a salt-dependent accumulation of anions in the vicinity of the protein.

The fact that the largest effects of salt on $\Delta H_{\text{obs}}$ occur at high anion concentrations and do not appear to saturate with respect to salt concentration rules out a major role for site-specific binding of anions to the SSB protein. Rather, the results suggest that weak, preferential binding of anions to SSB is responsible, although some contribution from coupling of protonation to anion binding may also occur. We have previously shown that some binding of Cl– to SSB is coupled to protonation, since net Cl– release increases with decreasing pH (Overman & Lohman, 1994). In fact, there is a significant effect of pH on $\Delta H_{\text{obs}}$ such that $\Delta H_{\text{obs}}$ becomes less negative with decreasing pH (unpublished data). However, we have performed these experiments at only one salt concentration (0.2 M NaBr, 25.0°C), whereas the salt dependence of $\Delta H_{\text{obs}}$ needs to be examined as a function of pH in order to determine the contribution due to coupling of protonation to anion binding.

The large salt effects on $\Delta H_{\text{obs}}$ may also have contributions from the coupling of anion binding to a protein conformational transition. Although, we were not able to detect NaCl-dependent conformational changes within the SSB tetramer using CD spectroscopy, we cannot rule out this possibility. Salt effects on SSB protein conformation are likely to be important at low salt concentrations, where cation type does effect $\Delta H_{\text{obs}}$. Bujalowski & Lohman (1989b) have previously shown that cation uptake accompanies the binding of ssDNA to the third and fourth subunits of the SSB tetramer and have suggested that this results from an allosteric effect of cation binding on the structure of the SSB tetramer. Therefore, changes in cation concentration in this low salt region may modulate the fraction of SSB in a particular conformation and thus the resulting overall $\Delta H_{\text{obs}}$ if the two SSB conformations bind ssDNA with different values of $\Delta H$. This is a subject for further investigation.

Weak ion–macromolecule interactions of the type observed here at high salt concentrations are often described as Hofmeister effects (Hofmeister, 1888) and the possible molecular basis for such weak anion–protein interactions has been discussed, although this is still the subject of some debate (von Hippel & Schleich, 1969; Record et al., 1978; Collins & Washabaugh, 1985; Collins, 1995, 1997). Regardless of molecular basis, the studies presented here, as well as our previous results (Lohman et al., 1996), indicate that such anion–protein interactions decrease with increasing temperature and that the temperature dependence increases in the order $\text{F}^- < \text{Cl}^- < \text{Br}^-$. Interestingly, model studies of the interaction of anions (relative to water) with Sephadex G-10, which has been used to mimic these weak interactions, show the same ordering of the temperature dependences.
of these interactions, with $I^- > Br^- > Cl^- > F^- \ (Washabaugh \& Collins, 1986)$.

**Thermodynamics of SSB-dT(pT)$_{69}$ binding**

The experiments reported here have focused on the salt dependence of $\Delta H_{\text{obs}}$ for SSB binding to ssDNA. In fact, under most of the conditions used in these studies, accurate measurements can only be made of $\Delta H_{\text{obs}}$ since the binding affinity of SSB for dT(pT)$_{69}$ is too high to estimate $\Delta G_{\text{obs}}$ and thus $\Delta S_{\text{obs}}$. However, under a limited set of solution conditions (1 to 2 M NaBr, pH 8.1, 25 °C), we have been able to determine accurately all three thermodynamic quantities and thus can comment on the thermodynamic profile for SSB tetramer binding to dT(pT)$_{69}$. In general, binding is enthalpically driven ($\Delta H_{\text{obs}} < T \Delta S_{\text{obs}}$), with $\Delta H_{\text{obs}}$ being considerably more negative than has been reported for any other protein–nucleic acid system, whether specific or non-specific. However, the net entropic contribution to binding ($\Delta S^*$) is also large and negative. Although there is a favorable entropic contribution to binding due to the release of monovalent cations from the ssDNA, this does not dominate the overall $\Delta S_{\text{obs}}$. One factor that might contribute to the large unfavorable $\Delta S_{\text{obs}}$ may be the loss of conformational entropy of the ssDNA, which should be much more flexible when free in solution than when bound to the SSB tetramer. However, studies of the temperature dependence of $\Delta H_{\text{obs}}$ will be needed to more completely assess the relative enthalpic and entropic contributions to the overall binding interaction.

