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Apparent Heat Capacity Change Accompanying a Nonspecific Protein–DNA Interaction. *Escherichia coli* SSB Tetramer Binding to Oligodeoxyadenylates†

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ABSTRACT: We have examined the effects of temperature on the equilibrium constant, K_{obs}, for *Escherichia coli* SSB tetramer binding to a series of single-stranded (ss) oligodeoxyribonucleotides, $dT(pT)_n$, $dC(pC)_n$, and $dA(pA)_n$ (n = 34, 55, and 69) in order to investigate the thermodynamic basis for the strong preference of *E. coli* SSB (and other SSB proteins) for binding polypyrimidine stretches of ss-DNA. In addition to the expected base-dependent differences in the magnitude of K_{obs}, we also observe qualitatively different temperature dependencies for the binding of the SSB tetramer to oligodeoxyadenylates. Linear van't Hoff plots are obtained for SSB tetramer binding to $dT(pT)_n$ and $dC(pC)_n$, with $\Delta H°_{obs}$ ranging from −50 to −100 kcal/mol depending on the oligodeoxynucleotide length and salt concentration. In contrast, all van't Hoff plots for SSB tetramer binding to $dA(pA)_N$ are distinctly nonlinear with maxima in K_{obs} occurring near 25 °C, indicative of an *apparent* large negative change in molar heat capacity ($\Delta C°_{P,obs} < 0$). Thus for the SSB–$dA(pA)_n$ interaction, $\Delta H°_{obs}$ and $\Delta S°_{obs}$ are both highly temperature dependent, but compensate such that $\Delta G°_{obs}$ is relatively insensitive to temperature. These nonlinear van't Hoff plots are *not* due to coupling of SSB assembly to $dA(pA)_n$ binding or to temperature-dependent shifts in the formation of other SSB–DNA binding modes. The nonlinear van't Hoff plots for SSB tetramer binding to $dA(pA)_n$ appear to result from the coupling of two processes: (1) the unstacking of the $dA(pA)_n$ bases (occurring with $\Delta H° > 0$ and $\Delta C°_P = 0$) and (2) the binding of SSB to the unstacked DNA (occurring with $\Delta H° < 0$ and $\Delta C°_P = 0$). Therefore, although each isolated equilibrium occurs with $\Delta C°_P \approx 0$, the overall equilibrium displays an *apparent* $\Delta C°_{P,obs} < 0$ due to the coupled equilibrium. The binding of SSB to $dT(pT)_n$ and $dC(pC)_n$ occurs with $\Delta H° < 0$ and $\Delta C°_{P,obs} = 0$, since the bases in these ss-DNA molecules do not stack appreciably. These results indicate that a nonspecific protein–DNA interaction can display a large negative *apparent* $\Delta C°_P$; however, this effect appears not to be due to the hydrophobic effect, but rather to a temperature-dependent conformational transition in the DNA that is coupled to protein binding. Implications of these observations for other protein–nucleic acid systems are discussed.

An understanding of the stabilities and specificities of noncovalent protein–nucleic acid interactions requires thermodynamic information ($\Delta G°_{obs}$, $\Delta H°_{obs}$, $\Delta S°_{obs}$, $\Delta C°_{P,obs}$), which can be obtained, in principle, from studies of the temperature dependence of the equilibrium binding constant, K_{obs} (van't Hoff analyses), or from calorimetric studies. van't Hoff analyses have shown that the binding of a number of sequence-specific DNA binding proteins (e.g., *Escherichia coli* lac repressor, *Eco*RI endonuclease, *E. coli* Trp repressor) to their specific DNA sites is accompanied by large, negative heat capacity changes ($\Delta C_{P,obs} < 0$) (Ha et al., 1989; Jin et al., 1993; Spolar & Record, 1994; Lundback et al., 1993). Recent calorimetric studies also indicate large negative heat capacity changes for the DNA sequence specific binding of phage λ Cro protein (Takeda et al., 1992) and *E. coli* Trp repressor (Ladbury et al., 1994). These negative heat capacity changes have recently been interpreted as resulting mainly from the hydrophobic effect, i.e., the release of "hydrophobically bound" water upon burial of water-accessible nonpolar surfaces of the protein and DNA upon formation of the protein–DNA complex (Ha et al., 1989), and it has been suggested that such large negative heat capacity changes may be distinctive features of sequence-specific interactions of proteins with DNA (Spolar & Record, 1994). However, systematic determinations of equilibrium binding constants for nonspecific protein–DNA interactions over the wide range of temperatures needed to assess curvature in van't Hoff plots have been limited. On the other hand, calorimetric studies of phage λ Cro protein (Takeda et al., 1992) and *E. coli* Trp repressor (Ladbury et al., 1994) suggest that $\Delta C_{P,obs} \sim 0$ for the nonspecific binding of these proteins to duplex DNA.

In order to compare with the results of studies of site-specific DNA binding proteins, we have examined the thermodynamics of binding to single-stranded (ss) DNA of the *E. coli* single-stranded DNA binding (SSB) protein, a well-characterized nonspecific DNA binding protein. The *E. coli* SSB protein is an essential helix-destabilizing protein that functions in DNA replication, recombination, and repair [for reviews, see Chase and Williams (1986), Lohman et al. (1988), Meyer and Laine (1990), Lohman and Bujalowski (1990), Lohman and Ferrari (1994)]. This protein binds to ss-DNA as a homotetramer [subunit M_r = 18 843 (Sancar et al., 1981; Chase et al., 1984)] in a number of different binding modes, depending on solution conditions, particularly the salt concentration and type (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988). These ss-DNA binding modes are referred to as $(SSB)_n$, where n represents the average number of nucleotides occluded per SSB tetramer (n = 35, 56, or 65). In the $(SSB)_{35}$ mode, ss-DNA binds to only two subunits of the SSB tetramer, whereas in both the $(SSB)_{56}$ and $(SSB)_{65}$ modes, all four subunits of the tetramer

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bind ss-DNA (Lohman & Bujalowski, 1988; Bujalowski & Lohman, 1989a,b). Furthermore, the switch between binding modes is due at least in part to a salt-dependent negative cooperativity among the DNA binding sites within individual tetramers. This negative cooperativity decreases the apparent affinity of the third and fourth SSB subunits for ss-DNA and is greatest at low salt concentrations (Lohman & Bujalowski, 1988, 1994; Bujalowski & Lohman, 1989a,b).

Although the *E. coli* SSB protein is a "nonspecific" DNA binding protein, it displays a hierarchy of affinities for ss-homopolynucleotides of different base and sugar composition (Weiner et al., 1975; Overman et al., 1988; Bobst et al., 1991). Similar hierarchies have also been observed for other prokaryotic SSB proteins, such as the gene V proteins from filamentous phages (Alma et al., 1983; Porschke & Rau, 1983; Bulsink et al., 1985; Sang & Gray, 1989) and the phage T4 gene 32 protein (Newport et al., 1981; Bobst & Pan, 1975), as well as the heterotrimeric eukaryotic SSB (RP-A, RF-A) proteins (Kim et al., 1992; Mignotte et al., 1985). All of these SSB proteins bind with greater affinity to polydeoxypyrimidines relative to poly(dA) and poly(A). Overman et al. (1988) showed the following base specificity for $K_{65,obs}$, the equilibrium constant for *E.coli* SSB tetramer binding to a series of ss-homopolynucleotides in its $(SSB)_{65}$ binding mode (0.20 M NaCl, pH 8.1, 25.0 °C): $K_{65,obs}$ poly(dT) > poly(dC) ≫ M13 ss-DNA > poly(I) > poly(U) > poly(dA) > pol(y)A ≫ poly(C). In this mode, the SSB tetramer binds with ~10^9-fold higher affinity to poly(dT) than to poly(dA). Although the molecular basis for this large base preference is not yet understood, nearly all SSB proteins show a decreased affinity for poly(dA) as compared to poly(dT) and poly(dC).

In an attempt to understand the thermodynamic basis for this base specificity, we have measured equilibrium binding constants as a function of temperature for the nonspecific binding of *E. coli* SSB tetramer to the oligodeoxynucleotides $dA(pA)_n$, $dC(pC)_n$, and $dT(pT)_n$ (n = 34, 55, or 69). We find that the binding of the SSB tetramer to both $dT(pT)_n$ and $dC(pC)_n$ is characterized by a large negative $\Delta H°_{obs}$ but a negligible change in heat capacity ($\Delta C°_{P,obs} \approx 0$). In contrast, binding to $dA(pA)_n$ is accompanied by a large negative heat capacity change; however, this appears to result from the coupling of two processes, each with $\Delta C°_{P,obs} = 0$, but with $\Delta H°_{obs}$ values of opposite sign, these being the unstacking of the adenine bases and protein–DNA binding. Therefore, the *E. coli* SSB tetramer–$dA(pA)_n$ interaction provides an example of a nonspecific protein–DNA interaction with a large negative *apparent* $\Delta C°_{P,obs}$; however, the molecular basis for this phenomenon does not appear to be the release of water due to the hydrophobic effect, as has been suggested for a number of specific protein–DNA interactions (Ha et al., 1989; Spolar & Record, 1994), but rather results from a coupling of two equilibria with $\Delta H°$ values of opposite sign and $\Delta C°_{P,obs} \approx 0$.

MATERIALS AND METHODS

Reagents and Buffers. All solutions were prepared with reagent grade chemicals and distilled water that was subsequently passed through a Milli-Q system (Millipore, Bedford, MA). All titrations were performed in buffer H (10 mM HEPES (4-(2-hydroxethyl)-1-piperazineethanesulfonic acid) and 0.1 mM Na_3EDTA (ethylenediaminetetraacetic acid)). Stock solutions of 0.1 M HEPES, free acid, and 0.1 M HEPES, sodium salt, were mixed in the appropriate ratios to obtain a final solution of pH 8.1 at the indicated salt concentration and temperature. The pH of the solutions was verified by direct pH measurement. Buffer T is 10 mM Tris (tris-(hydroxymethyl)aminomethane), pH 8.1, and 0.1 mM Na_3EDTA.

SSB Protein and Oligodeoxynucleotides. *E. coli* SSB protein was purified as described (Lohman et al., 1986a), and its 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} (1.5 mL mg^{-1} cm^{-1}) (Lohman & Overman, 1985). The oligodeoxynucleotides, $dA(pA)_n$, $dT(pT)_n$, and $dC(pC)_n$ (n = 69, 55, or 34), were synthesized using phosphoramidite chemistry with an Applied Biosystems 391 PCR mate automated synthesizer and purified by electrophoresis through polyacrylamide gels as described (Ferrari et al., 1994). The DNA was resuspended in buffer H containing the appropriate NaCl concentration and dialyzed extensively before use. The DNA was ≥98% pure as judged 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 spectrophotometrically in buffer T (pH 8.1) and 0.1 M NaCl, using the following extinction coefficients (per nucleotide): $(dT)_n$, $\epsilon_{260} = 8.1 \times 10^3$ M^{-1} cm^{-1}; $(dA)_n$, $\epsilon_{257} = 1.0 \times 10^4$ M^{-1} cm^{-1}; $(dC)_n$, $\epsilon_{270} = 7.2 \times 10^3$ M^{-1} cm^{-1} (Kowalczykowski et al., 1981).

Fluorescence Titrations. Equilibrium binding of oligodeoxynucleotides to the SSB tetramer was studied by monitoring the quenching of the SSB tryptophan fluorescence, using an SLM 8000C spectrofluorometer (λ_{ex} = 300 nm, 2-nm excitation band pass; λ_{em} = 347 nm, 8-nm emission band pass) as described (Bujalowski & Lohman, 1989a,b). The sample was maintained at the indicated temperature to within ±0.1 °C using a refrigerated water bath. Experiments were performed by titrating an SSB solution (generally 0.14 μM (tetramer) unless noted otherwise) with a concentrated oligodeoxynucleotide stock, both made up in buffer H containing the indicated NaCl concentration. The observed quenching of the SSB fluorescence was calculated as $Q_{obs} = (F_0 - F_i)/F_0$, where F_0 is the initial protein fluorescence of the free protein, and F_i is the fluorescence after addition of the ith aliquot of DNA. Values of F_0 and F_i were corrected for minor contributions to the background fluorescence from the buffer and the DNA as well as for dilution and inner filter effects as described previously (Bujalowski & Lohman, 1989b; Lohman & Mascotti, 1992b). Detectable photobleaching was not observed under the conditions of these experiments.

Determination of K_{70} and K_{56}. Under high-salt conditions (>0.15 M NaCl), SSB tetramers bind to both $dX(pX)_{69}$ and $dX(pX)_{55}$ (X = T, C, A) and form exclusively 1:1 complexes, with equilibrium constants K_n (n = 56 or 70, respectively) (Lohman & Bujalowski, 1988; Bujalowski & Lohman, 1989a,b; Ferrari et al., 1994). Values of K_n and Q_{max}, the maximum fluorescence quenching at saturating DNA concentrations, were determined by nonlinear least squares regression analysis of experimental isotherms using Kaleidagraph (Synergy Software, Reading, PA) based on a 1:1 binding model, where $Q_{obs}/Q_{max} = [PD]/P_T = K_n D_f/(1 + K_n D_f)$; $[PD]/P_T$ is the fraction of SSB tetramers bound to DNA, and D_f is the free DNA concentration. An expression for Q_{obs} written in terms of total SSB tetramer concentration, P_T, and total DNA concentration, D_T, is given in eq 1, where $K_D = 1/K_n$.

$$Q_{obs} = \frac{Q_{max}\left(D_T + K_D + P_T - \sqrt{(D_T + K_D + P_T)^2 - 4P_T D_T}\right)}{2P_T} \quad (1)$$

The salt dependence of K_{70} was determined using "salt back" titrations in which the SSB–dA(pA)$_{69}$ complex is first formed by titration of protein with DNA, followed by dissociation of the complex by titrating with buffer containing 4 M NaCl (Overman et al., 1988; Lohman & Mascotti, 1992b). Since the free SSB fluorescence is known, and since Q_{max} is independent of [NaCl] (Lohman & Overman, 1985; Overman et al., 1988), the concentration of bound SSB ([PD]) can be calculated from the value of Q_{obs}/Q_{max} at each salt concentration as [PD] = $Q_{obs}P_T/Q_{max}$. K_{70} is then calculated at each NaCl concentration using eq 2, where $D_f = D_T - [PD]$ and $P_f = P_T - [PD]$.

$$K_{70} = \frac{[PD]}{D_f P_f} \quad (2)$$

Analysis of SSB–dX(pX)$_{34}$ Titrations. Equilibrium titrations of SSB with dX(pX)$_{34}$ (X = T,A) were analyzed using the square model (Bujalowki & Lohman, 1989a; see eq 3). Values of K_{35}, the intrinsic equilibrium constant for binding dX(pX)$_{34}$ to two SSB subunits, and σ_{35}, a parameter reflecting negative cooperative interactions between two DNA-ligated SSB subunits, were obtained using the nonlinear least squares algorithm NONLIN (Johnson & Frasier, 1985) on a Hewlett-Packard Apollo 9000/730 computer. Theoretical isotherms were simulated based on eq 3 using Kaleidagraph software (Synergy Software, Reading, PA).

$$Q_{obs} = \frac{Q_{1/35}(4\sigma_{35} + 2)K_{35}L_f + Q_{2/35}3\sigma_{35}^4(K_{35}L_f)^2}{1 + 4\sigma_{35}K_{35}L_f + 2K_{35}L_f + 3\sigma_{35}^4(K_{35}L_f)^2} \quad (3)$$

In eq 3, $Q_{1/35}$ and $Q_{2/35}$ are the extents of tryptophan fluorescence quenching associated with binding one and two molecules of dX(pX)$_{34}$, respectively, and the denominator is the macromolecule partition function, Z. In order to fit the experimental binding curves to eq 3 using nonlinear least squares analysis, expressions for the concentrations of free protein (P_f) and free DNA (L_f) are required. The free protein concentration is given by $P_f = P_T/Z$, where P_T is the total protein concentration, and the total DNA concentration, L_T, is given in eq 4:

$$L_T = L_f + 4\sigma_{35}K_{35}L_fP_f + 2K_{35}L_fP_f + 6\sigma_{35}^4(K_{35}L_f)^2P_f \quad (4)$$

Substitution of $P_f = P_T/Z$ into eq 4, followed by rearrangement, gives eq 5, which is the root-finding function for L_f used in the NONLIN analysis.

$$6K_{35}^2L_f^2\sigma_{35}^4 + K_{35}L_f(2 + 4\sigma_{35}) - \frac{L_TZ}{P_T} + \frac{L_fZ}{P_T} = 0 \quad (5)$$

The quenching constants ($Q_{1/35}$ and $Q_{2/35}$) were fixed in the analysis of the dT(pT)$_{34}$ data, based on values obtained previously (Bujalowski & Lohman, 1989a,b), and K_{35} and σ_{35} were determined by nonlinear least squares fitting. We were not able to obtain estimates of σ_{35} for the SSB–dA(pA)$_{34}$ interaction, since the titrations were not carried out to the high [dA(pA)$_{34}$] (>10^{-5} M) needed to bind two dA(pA)$_{34}$ molecules per SSB tetramer, due to the extensive negative cooperativity for dA(pA)$_{34}$ (Lohman & Bujalowski, 1994). Therefore, to obtain estimates of K_{35}, we fixed σ_{35} at 0.28 (Lohman & Bujalowski, 1994). $Q_{1/35}$ was fixed at 0.4 (5–30 °C) or 0.39 (37 °C) on the basis of the observed quenching for the first plateau in the titration curve (see Figure 7). $Q_{2/35}$ was fixed at the value of Q_{max} obtained for the SSB–dA(pA)$_{69}$ interaction at 0.17 M NaCl. Varying $Q_{2/35}$ did not alter the value of K_{35} determined from the fitting procedure.

SSB Denaturation Studies. Equilibrium denaturation studies using guanidine hydrochloride (Gdn·HCl) were performed to determine the stability of the SSB tetramer at 37 °C while monitoring the decrease in SSB tryptophan fluorescence intensity (λ_{ex} = 296 nm, 2-nm band pass; λ_{em} = 347 nm, 8-nm band pass). Measurements were made in buffer H (pH 8.1) and 0.2 M NaCl at 37 °C by adding Gdn·HCl from a 6 M stock solution in buffer H, pH 8.1. The Gdn·HCl stock concentration was determined by refractive index measurements (Pace, 1986). Each measurement was performed by adding protein to a Gdn·HCl solution that was pre-equilibrated for 10 min. The SSB fluorescence intensity was measured after equilibration for an additional 12 min, after which time no further change in fluorescence was observed. Each point in the denaturation curve was obtained from a separate experiment at each [Gdn·HCl] and was corrected for background fluorescence from the Gdn·HCl solution. The denaturation curve was shown to be reversible by diluting a concentrated solution of SSB in 6 M Gdn·HCl into pre-equilibrated buffer containing the desired final [Gdn·HCl].

Equilibrium denaturation curves were analyzed using a two-state model assuming that only native SSB tetramers and denatured monomers occur in solution as described in eq 6,

$$T \overset{K_D}{\leftrightarrow} 4D \quad (6)$$

with equilibrium constant, $K_D = [D]^4/[T]$. At equilibrium, the observed protein fluorescence signal can be expressed as $F_{obs} = F_Df_D + F_Nf_N$, where F_N and F_D are the molar fluorescence signals for the native and denatured proteins (per monomer), respectively, and f_N and f_D are the fraction of native tetramer (T) and denatured monomer (D), respectively as given in eq 7, where $[T_0]$ is the total tetramer concentration.

$$f_D = \frac{[D]}{4[T_0]} \quad (7a)$$

$$f_N = \frac{[T]}{[T_0]} \quad (7b)$$

K_D can be expressed in terms of T_0 and f_D as in eq 8 and in terms of ΔG_0, the free energy of unfolding in the absence of denaturant, and m, the gradient of the free energy as a function of denaturant concentration (Pace, 1986; Santoro & Bolen, 1988) as in eq 9.

$$K_D = 256T_0^3\left(\frac{f_D^4}{1 - f_D}\right) \quad (8)$$

$$K_D = \exp\{-(m[\text{Gdn·HCl}] + \Delta G_0)/RT\} \quad (9)$$

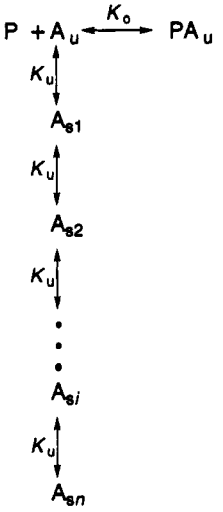
The value of f_D ($0 < f_D < 1$) can be determined as the real root of eq 10.

$$256T_0^3f_D^4 + K_Df_D - K_D = 0 \quad (10)$$

Denaturation curves obtained at three protein concentrations were analyzed to obtain f_N, f_D, m, and ΔG_0, using a global implementation of the Marquardt–Levenberg nonlinear least squares algorithm (Press et al., 1990).

Sedimentation Studies. Sedimentation velocity experiments were performed at several SSB concentrations (0.14–1.16 μM (tetramer)) in 10 mM Tris, pH 8.1, and 0.2 M NaCl

Scheme 1



at 37 °C using an Optima XL-A analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA). The absorbance profile as a function of radial distance was obtained by scanning each cell (at 220 nm). The sedimentation coefficient was determined from analysis of the first derivative (dA/dr) using the XLAVEL software provided by the manufacturer. The sedimentation coefficients, obtained at 37 °C, are reported as $s_{20,w}$.

THERMODYNAMIC BACKGROUND

Analysis of Apparent Heat Capacity Change Associated with SSB Tetramer Binding to dA(pA)$_n$. We consider two models to explain the apparent negative heat capacity change ($\Delta C°_{P,obs}$) associated with the equilibrium binding of SSB tetramer to dA(pA)$_n$. One model, shown in Scheme 1, considers $\Delta C°_{P,obs} < 0$ to result from the coupling of two equilibria, the unstacking of the adenine bases occurring with $\Delta H° > 0$ and the binding of SSB to unstacked dA(pA)$_n$ occurring with $\Delta H° < 0$. The second model assumes that a temperature-independent heat capacity change accompanies the binding of SSB to dA(pA)$_n$ but not to dT(pT)$_n$ or dC(pC)$_n$.