**Possible origins of the large negative $\Delta H_{\text{obs}}$ for SSB binding to ssDNA**

Independent of the large effect of salt concentration on $\Delta H_{\text{obs}}$, our studies show that SSB binding to ss-nucleic acids is accompanied by an extremely large and negative $\Delta H_{\text{obs}}$ at all salt concentrations. The largest value of $\Delta H_{\text{obs}} = -150(\pm 5)$ kcal/mol is obtained at monovalent salt concentrations $\leq 0.1$ M. This is nearly a factor of 5 larger than any value of $\Delta H_{\text{obs}}$ reported for any other protein–nucleic acid interaction, either specific or non-specific (de Haseth et al., 1977; Ha et al., 1989; Jin et al., 1993; Ladbury et al., 1994; Merabet & Ackers, 1995). Although we are not currently able to identify each of the factors that might contribute to such a large negative $\Delta H_{\text{obs}}$, it is worth considering some of the likely factors.

Major contributions to the large, negative $\Delta H_{\text{obs}}$ likely come from a number of aromatic amino acids that seem to be involved in stacking interactions with nucleic acid bases. There is evidence that at least three tryptophan residues (Trp40, Trp54 and Trp88) and one phenylalanine (Phe60) per SSB subunit interact with the ssDNA bases (Curth et al., 1993; Khamis et al., 1987; Casas-Finet et al., 1987). In fact, the recent crystal structure of the chymotryptic DNA binding core of the SSB protein (Raghunathan et al., 1997) shows that these residues are clustered within a region in each SSB subunit that likely forms part of the ssDNA binding site. Thermodynamic studies of ss-nucleic acid binding to simple oligolysine peptides containing tryptophan indicate that each Trp can contribute approximately $\sim -2$ to $-3$ kcal/mol (Mascotti & Lohman, 1992, 1993) to the binding enthalpy. Therefore, a total of 12 aromatic amino acids could potentially contribute as much as $-24$ to $-36$ kcal/mol to the overall $\Delta H_{\text{obs}}$, assuming these effects are additive. In addition, although lysine–phosphate interactions contribute only $\sim -0.5$ kcal/mol to $\Delta H_{\text{obs}}$, arginine–phosphate interactions contribute a significantly larger negative $\Delta H$, as much as $-2$ kcal/mol more than do lysine–phosphate interactions (Mascotti & Lohman, 1997). Any coupling of protonation of the protein to nucleic acid binding, which is known to occur for SSB (Overman & Lohman, 1994), will also contribute a large negative $\Delta H$ (at high pH), as shown for E. coli lac repressor binding non-specifically to duplex DNA (de Haseth et al., 1977). These interactions alone can be responsible for a substantial negative $\Delta H_{\text{obs}}$ for SSB binding, approaching $-50$ kcal/mol (SSB tetramer). However, other factors including conformational changes (Spolar & Record, 1994), may also contribute to produce values as large as $-150$ kcal/mol.

As is the case for the E. coli SSB protein, many other protein–ss-nucleic acid interactions seem to involve stacking interactions between nucleic acid bases and aromatic amino acids (Korolev et al., 1997; Oubridge et al., 1994; Shamoo et al., 1995; Bochkarev et al., 1997). Therefore, we suggest that these and other proteins that bind to single-stranded nucleic acids will generally display larger negative values of $\Delta H_{\text{obs}}$ than observed for protein–duplex DNA interactions due to contributions from stacking interactions between nucleic acid bases and aromatic amino acids. Unfortunately, not many measurements of $\Delta H_{\text{obs}}$ have been reported for other protein–ss-nucleic acid interactions. Furthermore, although the few that have been studied do not show extremely large negative values of $\Delta H_{\text{obs}}$ (Kowalczykowski et al., 1981; Alma et al., 1983; Bulsink et al., 1985), this may be due to the fact that these other studies were performed using homopolyadenylates or oligoadenylates, which undergo substantial base stacking. As we have discussed previously (Ferrari & Lohman, 1994), the overall $\Delta H_{\text{obs}}$ for proteins binding to ss-polyadenylates will generally be less negative than for other homopolynucleotides, since unstacking of the adenylates generally accompanies protein binding and the unstacking of the adenylates will make a positive contribution to the overall $\Delta H_{\text{obs}}$ for binding. Other protein–ss-nucleic acid binding systems need to be examined in more detail to test this proposal.

The large effects of salt concentration on $\Delta H_{\text{obs}}$ reported here for SSB–ssDNA interactions are unprecedented, although we emphasize that few
protein–nucleic acid interacting systems have been examined with these effects in mind. Therefore, there is a need to examine the effects of salt concentration on $\Delta H_{\text{obs}}$ for other protein–nucleic acid systems, especially those interacting with ss-nucleic acids, in order to determine the generality of the effects reported here. However, the potential of such large salt effects on $\Delta H_{\text{obs}}$ needs to be considered when attempting to understand the molecular bases of thermodynamic profiles for protein–nucleic acid interactions, since these effects can be substantial.