The base-unstacking model is based on the fact that the bases within dA(pA)$_n$ undergo significant unstacking with increasing temperature (Leng & Felsenfeld, 1966; Riley et al., 1966; Poland et al., 1966; Ts'o et al., 1966; Vournakis et al., 1967), whereas dT(pT)$_n$ and dC(pC)$_n$ show negligible base stacking (Felsenfeld & Miles, 1967; Riley et al., 1966; Ts'o et al., 1966). However, when bound to SSB protein, the bases in dA(pA)$_n$ become unstacked, at least partially (Kuil et al., 1990). In this model, the DNA exists in equilibrium among a continuum of conformational states differing in the extent of base stacking; however, the SSB protein is assumed to bind only to the unstacked state of the DNA, A_u. The adenine base unstacking transition is assumed to be non-cooperative on the basis of spectroscopic studies of poly(A) unstacking (Riley et al., 1966; Poland et al., 1966; Leng & Felsenfeld, 1966), although one study suggests that this transition displays negative cooperativity (Vournakis et al., 1967). In contrast, calorimetric and spectroscopic studies of Breslauer and Sturtevant (1977) indicate that the unstacking of the oligoribonucleotide A_7 shows two-state behavior. However, a fully cooperative unstacking model does not provide an adequate fit to the SSB–dA(pA)$_n$ binding data (see Discussion).

As shown below, a nonzero $\Delta C°_{P,obs}$ can result from the base-unstacking model, even if the separate equilibria, i.e., DNA base unstacking and protein binding to unstacked DNA, have $\Delta C°_P = 0$ as seems to be the case, as we show here, for SSB binding to unstacked ss-DNA and as has been shown for poly(A) unstacking (Filimonov & Privalov, 1978). The coupling of base unstacking to SSB–dA(pA)$_n$ binding predicts a nonlinear van't Hoff plot and also explains the absence of a heat capacity change for the binding of SSB protein to either dT(pT)$_n$ or dC(pC)$_n$. The suggestion that coupled equilibria can result in apparent heat capacity changes even for systems that show no heat capacity changes for the separate equilibria has been discussed previously for other systems (Eftink et al., 1983; Gill et al., 1985; Wyman & Gill, 1990).

Non-Cooperative Base-Stacking Model. Scheme 1 assumes that the free oligodeoxyadenylate exists in equilibrium among a continuum of stacked states, A_{si}, each containing i stacked bases, and an unstacked state, A_u, in which all the bases are unstacked. Base unstacking is assumed to be non-cooperative, so that the equilibrium constants for all base-unstacking reactions are identical, with equilibrium constant, K_u, for *unstacking*. We also assume that only the fully unstacked state, A_u, can bind SSB tetramer, P, with equilibrium constant $K_0 = [PA_u]/[P][A_u]$.

The apparent equilibrium constant, K_n, for the coupled equilibrium in Scheme 1 is given by eq 11.

$$K_n = K_o[K_u/(1 + K_u)]^n \tag{11}$$

Upon differentiating eq 11 with respect to T^{-1}, we obtain the expression for $\Delta H°_{obs}$ in eq 12.

$$\Delta H°_{obs} = \Delta H°_0 + n\Delta H°_u(1 + K_u)^{-1} \tag{12}$$

The expression for the heat capacity change, $\Delta C°_{P,obs} = (d\Delta H°_{obs}/dT)_P$, can be obtained from eq 12 and is given in eq 13a.

$$\Delta C°_{P,obs} = \Delta C°_{P,0} + n\Delta C°_{P,u}(1 + K_u)^{-1} - n(\Delta H°_u)^2(K_u/(1 + K_u)^2)(1/RT^2) \tag{13a}$$

Note that even if there is no heat capacity change associated with either the unstacking equilibrium or the protein–DNA binding equilibrium (i.e., $\Delta C°_{P,o} = 0$ and $\Delta C°_{P,u} = 0$), an *apparent* heat capacity change can still result due to the third term in eq 13a (if $\Delta H°_u \neq 0$) (Eftink et al., 1983). However, this term results solely from the coupling of the base-unstacking process to protein–DNA binding. When $\Delta C°_{P,o} = 0$ and $\Delta C°_{P,u} = 0$, eq 13a reduces to eq 13b.

$$\Delta C°_{P,obs} = -n(\Delta H°_u)^2(K_u/(1 + K_u)^2)(1/RT^2) \tag{13b}$$

Upon substituting $K_0 = \exp[(\Delta S°_0/R) - (\Delta H°_0/RT)]$ and $K_u = \exp[(-\Delta H°_u/R)((1/T) - (1/T_m))]$ into eq 11, we obtain eq 14,

$$\ln K_n = \left(\frac{\Delta S°_0}{R} - \frac{\Delta H°_0}{RT}\right) - \left(N \ln\left(1 + \exp\left(\frac{\Delta H°_u}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right)\right)\right)\right) \tag{14}$$

where $\Delta S°_0$ and $\Delta H°_0$ are the entropy and enthalpy changes, respectively, for SSB binding to the fully unstacked DNA, and $\Delta H°_u$ and T_m are the enthalpy change (per nucleotide) and the melting temperature for the base *unstacking* transition. (Note that, at T_m, $\Delta S°_u = \Delta H°_u/T_m$). The experimental data ($\ln K_n$ vs $1/T$) were analyzed according to eq 14 to obtain $\Delta S°_0$ and $\Delta H°_0$ using the nonlinear least squares regression

software in Kaleidagraph (Synergy Software, Reading, PA), while the values $\Delta H°_u$ and the T_m were constrained (see Table 5, and Discussion).

Models Assuming a Temperature-Independent Heat Capacity Change Due to Hydrophobic Effects. A nonlinear van't Hoff plot will also result if the binding process is accompanied by heat capacity changes arising from other sources such as hydrophobic effects. If $\Delta C°_{P,obs}$ is independent of temperature, the temperature dependence of the equilibrium association constant can be described by eq 15,

$$\ln K_n = \left(\frac{\Delta C°_P}{R}\right)\left[\left(\frac{T_H}{T}\right) - \ln\left(\frac{T_S}{T}\right) - 1\right] \qquad (15)$$

where T_H and T_S are the characteristic temperatures where $\Delta H°_{obs} = 0$ and $\Delta S°_{obs} = 0$ (Becktel & Schellman, 1987; Ha et al., 1989). This expression has been used to interpret the nonlinear van't Hoff plots for the equilibrium binding of a number of sequence-specific DNA binding proteins to their specific DNA sites (Ha et al., 1989; Spolar & Record, 1994). Experimental data ($\ln K_n$ vs $1/T$) were analyzed using eq 15 to obtain $\Delta C°_{P,obs}$, T_H, and T_S by nonlinear least squares regression using Kaleidagraph (Synergy Software, Reading, PA).

RESULTS

Temperature Dependence of the Equilibrium Constant for SSB Tetramer Binding to dX(pX)$_{69}$. Previous experiments with dT(pT)$_{69}$ and dA(pA)$_{69}$ (Bujalowski & Lohman, 1989b; Ferrari et al., 1994) indicate that, at NaCl concentrations < 10 mM, two SSB tetramers can bind to a single DNA at sufficiently high SSB concentrations. In this 2:1 complex each SSB tetramer interacts with DNA using only two of its protomers (subunits), covering ~35 nucleotides per tetramer. However, at higher salt concentrations (>150 mM NaCl) or at lower SSB:DNA ratios, a 1:1 complex is formed in which all four subunits of the SSB tetramer interact with the DNA, covering ~65 nucleotides. We used dX(pX)$_{69}$ (X = T, C, A) to examine the temperature dependence of the binding under high-salt conditions that favor formation of only the 1:1 complex in which all four subunits interact with dX(pX)$_{69}$. The use of dX(pX)$_{69}$ requires determination of only two parameters, the equilibrium constant for the reaction, K_{70}, and the maximum quenching, Q_{max}. This represents the simplest system for comparing the temperature dependencies of SSB binding to dA(pA)$_n$ vs dC(pC)$_n$ and dT(pT)$_n$, since use of polydeoxynucleotides requires consideration of inter-tetramer cooperativity (Bujalowski & Lohman, 1987; Overman et al., 1988).

The dA(pA)$_{69}$ titrations were performed in buffer H, pH 8.1, and 0.17 M NaCl, conditions under which a 1:1 complex forms (Ferrari et al., 1994). However, since the affinity of SSB for dC(pC)$_{69}$ and dT(pT)$_{69}$ in NaCl is too high to allow accurate determination of the binding constant,[1] measurements had to be made in buffer H, pH 8.1, containing NaBr, in order to lower the binding constant for the SSB–DNA interaction (Overman et al., 1988; Bujalowski & Lohman, 1989a,b). The particular NaBr concentration was chosen to enable us to obtain measurements of K_{obs} for dT(pT)$_{69}$ and dC(pC)$_{69}$ over the widest temperature range. However, due to the different affinities, the equilibrium constants for SSB binding to dT(pT)$_{69}$ and dC(pC)$_{69}$ could not be performed under identical NaBr concentrations.

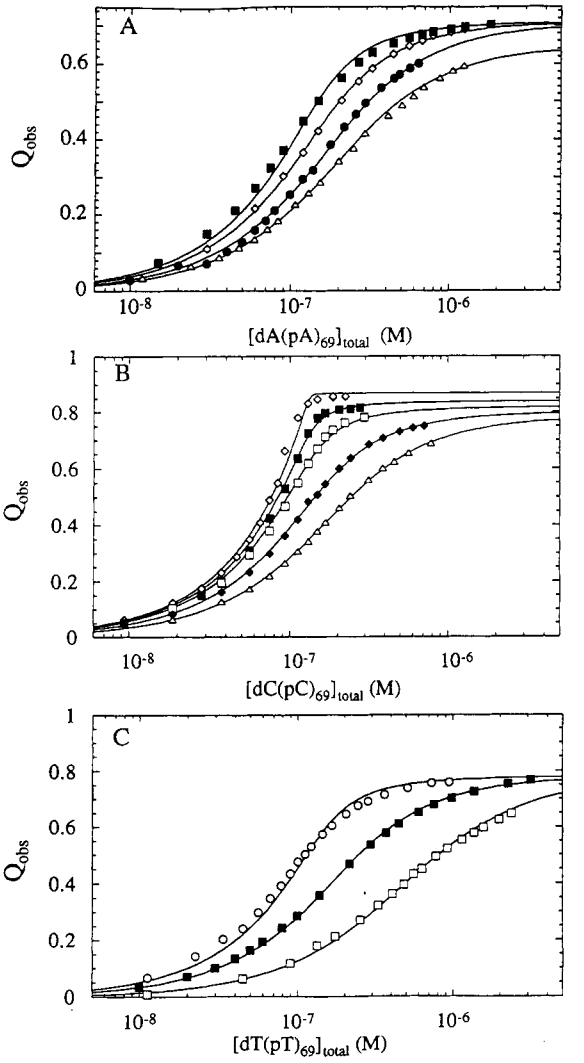


FIGURE 1: Equilibrium titrations of SSB protein (0.14 μM tetramer) with dA(pA)$_{69}$, dT(pT)$_{69}$, and dC(pC)$_{69}$ at several temperatures. Binding was monitored by the quenching of the SSB tryptophan fluorescence upon addition of DNA (buffer H, pH 8.1). (A) SSB tetramer binding to dA(pA)$_{69}$ (0.17 M NaCl) at 5 (●), 10 (◇), 25 (■), and 37 °C (△). (B) SSB tetramer binding to dC(pC)$_{69}$ (1 M NaBr) at 10 (◇), 25 (■), 31 (□), 35 (◆), and 37 °C (△). (C) SSB tetramer binding to dT(pT)$_{69}$ (2 M NaBr) at 23 (○), 25 (■), and 30 °C (□). The solid curves are nonlinear least squares fits of the data to eq 4; the best-fit values for K_{70} and Q_{max} are given in Table 1.

Figure 1 shows a series of equilibrium titrations of SSB protein (0.14 μM tetramer) with dX(pX)$_{69}$ in which the quenching of the SSB tryptophan fluorescence (Q_{obs}) was monitored as a function of dX(pX)$_{69}$ concentration. The solid curves in Figure 1 are simulated 1:1 binding isotherms using the values of K_{70} and Q_{max} (Table 1) determined from nonlinear least squares fitting of the data to eq 1. A marked difference is observed upon comparing the temperature dependence of SSB binding to dA(pA)$_{69}$ vs dC(pC)$_{69}$ or dT(pT)$_{69}$. For both dC(pC)$_{69}$ and dT(pT)$_{69}$, SSB affinity decreases as the temperature is increased (Figure 1B,C). These results are in agreement with previous van't Hoff analyses of SSB binding to both poly(U) and dT(pT)$_{15}$. For the SSB–dT(pT)$_{15}$ interaction (20 mM phosphate and 0.2 M KCl, pH 7.4), $\Delta H°_{obs}$ = −26 ± 3 kcal/mol (Bujalowski & Lohman, 1989b), whereas $\Delta H°_{obs}$ for SSB binding to poly(U) in the (SSB)$_{65}$ mode increases from −67 ± 2 kcal/mol at 0.12 M NaCl (pH 8.1) to −31 ± 3 kcal/mol at 0.48 M NaCl (pH 8.1) (Overman,

[1] The affinity of the SSB tetramer for poly(dT) is so high that accurate binding constants cannot be measured using fluorescence titrations in solutions containing even 5 M NaCl (Lohman & Overman, 1985).

Table 1: Equilibrium Constants for SSB Tetramer Binding to $dX(pX)_{69}$[a]

T (°C)	$K_{70} \times 10^{-7}$ M^{-1}[b]	Q_{max}[c]	T (°C)	$K_{70} \times 10^{-7}$ M^{-1}[b]	Q_{max}[c]
$dA(pA)_{69}$, 0.17 M NaCl			$dC(pC)_{69}$, 1 M NaBr		
5	1.17 (±0.05)	0.72	25	73.0 (±37.0)	0.81
5	1.15 (±0.06)	0.71	31	10.7 (±0.07)	0.82
10	2.22 (±0.06)	0.72	35	2.75 (±0.11)	0.81
15	2.66 (±0.18)	0.71	37	1.01 (±0.03)	0.78
25	3.92 (±0.50)	0.70	$dT(pT)_{69}$, 2 M NaBr		
25	4.69 (±0.70)	0.69	20	52.1 (±20.1)	0.80
25	5.71 (±0.67)	0.70	23	7.18 (±0.42)	0.78
31	3.80 (±0.52)	0.68	25	1.13 (±0.01)	0.78
34	0.99 (±0.02)	0.69	30	0.24 (±0.002)	0.78
37	1.11 (±0.04)	0.66	$dT(pT)_{69}$, 2.4 M NaBr		
37	0.88 (±0.01)	0.65	20	1.67 (±0.05)	0.78
37	0.90 (±0.01)	0.67	25	0.24 (±0.005)	0.78
			30	0.03 (±0.007)	0.78

[a] All measurements were performed in buffer H, pH 8.1; [SSB] = 0.14 μM (tetramer). [b] Errors in K_{70} are determined from the fitting routine. [c] Errors in Q_{max} are ±2%.

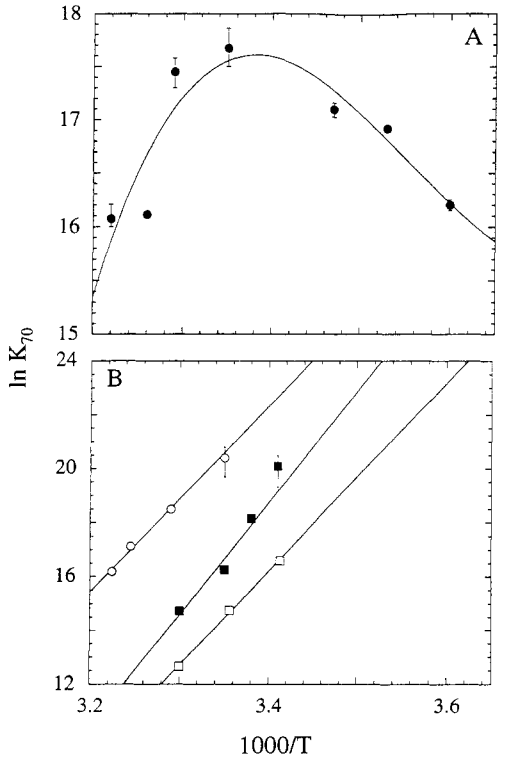


FIGURE 2: Comparison of the temperature dependences (van't Hoff plots) of K_{70} for SSB binding to $dA(pA)_{69}$, $dT(pT)_{69}$, and $dC(pC)_{69}$. (A) A nonlinear van't Hoff plot is obtained for SSB tetramer binding to $dA(pA)_{69}$ (0.17 M NaCl, pH 8.1). The solid curve is included for clarity and has no theoretical basis. (B) Linear van't Hoff plots are obtained for SSB tetramer binding to $dT(pT)_{69}$ and $dC(pC)_{69}$: (○) $dC(pC)_{69}$ (1 M NaBr, pH 8.1); (■), $dT(pT)_{69}$ (2 M NaBr, pH 8.1); (□) $dT(pT)_{69}$ (2.4 M NaBr, pH 8.1). The values of $\Delta H°_{obs}$ calculated from the slopes of these plots are given in Table 4.

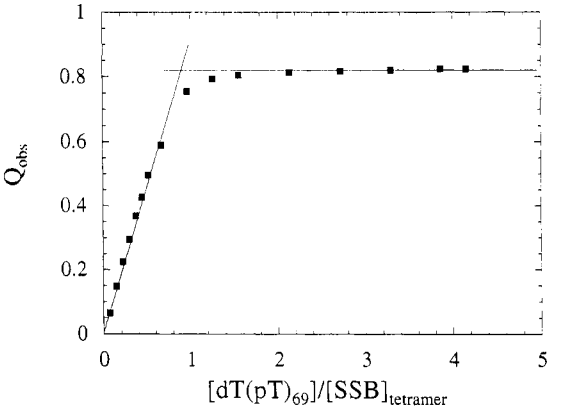


FIGURE 3: The binding of SSB to $dT(pT)_{69}$ is stoichiometric at 15 °C in buffer H, pH 8.1 and 2 M NaBr. The quenching increases linearily until Q_{max} = 0.82 (±0.02) with saturation occurring at ≈1 tetramer per $dT(pT)_{69}$. [SSB] = 1.4 × 10^{-7} M (tetramer).

1989; Lohman & Mascotti, 1992a). By contrast, the SSB–$dA(pA)_{69}$ equilibrium constant, K_{70}, is largest at 25 °C and decreases at both higher and lower temperatures (see Figure 1A).

The differences in the binding of SSB to $dC(pC)_{69}$ and $dT(pT)_{69}$ vs $dA(pA)_{69}$ are more apparent when the data are displayed as van't Hoff plots, as in Figure 2. The binding constants for the SSB–$dC(pC)_{69}$ and SSB–$dT(pT)_{69}$ interactions show linear van't Hoff plots (Figure 2B) with large, negative values of $\Delta H°_{obs}$ (−70 to −80 kcal/mol) that appear to be independent of temperature ($\Delta C°_{P,obs}$ = 0) over the range investigated. The values of $\Delta H°_{obs}$ calculated from the slopes of the lines in Figure 2b are given in Table 4. However, Figure 2A shows a distinctly nonlinear van't Hoff plot for the SSB–$dA(pA)_{69}$ interaction indicating a large negative *apparent* heat capacity change ($\Delta C°_{P,obs} < 0$). $\Delta H°_{obs}$ is positive (~10 kcal/mol) below 25 °C, whereas it becomes negative (~−25 kcal/mol) above 25 °C.

Note that the equilibrium constants for SSB binding to $dT(pT)_{69}$ and $dC(pC)_{69}$ were obtained over a smaller temperature range than those for the SSB–$dA(pA)_{69}$ interaction. This is a consequence of the relatively large, negative $\Delta H°_{obs}$ associated with the $dT(pT)_{69}$ and $dC(pC)_{69}$ interactions which limits the temperature range over which the binding constants can be determined accurately. Figure 3 shows the results of a titration of SSB protein with $dT(pT)_{69}$ performed at 15 °C in 2 M NaBr, which indicates that binding is stoichiometric at 0.9 ± 0.04 SSB tetramer per $dT(pT)_{69}$. Therefore, although the binding constant cannot be measured accurately for the SSB–$dT(pT)_{69}$ interaction at this lower temperature, the binding constant continues to increase upon decreasing the temperature below 25 °C, rather than *decrease* as it does for the SSB–$dA(pA)_{69}$ interaction.

It is possible that the nonlinear van't Hoff plot observed for the SSB–$dA(pA)_{69}$ interaction results from a linkage of SSB–$dA(pA)_{69}$ binding with other processes, such as tetramer denaturation or dissassembly, or changes in the binding mode of SSB when bound to the DNA (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988; Kuil et al., 1990). It is also possible that the qualitatively different temperature dependencies may be due to the differences in salt type (NaBr vs NaCl) or concentration that were required to measure the binding constants. We examine each of these possibilities below and conclude that these effects do not explain

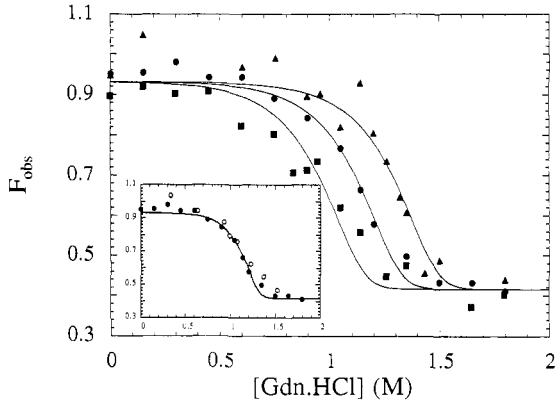


FIGURE 4: Denaturation of the SSB tetramer at 37 °C. The fluorescence of the SSB tetramer at 347 nm was monitored as a function of guanidine hydrochloride concentration in buffer H and 0.2 M NaCl, pH 8.1, at three different SSB tetramer concentrations: (■) 0.04 μM, (●) 0.14 μM, (▲) 0.54 μM. The solid curves are global nonlinear least squares fits of the data to a two-state unfolding transiton (see Thermodynamic Background) with $m = -13.9$ (±0.13) kcal/(mol M) and $\Delta G_0 = 42.8$ (±0.2) kcal/mol. Inset: Comparison of unfolding (●) and refolding (○) curves for SSB tetramer concentration of 0.14 μM.

the qualitatively different temperature dependence observed for the SSB–$dA(pA)_{69}$ equilibrium.

SSB Protein Remains Tetrameric. In principle, it is possible that the nonlinear van't Hoff plots observed for SSB tetramer–$dA(pA)_n$ binding may result from a temperature-dependent change in the fraction of free SSB protein that is tetrameric. Since DNA binding stabilizes the tetramer (Bujalowski & Lohman, 1991a,b), this would result in a linkage between tetramer assembly and DNA binding, which could yield nonlinear van't Hoff plots. Although this possibility is unlikely since this effect should be independent of the oligodeoxynucleotide, we have examined the stability of the SSB tetramer under the conditions of our experiments.