Materials and Methods

Reagents and buffers

All solutions were prepared with reagent grade chemicals and distilled water that was subsequently treated with a Milli Q (Millipore, Bedford, MA) water purification system. Highly hygroscopic choline chloride (ChCl–[2-hydroxyethyl] trimethyl-ammonium, chloride salt) was dried under vacuum prior to use. Buffer T is 10 mM Tris (tris(hydroxymethyl) aminomethane), 0.1 mM Na$_3$EDTA (ethylenediaminetetraacetic acid), pH 8.1. All titrations were performed in buffer H (10 mM Hepes (4-(hydroxyethyl)-1-piperazineethanesulfonic acid), 0.1 mM Na$_3$EDTA), pH 8.1. Buffers were prepared by mixing the appropriate volumes of the free acid (1.0 M) and base (1.0 M) forms of Hepes or Tris to obtain the appropriate pH, such that upon dilution to 10 mM yields pH 8.1 at the indicated salt concentration as verified by direct measurements of the final pH. The contribution to the overall [Na$^+$] from the sodium salt of Hepes is approximately 8 mM.

E. coli SSB protein and nucleic acids

SSB protein was purified as described (Lohman et al., 1986a) with the addition of a double-stranded DNA-cellulose column to remove a minor exonuclease contaminant (Bujalowski & Lohman, 1991). SSB protein concentration was determined spectrophotometrically in buffer T, 0.20 M NaCl using an extinction coefficient of $\varepsilon_{260} = 1.13 \times 10^5$ M$^{-1}$ (tetramer) cm$^{-1}$ (Lohman & Overman, 1985). The SSB tetramer is stable at all protein concentrations and NaCl, NaBr and NaF concentrations used in this study (Overman et al., 1988; Ferrari & Lohman, 1994). The oligodeoxynucleotides, dT(pT)$_{34}$ and dT(pT)$_{69}$, were synthesized and purified as described (Ferrari et al., 1994) and were $\geq 98\%$ pure as judged by denaturing gel electrophoresis and autoradiography of a sample that was 5’ end-labeled with $^{32}$P using polyuridine kinase. DNA concentrations were determined spectrophotometrically in buffer T, 0.1 M NaCl using an extinction coefficient of $\varepsilon_{260} = 8.1 \times 10^4$ M$^{-1}$ (nucleotide) cm$^{-1}$. DNA and SSB samples were dialyzed extensively against buffer H at the indicated salt concentration for use in ITC experiments.

Isothermal titration calorimetry (ITC)

ITC experiments were performed using an OMEGA titration microcalorimeter (MicroCal Inc., Northampton, MA; Wiseman et al., 1989). For the SSB-dT(pT)$_{69}$ interaction we have shown previously that $\Delta H_{\text{obs}}$ is independent of the protein and DNA concentrations and the order of titration (Lohman et al., 1996). Generally, experiments were carried out by titrating SSB solutions (1 to 2 $\mu$M tetramer) with oligodeoxynucleotide (generally stock concentrations ranging from 20 to 80 $\mu$M for dT(pT)$_{34}$ and dT(pT)$_{69}$). However, lower concentrations of SSB protein (0.1 to 1 $\mu$M tetramer) had to be used in the experiments in NaF due to the lower protein solubility in NaF, which also decreases with increasing [NaF]. The heats of dilution obtained from reference titrations of DNA into buffer were independent of DNA concentration at all salt conditions and temperatures. All corrections for heats of dilution were applied as previously described without correcting for the heats of buffer ionization, since these are negligible within our experimental uncertainty, especially in Hepes buffer, due to the very large values of $\Delta H_{\text{obs}}$ for SSB binding to ssDNA (Lohman et al., 1996).

Analysis of ITC data for SSB binding to dT(pT)$_{69}$

The affinity of SSB tetramer for dT(pT)$_{69}$ is too high to be measured accurately by ITC under most of the solution conditions used in our studies. Only stoichiometric binding (one tetramer per dT(pT)$_{69}$) was observed at all [NaCl] studied (0.17 to 2.0 M) and all [NaBr] < 1 M. Therefore, under these conditions only $\Delta H_{\text{obs}}$ can be measured accurately as described (Lohman et al., 1996). At [NaBr] > 1 M the affinity of SSB for dT(pT)$_{69}$ is lowered sufficiently so that an equilibrium binding constant ($K_{\text{eq}}$) can be measured and thus determinations of $\Delta G_{\text{obs}}$ and $\Delta S_{\text{obs}}$ can also be obtained from the standard relationships $\Delta G_{\text{obs}} = -RT \ln K_{\text{eq}} = \Delta H_{\text{obs}} - T \Delta S_{\text{obs}}$. Values of $\Delta H_{\text{obs}}$ and $K_{\text{eq}}$ for SSB tetramer binding to dT(pT)$_{69}$ were obtained by fitting the experimental titration curve to a model for a 1:1 interaction. For this model, the total heat after the $i$th injection ($Q_{t}^i$) is given by equation (1):

$$Q_{t}^i = \frac{nM_{\text{obs}}^{i} \Delta H_{o}^{i}}{2} \left[ 1 + \frac{X_{\text{tot}}^{i} \cdot \frac{1}{nKM_{\text{obs}}^{i} + \frac{1}{nKM_{\text{obs}}^{i}}} \sqrt{1 + \frac{X_{\text{tot}}^{i} \cdot \frac{1}{nKM_{\text{obs}}^{i} + \frac{1}{nKM_{\text{obs}}^{i}}}^{2} \frac{4X_{\text{tot}}^{i} \cdot \frac{1}{nKM_{\text{obs}}^{i}}}{nKM_{\text{obs}}^{i}}} \right]$$