Previous studies indicate that, in the absence of DNA, the SSB tetramer is stable at concentrations as low as 30 nM (tetramer) at 25 °C (Bujalowski & Lohman, 1991a) over a range of solution conditions (Williams et al., 1984; Bujalowski & Lohman, 1991a; Lohman et al., 1986b; Ruyechan & Wetmur, 1976; Overman et al., 1988). Williams et al. (1984) have also shown by gel filtration that the tetramer is stable at concentrations as low as 0.125 μM at 45 °C. However, we wished to examine the stability of the SSB tetramer under the conditions and at the protein concentrations used in the experiments reported here.

To examine tetramer stability, we performed guanidine hydrochloride (Gdn·HCl) denaturation studies as well as sedimentation velocity studies as a function of SSB protein concentration in 0.2 M NaCl at 37 °C, pH 8.1. Of all of the conditions used in our experiments, the SSB tetramer should be least stable under these conditions. On the basis of the expected stability of the SSB tetramer, it would be necessary to carry out experiments at subnanomolar protein concentrations in order to detect dissociation of the tetramer in 0.2 M NaCl at 37 °C, pH 8.1; however, this is not possible using our fluorescence techniques. Therefore, we examined the stability of the tetramer under our solution conditions (0.2 M NaCl, pH 8.1, 37 °C) in the presence of increasing amounts of Gdn·HCl. The stability of the tetramer in the absence of denaturant was then estimated by extrapolation of these data to zero Gdn·HCl concentration (Pace, 1986).

Figure 4 shows the results of Gdn·HCl-induced denaturation studies performed at three SSB concentrations (0.54, 0.14, and 0.04 μM tetramer), monitored by the decrease in SSB tryptophan fluorescence upon denaturation. Each transition was reversible as determined from protein refolding experiments (Figure 4, inset). Figure 4 indicates that the midpoint of the unfolding transition shifts to higher [Gdn·HCl] upon increasing the protein concentration, as expected for a tetrameric protein for which disassembly is linked to denaturation. We have analyzed these denaturation curves assuming a two-state transition between native tetramer and denatured monomers (see Materials and Methods) which provides a good description of the unfolding transitions as shown in Figure 4. Simultaneous analysis of the three denaturation curves in Figure 4 according to the two-state model yields $\Delta G°_0 = 42.8$ (±0.2) kcal mol^{-1} for native tetramer unfolding to denatured monomers in the absence of denaturant. This value indicates that >95% of the SSB protein is tetrameric at protein concentrations as low as 0.5 nM (tetramer) at pH 8.1 and 37 °C in 0.2 M NaCl.

We also performed sedimentation velocity experiments as a function of SSB concentration in the range 0.14–1.16 μM (tetramer) (10 mM Tris, pH 8.1, and 0.2 M NaCl, 37 °C). The value of $s_{20,w} = 4.03$ (±0.08) was independent of protein concentration and in agreement with the value obtained for tetrameric SSB at protein concentrations >1 μM (Weiner et al., 1975; Ruyechan & Wetmur, 1976; Overman et al., 1988; Overman & Lohman, 1994).

These results indicate that the free SSB tetramer is stable under the conditions of our experiments and that the nonlinear van't Hoff plot observed for the $dA(pA)_{69}$ interaction does not result from a temperature-dependent change in the stability of the SSB tetramer. The fact that linear van't Hoff plots are observed for SSB tetramer binding to $dT(pT)_{69}$ and $dC(pC)_{69}$ also supports this conclusion. If disassembly of free SSB tetramers were the cause of the nonlinear van't Hoff plots in the $dA(pA)_{69}$ binding experiments, then similar results should be apparent in the $dT(pT)_{69}$ and $dC(pC)_{69}$ experiments unless SSB tetramer stability increases with increasing salt concentration. However, previous studies of the SSB-1 mutant protein (SSB-1 shows decreased tetramer stability, relative to wild-type SSB) indicate that the SSB-1 monomer–tetramer equilibrium is independent of [NaCl] from 50 mM to 1 M (Bujalowski & Lohman, 1991a).

Nonlinear van't Hoff Plots Are Not Due to a Temperature-Dependent Shift in SSB Binding Modes. Equilibrium binding of *E. coli* SSB tetramer to ss-DNA can be complex since tetramers can bind to ss-DNA in multiple binding modes and the relative stabilities of these modes are sensitive functions of solution conditions (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988). In fact, we have recently shown that at low [NaCl], SSB tetramers can even bind to $dA(pA)_{69}$ in at least two different binding modes (Ferrari et al., 1994). Therefore, it is possible, in principle, that the nonlinear van't Hoff plot is due to a temperature-dependent shift in the relative stability of a different SSB binding mode, which possesses a different $\Delta H°$. For example, a temperature-dependent increase in the relative population of the $(SSB)_{35}$ mode relative to the $(SSB)_{65}$ mode might occur. We examine this possibility below and conclude that such shifts in the relative population of different SSB–ss-DNA binding modes do not occur under the conditions of our experiments.

At intermediate salt concentrations (0.125 M NaCl, pH 8.1, 25 °C), an equilibrium mixture of 2:1 and 1:1 SSB–$dA(pA)_{69}$ complexes can form (Ferrari et al., 1994). At low SSB binding densities, 1:1 SSB–$dA(pA)_{69}$ complexes can form

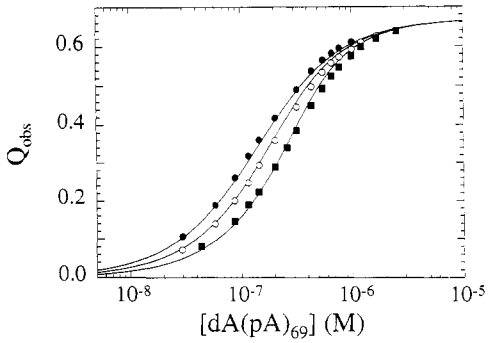


FIGURE 5: Equilibrium titrations of SSB with dA(pA)$_{69}$, plotted as a function of the quenching of the SSB tryptophan fluorescence. Experiments were performed in buffer H and 0.17 M NaCl, pH 8.1, at 37 °C and the following SSB concentrations: (●) 0.074, (○) 0.148, and (■) 0.295 μM. The binding is well represented by a 1:1 binding; the solid lines are simulated according to a simple 1:1 model (eq 1) with K_{70} = 1.0 × 10^7 M^{-1} and Q_{max} = 0.67.

in which dA(pA)$_{69}$ interacts with either two or four protomers within the SSB tetramer, whereas higher SSB binding densities favor formation of a 2:1 complex in which two SSB tetramers bind to dA(pA)$_{69}$, where only two subunits of each SSB tetramer interact with the DNA. From equilibrium binding studies we have estimated that the nearest neighbor equilibrium cooperativity parameter for the (SSB)$_{35}$ binding mode, ω_{35}, is greater than 10^5 (Ferrari et al., 1994). On the basis of these studies, we can calculate the distribution of these different binding modes in 0.125 M NaCl, pH 8.1, 25 °C; however, we have not previously examined how these distributions are influenced quantitatively by temperature. Overman (1989) showed that the nearest neighbor equilibrium cooperativity parameter for the (SSB)$_{65}$ binding mode, ω_{65}, increases with increasing temperature; hence it is possible that ω_{35} possesses a similar temperature dependence. If this were the case, a mixture of 2:1 and 1:1 SSB–dA(pA)$_{69}$ complexes might occur at higher temperature, resulting in a nonlinear van't Hoff plot.

Although the binding curves shown in Figure 1 are well described by a simple 1:1 interaction, the possibility that multiple SSB binding modes could form at equilibrium under these conditions (buffer H, pH 8.1, 0.17 M NaCl, 37 °C) was investigated further by performing equilibrium titrations at a series of SSB protein concentrations ranging from 0.074 to 0.295 μM (tetramer). Figure 5 shows the results of these experiments along with simulations for a 1:1 binding equilibrium, using the same binding constant, K_{70} = 1 × 10^7 M^{-1} (Q_{max} = 0.67), for each simulation. These results indicate that the 1:1 complex in which all four SSB subunits interact with dA(pA)$_{69}$ is the dominant form and that other SSB binding modes, if present, are negligible under these conditions. This indicates that the nonlinear van't Hoff plot for the SSB–dA(pA)$_{69}$ equilibrium binding interaction is not due to a stabilization of the (SSB)$_{35}$ mode at higher temperature. This conclusion is further supported by equilibrium binding studies of shorter oligodeoxyadenylates, as discussed below.

We have also investigated the possibility that the nonlinear van't Hoff plots for the SSB–dA(pA)$_{69}$ interaction might result from stabilization of the (SSB)$_{56}$ mode (Bujalowski & Lohman, 1986; Bujalowski et al., 1988). Studies with polynucleotides have shown that the salt concentration required to stabilize each binding mode varies slightly with polynucleotide type (Lohman & Bujalowski, 1994). However, the linkage of effects of temperature and polynucleotide type on the relative stability of the (SSB)$_{56}$ mode has not been fully investigated. To investigate the possibility that a temperature-dependent shift in the relative stabilities of the (SSB)$_{65}$ and (SSB)$_{56}$ binding modes might be causing the nonlinear van't Hoff plots with dA(pA)$_{69}$, we performed equilibrium titrations as a function of temperature with the series dX(pX)$_{55}$ (X = A, T, or C).

The experimental isotherms (Q_{obs} vs total dX(pX)$_{55}$ concentration) were all consistent with a 1:1 binding model (data not shown) and were analyzed using eq 1 to obtain values of K_{56} and Q_{max} (see Table 2). The van't Hoff plots for the SSB–dX(pX)$_{55}$ interactions shown in Figure 6 are qualitatively similar to those obtained with dX(pX)$_{69}$. The van't Hoff plot for the SSB–dA(pA)$_{55}$ interaction is clearly nonlinear; however, the van't Hoff plots for SSB tetramer binding to dT(pT)$_{55}$ or dC(pC)$_{55}$ are linear, with $\Delta H°_{obs}$ = −70 ± 2 and −100 ± 6 kcal/mol, respectively. These values of $\Delta H°_{obs}$ are of similar sign and magnitude to those obtained with dT(pT)$_{69}$ and dC(pC)$_{69}$ (see Table 4).

It is interesting that $\Delta H°_{obs}$ for the SSB–dC(pC)$_{55}$ interaction is significantly larger in absolute magnitude (−100 ± 6 kcal/mol) than the $\Delta H°$ for the SSB–dC(pC)$_{69}$ interaction (−68 ± 7 kcal/mol) (1 M NaBr, pH 8.1). In this regard we also note that the absolute magnitude of the salt dependencies of the equilibrium constants for SSB tetramer binding to dC(pC)$_{34}$ and dC(pC)$_{69}$ are unusual in that both d log K_n/d log[NaCl] and d log K_n/d log[NaBr] are larger in absolute magnitude for dC(pC)$_{34}$ (−6.3 ± 0.5) than for dC(pC)$_{69}$ (−3.8 ± 0.6) (25 °C, pH 8.1) (Lohman & Bujalowski, 1994). These results likely reflect the fact that differential anion and cation binding to the SSB protein also contributes to the salt dependence of the equilibrium constants (Lohman & Bujalowski, 1994). Thus, the expected result that net ion release should increase with increasing DNA length if only cation release from the DNA occurs (Record & Lohman, 1978; Olmsted et al., 1989) is not observed. These results emphasize that most thermodynamic properties of SSB–DNA interactions are not additive.

Effects of NaBr vs NaCl. As discussed above, the large differences in relative affinities of SSB tetramer for the different oligonucleotides makes it impossible to compare directly the temperature dependencies under the same set of solution conditions. As a result, the dA(pA)$_{69}$ and dA(pA)$_{55}$ experiments were performed in 0.17 M NaCl (pH 8.1), whereas the dT(pT)$_n$ and dC(pC)$_n$ data were obtained in solutions containing NaBr (1–2.4 M NaBr, pH 8.1). Therefore, it is possible that the qualitatively different temperature dependencies we observe for dT(pT)$_n$ and dC(pC)$_n$ vs dA(pA)$_n$ are due to the different salt concentration ranges and anions (Cl^- vs Br^-) used for these experiments.

To test this further, we examined the temperature dependence of SSB tetramer binding to dA(pA)$_{34}$ vs dT(pT)$_{34}$ in solutions containing NaBr. The equilibrium constants for SSB binding to dT(pT)$_{34}$ and dA(pA)$_{34}$ can be measured in buffers containing NaBr over a wide temperature range; however, since the affinity of SSB for dA(pA)$_{34}$ is low relative to that for dT(pT)$_{34}$, significantly lower NaBr concentrations are required for the dA(pA)$_{34}$ studies (80 mM vs 0.6 M NaBr (pH 8.1)). However, the main purpose of this comparison is to determine whether the nonlinear van't Hoff plot observed for the SSB–dA(pA)$_n$ interaction results from some specific effect of Cl^- vs Br^-.

Equilibrium binding of oligodeoxynucleotides to SSB has been examined as a function of DNA length, salt concentration, and temperature and analyzed using a statistical thermodynamic "square" model that accounts for the negative cooperativity which exists among ss-DNA binding sites on

Table 2: Equilibrium Constants for SSB Tetramer Binding to $dX(pX)_{55}$[a]

T (°C)	$K_{56} \times 10^{-7}$ M^{-1}[b]	Q_{max}[c]	T (°C)	$K_{56} \times 10^{-7}$ M^{-1}[b]	Q_{max}[c]
$dA(pA)_{55}$, 0.17 M NaCl			$dC(pC)_{55}$, 1 M NaBr		
5	0.66 (±0.01)	0.64	30	17.25 (±4.18)	0.66
5	0.77 (±0.01)	0.64	32	4.86 (±0.31)	0.66
15	1.42 (±0.06)	0.63	34	1.52 (±0.05)	0.65
25	1.48 (±0.04)	0.64	37	0.40 (±0.01)	0.65
25	1.42 (±0.05)	0.64	$dT(pT)_{55}$, 2 M NaBr		
30	1.47 (±0.08)	0.63	15	15.54 (±5.74)	0.66
37	0.65 (±0.02)	0.64	18	4.28 (±0.30)	0.65
37	1.02 (±0.04)	0.64	20	1.78 (±0.09)	0.66
			23	0.57 (±0.01)	0.65
			25	0.26 (±0.01)	0.65

[a] All measurements were performed in buffer H, pH 8.1; [SSB] = 0.14 μM (tetramer). [b] Errors in K_{56} are determined from the fitting procedure. [c] Errors in Q_{max} are ±2%.

Table 3: Equilibrium Constants for SSB Tetramer Binding to $dX(pX)_{34}$[a]

T (°C)	$K_{35} \times 10^{7}$ M^{-1}	σ_{35}	Q_1	Q_2
$dT(pT)_{34}$, 0.6 M NaBr				
15	58.9 (±2)	0.42[b]	0.50[b]	0.90[b]
20	7.9 (±1)	0.42 (±0.04)	0.52	0.90
25	3.08 (±1)	0.39 (±0.03)	0.50	0.90
30	0.7 (±0.1)	0.41 (±0.03)	0.50	0.90
35	0.13 (±0.02)	0.43 (±0.04)	0.49	0.90
$dA(pA)_{34}$, 0.08 M NaBr[c]				
5	0.47 (±0.03)	0.28	0.40	0.7
15	0.56 (±0.02)	0.28	0.40	0.7
20	0.83 (±0.07)	0.28	0.40	0.7
25	0.84 (±0.1)	0.28	0.40	0.7
30	1.5 (±0.2)	0.28	0.40	0.7
37	0.35 (±0.04)	0.28	0.39	0.67

[a] All measurements were performed in buffer H, pH 8.1; [SSB] = 0.14 μM (tetramer). [b] This value was fixed. [c] σ_{35}, Q_1, and Q_2 were fixed at the indicated values.

the tetramer (Bujalowski & Lohman, 1989 a,b). The square model is described by two parameters: the intrinsic equilibrium binding constant, K_n, and a negative cooperativity constant, σ_n (where n refers to the length of the oligodeoxynucleotide). Oligodeoxynucleotides with lengths of 35 nucleotides bind with maximum stoichiometries of 2 and display salt-dependent negative cooperativity (Lohman & Bujalowski, 1988, 1994; Bujalowski & Lohman, 1989a,b). However, the negative cooperativity parameter σ_{35} displays very little dependence on temperature for the SSB–$dT(pT)_n$ interaction (Bujalowski & Lohman, 1989a,b).

The results of equilibrium titrations of SSB with $dA(pA)_{34}$ and $dT(pT)_{34}$ performed at different temperatures are shown in Figure 7. The solid lines are simulations based on eq 3 and the values of K_{35} and σ_{35} obtained from nonlinear least squares analysis of the data according to the square model (see Table 3), with the values of $Q_{1/35}$ and $Q_{2/35}$ constrained to those determined previously (Bujalowski & Lohman, 1989a,b). In the case of $dT(pT)_{34}$, K_{35} decreases with increasing temperature over the full temperature range, while σ_{35} remains fairly constant over the temperature range investigated, in agreement with previous results with $dT(pT)_{16}$ (Bujalowski & Lohman, 1989b). However, for the SSB–$dA(pA)_{34}$ interaction, K_{35} is highest at 31 °C, with weaker binding observed at both 5 and 37 °C. These results are qualitatively similar to those obtained with $dA(pA)_{55}$ and $dA(pA)_{69}$ in 0.17 M NaCl. Due to the significantly higher degree of negative cooperativity associated with the binding of SSB to $dA(pA)_{34}$ relative to $dT(pT)_{34}$ (Lohman & Bujalowski, 1994), it is more difficult to bind a second molecule of $dA(pA)_{34}$. Thus we did not attempt to obtain an accurate estimate of σ_{35}, but rather fixed σ_{35} at 0.28, which is the same, within error, as the value determined

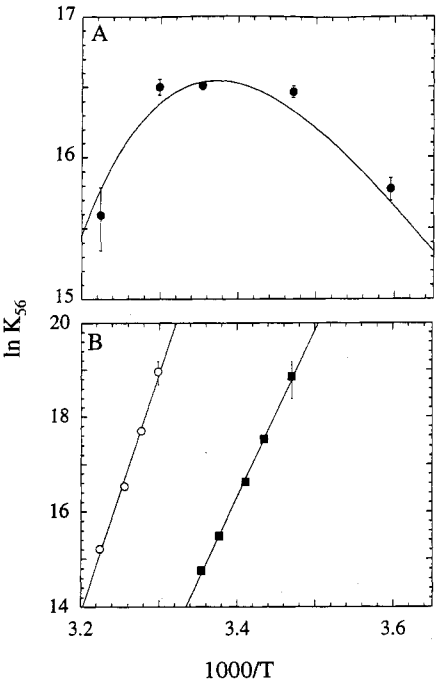


FIGURE 6: van't Hoff plots comparing the temperature dependence of K_{56} for the SSB–$dA(pA)_{55}$ interaction and the SSB–$dC(pC)_{55}$ and SSB–$dT(pT)_{55}$ interactions. (A) The temperature dependence of SSB–$dA(pA)_{55}$ interaction (0.17 M NaCl, pH 8.1) is nonlinear and shows the same qualitative behavior as the van't Hoff plot for the SSB–$dA(pA)_{69}$ interaction (Figure 1A). (B) Linear van't Hoff plots are obtained for the pyrimidines (○) $dC(pC)_{55}$ (1 M NaBr, pH 8.1) and (■) $dT(pT)_{55}$ (2 M NaBr, pH 8.1). The enthalpy values are given in Table 4. The SSB tetramer concentration is 1.4×10^{-7} M.

Table 4: Thermodynamic Parameters for SSB–Oligodeoxypyrimidine Interactions[a]

DNA	[NaBr]	$\Delta H°$, kcal (mol DNA)$^{-1}$	$\Delta S°$, cal (mol DNA)$^{-1}$ K^{-1}
$dC(pC)_{69}$	1.0	−68 (±7)	−188 (±23)
$dC(pC)_{55}$	1.0	−100 (±6)	−293 (±19)
$dT(pT)_{69}$	2.0	−82 (±17)	−242 (±60)
$dT(pT)_{69}$	2.4	−69 (±4)	−204 (±12)
$dT(pT)_{55}$	2.0	−70 (±2)	−205 (±5)
$dT(pT)_{34}$	0.6	−52 (±3)	−140 (±10)

[a] All measurements were performed in buffer H, pH 8.1; [SSB] = 0.14 μM (tetramer). Parameters were calculated from van't Hoff analysis.

previously from more extensive titrations at 25 °C (Lohman & Bujalowski, 1994). Figure 8 compares the van't Hoff plots for $dA(pA)_{34}$ and $dT(pT)_{34}$. For $dT(pT)_{34}$, the van't Hoff plot is linear, with $\Delta H°_{obs}$ = −52 ± 3 kcal/mol of DNA; however, curvature in the van't Hoff plot is clearly observed with $dA(pA)_{34}$. These results indicate that the nonlinear van't

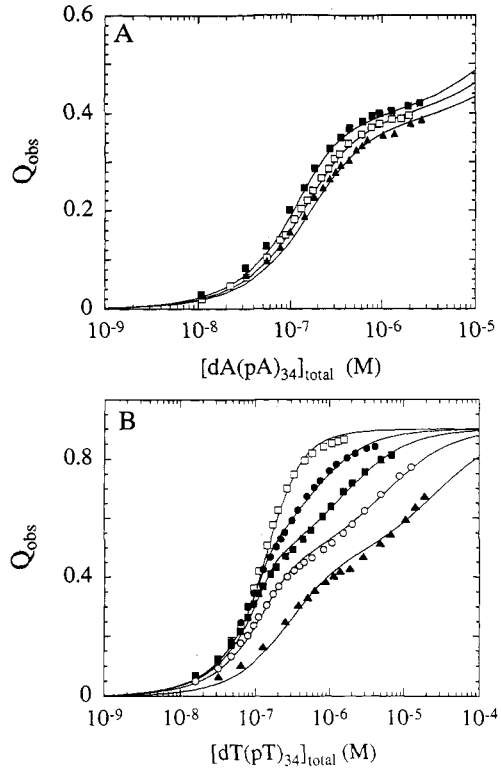


FIGURE 7: Fluorescence quenching of the SSB tetramer upon titration with $dA(pA)_{34}$ (panel A) and $dT(pT)_{34}$ (panel B) as a function of temperature. All titrations were performed in buffer H, pH 8.1, at an SSB tetramer concentration of 1.4×10^{-7} M. (A) SSB–$dA(pA)_{34}$ (80 mM NaBr). The temperature dependence of the binding shows the same trend as observed with $dA(pA)_{69}$ and $dA(pA)_{56}$. The binding is tighter at (■) 25 °C and weaker at (▲) 37 and (□) 5 °C. (B) SSB–$dT(pT)_{34}$ (0.6 M NaBr) shows that the binding decreases with increasing temperature: (□) 15, (●) 20, (■) 25, (○) 30, and (▲) 35 °C. The equilibrium binding parameters obtained from non-linear least squares analysis of the data according to eq 6 are given in Table 3.