After correcting for the heat contributed by the solution that is displaced from the cell during the $i$th injection, the expression for the heat released upon the $i$th injection is given in equation (2):

$$\Delta Q_{i} = Q_{t}^i - Q_{t}^{i-1} + \frac{dV_{i}}{2V_{o}} (Q_{t}^{i} + Q_{t}^{i-1})$$

where $V_{o}$ is the active cell volume (1.37 ml), $dV_{i}$ is the volume of the $i$th injection (displaced volume), $M_{\text{tot}}^{i}$ is the total concentration of macromolecule in the cell after the $i$th injection, $X_{\text{tot}}^{i}$ and $X_{i}$ are the total and free concentrations, respectively, of ligand in the cell after the $i$th injection, $K_{\text{eq}}$ is the equilibrium binding constant ($K_{\text{eq}}$), $n$ is the number of binding sites for DNA on the SSB tetramer ($n = 1$ for this case), and $\Delta H$ is the enthalpy change for SSB-DNA binding ($\Delta H_{\text{obs}}$). Nonlinear least squares fitting of the data to equation (2) was performed using the ITS Data Analysis in Origin software provided by the manufacturer. The uncertainties in $\Delta H_{\text{obs}}$ measured in NaF are larger than in NaCl or NaBr for the following reasons. In NaF solutions, we are constrained to using much lower SSB concentrations (approximately 0.1 $\mu$M) due to the low solubility of SSB protein in NaF solutions. This decreases the

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signal-to-noise in these experiments. In fact, in the NaF experiments, the heat evolved per 10 µl injection is less than −10 µcal whereas the heat of dilution is ~one-third of this value.

Analysis of ITC data for SSB binding to dT(pT)₃₄
A single SSB tetramer can bind two molecules of dT(pT)₃₄ at saturation, although the second DNA molecule binds with negative cooperativity (Lohman & Bujalowski, 1988; Bujalowski & Lohman, 1989a,b). The degree of negative cooperativity (σ), as defined quantitatively by the square model (Bujalowski & Lohman, 1989a), does not vary with [NaBr] and [NaCl] over the range from 0.3 to 3.0 M salt. We analyzed the dT(pT)₃₄-SSB tetramer ITC binding isotherms using a simple two-site binding model. The data were fit to this model using the ITS Data Analysis in Origin software provided by the manufacturer. For this model the total heat after ith injection, $Q^\text{tot}_i$, is given by equation (3):

$$Q^\text{tot}_i = M^\text{eq}V_i (\Delta H_1 + \Delta H_2) k_1 k_2 X_i^2$$

(3)

where $X_i$ can be obtained by solving equation (4):

$$X^\text{tot}_i = X_1 + v \cdot M^\text{eq}_i = X_1 + M^\text{eq}_i \frac{2k_1 X_i + 2k_2 X_i^2}{p}$$

(4)

and, $\Delta Q$, is given by equation (5):

$$\Delta Q = Q^\text{tot}_i - Q^\text{tot}_{i-1} = \frac{dV_i}{2V_o} (Q^\text{eq}_i - Q^\text{eq}_{i-1})$$

(5)

In equations (3) to (5), $k_1$ and $k_2$ are the microscopic binding constants for the first and the second sites, $\Delta H_1$ and $\Delta H_2$ are the enthalpy changes for the interaction with the first and the second sites, $p=1+2k_1X+k_1k_2X^2$ is the partition function for dT(pT)₃₄ binding to the SSB tetramer.

CD spectra
Circular dichroism (CD) spectra were recorded on a Jasco-600 spectropolarimeter (Jasco, Japan), calibrated with ammonium d-10-camphorsulfonate (0.06%, w/v). Because of the lower signal in the near UV region, the spectra were recorded separately for near and far UV regions using thermostatted cuvettes with 1.0 and 0.1 cm pathlengths, respectively. Each curve represents the average of six spectra. After subtraction of the reference spectrum, the data were expressed as molar ellipticity [θ] = deg cm²/dmol of SSB tetramer. Spectra were limited to wavelengths >204 nm due to the high absorbance of Hepes buffer at lower wavelengths.

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