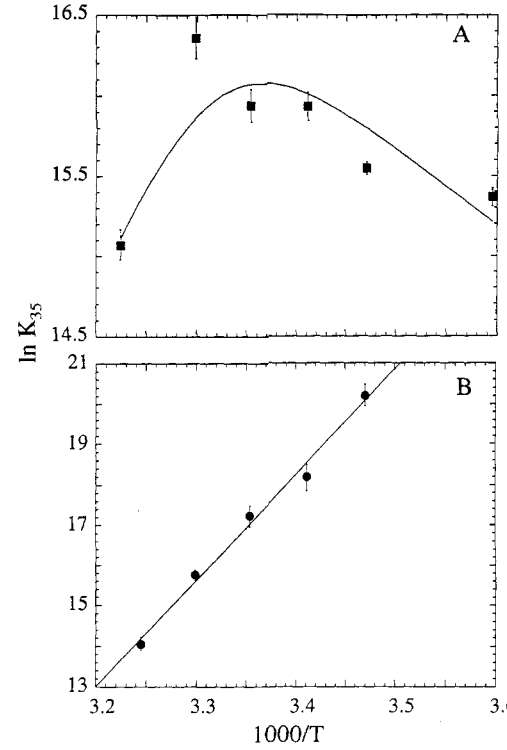


FIGURE 8: van't Hoff plots constructed from the data in Figure 7 comparing the temperature dependence of K_{35} in NaBr for the SSB–$dA(pA)_{34}$ and the SSB–$dT(pT)_{34}$ interactions. A nonlinear temperature dependence is obtained for the SSB–$dA(pA)_{34}$ interaction, whereas linear plots are obtained for the SSB–$dT(pT)_{34}$ interaction. (A) SSB–$dA(pA)_{34}$ (80 mM NaBr, pH 8.1). (B) SSB–$dT(pT)_{34}$ (0.6 M NaBr, pH 8.1); $\Delta H°_{obs} = -52 \pm 3$ kcal/mol, determined from the slope of the line.

Hoff plots observed for SSB–$dA(pA)_n$ binding are independent of anion type and appear to be solely a function of the DNA base type.

Since the experiments with $dT(pT)_n$ and $dC(pC)_n$ were performed at higher salt concentrations than those with $dA(pA)_n$, we also examined the [NaCl] dependence of K_{70} to determine whether the nonlinear van't Hoff plots observed for the SSB–$dA(pA)_n$ are also observed at higher NaCl concentrations. Salt back titrations were performed under conditions where a 1:1 SSB–$dA(pA)_{69}$ complex is formed, and K_{70} was determined as a function of NaCl concentration at several temperatures (5, 10, 15, 20, 25, 34, and 37 °C). At each temperature, log K_{70} is a linear function of log[NaCl] and K_{70} decreases with increasing [NaCl], indicating that a net release of ions (Na^+ plus Cl^-) accompanies complex formation (Record et al., 1976, 1978). However, the value of d log K_{70}/d log[NaCl] varies with temperature, increasing from –5.7 at 5 °C to –4.3 at 37 °C (data not shown), indicating that net ion release decreases at higher temperatures. These data were used to construct a van't Hoff plot at 0.35 M, NaCl which is the highest [NaCl] within the range used in the salt back titrations. The van't Hoff plot constructed from the 0.35 M NaCl data shows the same qualitative nonlinear behavior, with a broad optimum in K_{obs} in the range from 9–30 °C, indicating that this behavior is not dependent upon the lower [NaCl] used for the $dA(pA)_n$ experiments. On the other hand, there is a clear linkage between the temperature dependence and the salt dependence of K_{obs}, as we have observed previously for the interaction of SSB with poly(U) in the $(SSB)_{65}$ mode. However, d log K_{obs}/d log[NaCl] for the SSB–poly(U) interaction shows an even larger dependence on temperature, decreasing from –9.3 ± 0.3 at 10 °C to –5.1 ± 0.4 at 37 °C (Overman, 1989; Lohman & Mascotti, 1992a).

DISCUSSION

Our studies indicate qualitatively different behavior for the temperature dependence of the equilibrium constants for *E. coli* SSB tetramer binding to $dA(pA)_n$ vs $dC(pC)_n$ and $dT(pT)_n$ ($n = 35, 55, 69$). The binding of SSB to $dC(pC)_n$ and $dT(pT)_n$ is enthalpically driven with large negative values for $\Delta H°_{obs}$ that are apparently constant over the temperature range investigated, suggesting that $\Delta C°_{P,obs} = 0$ for these interactions. However, a distinctly nonlinear van't Hoff plot, with an optimum in K_{obs} occurring near 25–30 °C, is obtained for SSB tetramer binding to $dA(pA)_n$. For $T < 25$ °C, $\Delta H°_{obs}$ is small and positive, and the binding is entropically driven, whereas for $T > 25$ °C, $\Delta H°_{obs}$ is negative and binding is enthalpically driven. Therefore, the binding of the SSB tetramer to $dA(pA)_n$ is accompanied by a large negative heat capacity change ($\Delta C°_{P,obs} < 0$).

The possible origins of heat capacity changes accompanying macromolecular interactions or macromolecular conformational changes have been discussed previously for protein folding (Brandts, 1964a,b; Sturtevant, 1977; Baldwin, 1986; Privalov & Gill, 1988; Murphy & Freire, 1992), protein–ligand interactions (Sturtevant, 1977; Eftink et al., 1983; Gill et al., 1985; Wyman & Gill, 1990; Spolar & Record, 1994), and protein–nucleic acid interactions (Ha et al., 1989; Spolar & Record, 1994). The potential origins appear to be (1)

changes in water-accessible surface area upon complexation, resulting in release of water of "hydrophobic hydration" (i.e., the hydrophobic effect) (Kauzmann, 1959; Brandts, 1964a,b; Sturtevant, 1977; Ha et al., 1989; Spolar & Record, 1994); (2) changes in the internal vibrational states of the macromolecules (Sturtevant, 1977); and (3) linkage of two or more temperature-dependent equilibria (Sturtevant, 1977; Eftink et al., 1983; Gill et al., 1985; Wyman & Gill, 1990).

For the equilibrium binding of SSB tetramer to $dA(pA)_n$, we have shown that $\Delta C°_{P,obs}$ is *not* due to any of the following: (1) partial dissociation of the unliganded SSB tetramer with increasing temperature, (2) a temperature-dependent shift in SSB binding modes, or (3) effects due to the type and concentration of monovalent salt. On the basis of these results, we conclude that two possibilities remain. The first is that the large negative $\Delta C_{P,obs}$ is due to net removal of apolar groups from water and the resulting hydrophobic effects. This proposal has been used to explain similar observations for sequence-specific protein–DNA interactions (Ha et al., 1989; Spolar & Record, 1994). The second possibility, which we favor as discussed below, is that the overall process for SSB tetramer binding to $dA(pA)_n$ is composed of two linked equilibria: the unstacking of some number of adenine base pairs (occurring with $\Delta H° > 0$), which is coupled to SSB binding to the DNA (occurring with $\Delta H° < 0$) (see Scheme 1). Furthermore, since both of these separate processes occur with $\Delta C°_P \approx 0$, the observation of an *apparent* $\Delta C°_{P,obs} < 0$ is due solely to the coupling of these two equilibria rather than to a $\Delta C°_P$ resulting from other origins such as hydrophobic or vibrational effects. The apparent lack of a $\Delta C°_{P,obs}$ associated with SSB binding to $dC(pC)_n$ and $dT(pT)_n$ is due to the fact that the bases within these DNA molecules show negligible stacking, and thus no unstacking accompanies the overall binding equilibrium. Similar observations of apparent heat capacity changes in other systems have been explained in terms of such linked equilibria (Filimonov & Privalov, 1978; Eftink et al., 1983; Gill et al., 1985).

Base-Stacking Properties of Single-Stranded Homopolydeoxynucleotides. It is well established that extensive base stacking occurs within poly(dA) and poly(A) at low temperatures and that a broad temperature-dependent unstacking transition occurs for these homopolynucleotides (Leng & Felsenfeld, 1966; Riley et al., 1966; Applequist & Damle, 1965; Poland et al., 1966; Ts'o et al., 1966; Vournakis et al., 1967; Epand & Scheraga, 1967). The $\Delta H°$ for adenine unstacking in poly(A) is positive (3.0–3.4 kcal/mol (stack) (Breslauer & Sturtevant, 1977; Filimonov & Privalov, 1978), so that the fraction of unstacked bases increases with increasing temperature. The temperature-induced unstacking transition of poly(dA) is broad with a T_m near ~55 °C (Riley et al., 1966). The hyperchromism associated with the melting transition of poly(dA) is ~30% at 260 nm (pH 7.8), independent of [NaCl] between 0.05 and 0.5 M, although it is slightly higher at 0.013 M NaCl and slightly lower at 5 M NaCl (Riley et al., 1966). Our melting studies confirm these observations for the smaller oligodeoxynucleotides, $dA(pA)_{69}$ and $dA(pA)_{34}$ (M. E. Ferrari, unpublished experiments). By contrast, there is negligible base stacking in either poly(dT) or poly(dC) in the pH range > 7 (Riley et al., 1966; Ts'o et al., 1966; Bloomfield et al., 1974; Felsenfeld & Miles, 1967). At pH 7.8, poly(dT) shows <5% hyperchromism, with a midpoint near ~65 °C, independent of [NaCl] (Riley et al., 1966). Poly(dC) (pH 8.4, 0.05 M sodium phosphate) shows no hyperchromism between 23 and 90 °C at its absorption maximum of 267 nm and thus is considerably less ordered than its polyribonucleotide counterpart, poly(C) (Ts'o et al., 1966).

Electric field induced birefringence and circular dichroism studies indicate that the bases in poly(A) become unstacked, at least partially, upon binding *E. coli* SSB protein (Kuil et al., 1990). On the other hand, since the bases within free $dT(pT)_n$ or $dC(pC)_n$ are not stacked significantly, similar base unstacking should not occur upon SSB binding to $dT(pT)_n$ or $dC(pC)_n$. In addition, calorimetric evidence indicates $\Delta C_p = 0$ for the unstacking of adenine bases (Filimonov & Privalov, 1978), and we have shown that $\Delta C°_p \approx 0$ for the binding of SSB tetramer to $dT(pT)_{69}$. Furthermore, the linear van't Hoff plots obtained for the binding of SSB to $dC(pC)_n$ and $dT(pT)_n$ as well as for the binding of SSB to poly(U) (Overman, 1989) are consistent with $\Delta C_{P,obs} = 0$ for SSB tetramer binding to single-stranded nucleic acids containing unstacked bases.

Models for the Apparent Heat Capacity Change upon Binding SSB Tetramer to $dA(pA)_n$. The nonlinear van't Hoff plots observed for SSB–$dA(pA)_n$ binding can be explained by a model in which base unstacking within $dA(pA)_n$ is coupled to SSB binding to the DNA. The $\Delta H°_{obs}$ values for these two processes have opposite signs; the binding of SSB to unstacked DNA is negative, as we show here for SSB binding to $dT(pT)_n$ and $dC(pC)_n$, while $\Delta H°_{obs}$ for adenine base unstacking is positive (Breslauer & Sturtevant, 1977; Filimonov & Privalov, 1978). Therefore, the overall reaction displays a temperature-dependent $\Delta H°_{obs}$ and thus an *apparent* negative $\Delta C°_{P,obs}$ due to these coupled equilibria.

We have considered a simple coupled base unstacking model to interpret the temperature dependence of SSB–$dA(pA)_n$ binding (Scheme 1), which considers the DNA to exist in a continuum of states differing in the fraction of stacked bases and that SSB binds to the unstacked form of the DNA. The expressions for $\Delta H°_{obs}$, the *apparent* $\Delta C°_{P,obs}$, and $\ln K_n$ for a model which assumes the base-unstacking transition to be non-cooperative are given in eqs 12, 13, and 14. Figure 9 compares the results of nonlinear least squares fitting of the SSB tetramer–$dA(pA)_n$ binding data to the coupled base unstacking model (eq 14), and the model which assumes a constant $\Delta C°_{P,obs}$ (eq 15). The solid line represents the best fit to the base-unstacking model, with the unstacking enthalpy, $\Delta H°_u$, fixed at 2.9 kcal/mol of adenine (Breslauer & Sturtevant, 1977; Filimonov & Privalov, 1978) and the T_m fixed at 55 °C (Riley et al., 1966). The values of $\Delta H°_0$ and $\Delta S°_0$ for SSB tetramer binding to the fully unstacked $dA(pA)_n$ (0.17 M NaCl, pH 8.1) were determined from nonlinear least squares fitting and are summarized in Table 5. The values of $\Delta H°_0$ (−128 ± 3, −102 ± 2, and −63 ± 3 kcal/mol for SSB binding to $dA(pA)_{69}$, $dA(pA)_{55}$, and $dA(pA)_{34}$, respectively) are considerably more negative than the experimental values of $\Delta H°_{obs}$ determined from van't Hoff analysis of the SSB–$dT(pT)_n$ and SSB–$dC(pC)_n$ data at high NaBr concentrations (−50 to −100 kcal/mol) (see Table 4). However, this is partly due to the fact that $\Delta H°_{obs}$ for SSB tetramer binding to single-stranded nucleic acids is salt dependent, with $\Delta H°_{obs}$ decreasing with decreasing salt concentration. We have observed this for SSB binding to poly(U) in the $(SSB)_{65}$ mode (Overman, 1989; Lohman & Mascotti, 1992b) as well as for SSB binding to $dA(pA)_{69}$ (see Results). In fact, preliminary isothermal titration calorimetry studies of SSB binding to $dT(pT)_{69}$ performed at 0.17 M NaCl (pH 8.1, 25 °C) (the same conditions used for our SSB–$dA(pA)_{69}$ binding studies) yield $\Delta H°_{obs} = -130 \pm 11$ kcal/mol (M. E. Ferrari, unpublished experiments), consistent with the value of $\Delta H°_0 = -128 \pm 3$ kcal/mol reported in Table 5 for SSB binding to unstacked

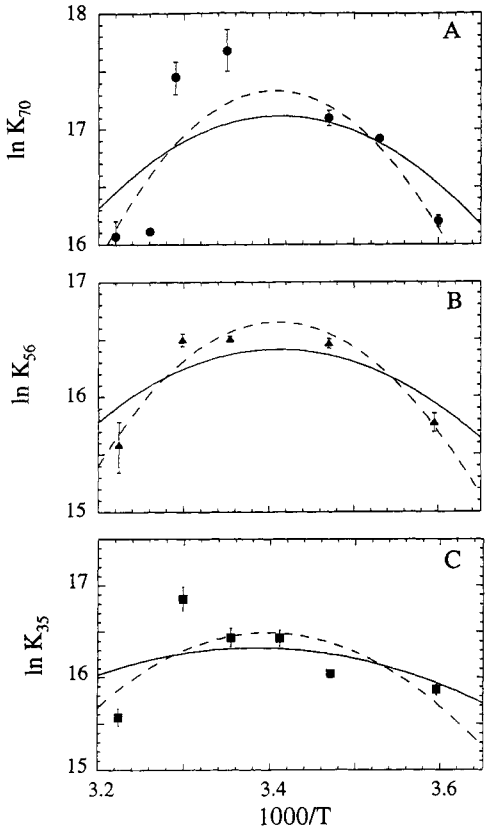


FIGURE 9: Comparison of the coupled stacking models and the constant $\Delta C^\circ_{P,obs}$ model to the van't Hoff plots for the SSB–dA(pA)$_n$ interaction. (A) n = 69; (B) n = 55; (C) n = 34. The data are fit to the continuous stacking model (eq 14) (—) and the constant $\Delta C^\circ_{P,obs}$ model (eq 15) (- - -). For the analysis with the coupled stacking model, the T_m and the unstacking enthalpy, ΔH°_u, are fixed at 328 K and 2.9 kcal/mol of adenine, respectively, and the enthalpy (ΔH°_0) and entropy (ΔS°_0) for SSB binding to the fully unstacked DNA are determined from the fitting routine (see Table 5). The best-fit parameters obtained from the constant $\Delta C^\circ_{P,obs}$ model (eq 15) are summarized in Table 6.

Table 5: Entropy and Enthalpy Change for SSB Binding to Fully Unstacked DNA Determined from the Coupled-Base Unstacking Model[a]

n	ΔS°_0, cal (mol DNA)$^{-1}$ K^{-1}	ΔH°_0, kcal (mol DNA)$^{-1}$
69	−264 (±8)	−128 (±3)
55	−205 (±6)	−102 (±2)
34	−114 (±10)	−63 (±3)

[a] Determined by fitting the temperature dependence of K_n for SSB–dA(pA)$_n$ binding (see Figure 9) to the coupled-base unstacking model (eq 14; Scheme 1). The unstacking enthalpy was fixed at 2.9 kcal/mol of adenine determined from calorimetric measurements (Breslauer & Sturdevant, 1977), and T_m was fixed at 55 °C (Riley et al., 1966).

dA(pA)$_{69}$. This agreement may be somewhat fortuitous since some differences in ΔH°_{obs} due to base composition are expected. However, these general results suggest that the adenine base unstacking model provides a plausible explanation for the nonlinear van't Hoff plots observed for SSB tetramer binding to dA(pA)$_n$.

We have also considered a modification of this model in which the base-unstacking transition is assumed to be fully cooperative (two states of dA(pA)$_n$), based on evidence that unstacking of the oligoribonucleotide A_7 shows two-state behavior (Breslauer & Sturtevant, 1977). Although this cooperative model can provide a good fit to the data, the fit requires a T_m near 30 °C for the dA(pA)$_n$ unstacking transition, whereas the observed T_m for dA(pA)$_n$ unstacking under these conditions is considerably higher (≥55 °C) (Riley et al., 1967; M. E. Ferrari, unpublished experiments). Therefore, we have not considered this model further. Although more complex models can be envisioned, these require additional adjustable parameters and would not provide any further explanation of the salient features of the temperature dependence of the SSB–dA(pA)$_n$ binding equilibrium.

Table 6: Thermodynamic Parameters for the SSB–dA(pA)$_n$ Interaction Determined Assuming a Constant ΔC°_p[a]

n	ΔC°_p (kcal mol^{-1} K^{-1})	T_H (°C)	T_s (°C)
69	−1.53 (±0.6)	20	27
55	−1.26 (±0.24)	20	28
34	−0.93 (±0.55)	21	31

[a] Determined by fitting the temperature dependence of K_n for SSB–dA(pA)$_n$ binding (see Figure 9), assuming a constant ΔC°_p (eq 15).

Comparison with a Model that Assumes a Constant Heat Capacity Change upon SSB Binding to dA(pA)$_n$. An alternative explanation for nonlinear van't Hoff plots such as we observe for SSB binding to dA(pA)$_n$ has been offered by Ha *et al.* (1989) and Spolar and Record (1994) to interpret the temperature dependencies of *E. coli* lac repressor and *Eco*RI protein binding to their specific DNA sites. This model attributes the large, negative $\Delta C^\circ_{P,obs}$ to the hydrophobic effect resulting from the burial of water-accessible surface area on the protein and DNA upon formation of the complex, which results in the concomitant "release of hydrophic water" and a positive entropy change as appears to be the case for protein folding (Kauzmann, 1959; Brandts, 1964a,b; Tanford, 1973; Sturtevant, 1977; Becktel & Schellman, 1987). In this model, the $\Delta C^\circ_{P,obs}$ is assumed to be independent of temperature, which is equivalent to assuming that the structures of the free protein, the free DNA, and the protein–DNA complex are independent of temperature. We have compared this model to the base-unstacking model for analysis of our SSB protein–dA(pA)$_n$ binding data.

Figure 9 compares the best fits of the coupled base-unstacking model (eq 14) and the constant $\Delta C^\circ_{P,obs}$ model (eq 15; dashed lines) for the SSB–dA(pA)$_n$ data. The values of $\Delta C_{P,obs}$ determined from fitting the dA(pA)$_{34}$, dA(pA)$_{55}$, and dA(pA)$_{69}$ data to eq 15 are −0.93 ± 0.55, −1.26 ± 0.24, and −1.53 ± 0.6 kcal/(mol deg), respectively (see Table 6). Both the constant $\Delta C^\circ_{P,obs}$ model and the base-stacking model provide adequate descriptions of the general features of the data. Even though the constant ΔC°_P model appears to fit the data slightly better, the two models cannot be differentiated on the basis of the comparisons in Figure 9.

We favor the explanation that the apparent negative heat capacity change, $\Delta C_{P,obs}$, results primarily from the coupling of adenine base unstacking to SSB–dA(pA)$_n$ binding, rather than from release of hydrophobically bound water. As discussed above, this is based on the fact dA(pA)$_n$ does undergo a temperature-dependent conformational transition over the temperature range used in our binding studies. Furthermore, $\Delta C^\circ_{P,obs} \approx 0$ for the binding of SSB to both dT(pT)$_n$ and dC(pC)$_n$ and for the adenine-unstacking transition. Therefore, either no significant change in water-accessible surface area occurs upon complex formation with dT(pT)$_n$ and dC(pC)$_n$ or a fortuitous cancellation of effects occurs, possibly due to burial of apolar and polar surfaces. For this reason it also seems unlikely that the $\Delta C^\circ_{P,obs}$ associated with the SSB–dA(pA)$_n$ interaction is due to differential burial of nonpolar surface upon DNA binding. Thus the coupling of the

temperature-dependent unstacking of the adenine bases provides the most likely explanation for the strikingly different dependencies of K_{obs} on temperature for SSB tetramer binding to $dA(pA)_n$ as compared to $dT(pT)_n$ and $dC(pC)_n$. Although we cannot rule out contributions from conformational changes in the SSB protein that accompany binding, these would be in addition to the base-unstacking transition in $dA(pA)_n$.

Implications for Other Single-Stranded Nucleic Acid Binding Proteins. The equilibrium binding of *E. coli* SSB tetramer to ss-DNA displays complex behavior that differs from that of some other SSB proteins (Lohman et al., 1988; Lohman & Bujalowski, 1990; Lohman & Ferrari, 1994); however, this mainly reflects its tetrameric assembly state and its cooperative binding properties. In fact, a number of prokaryotic and mitochondrial SSB proteins that share significant sequence similarities to *E. coli* SSB protein and are also tetrameric display many of the same qualitative DNA binding properties. On the other hand, the interactions of *E. coli* SSB protein with ss-DNA do share many similarities with those of other SSB proteins. In particular, SSB proteins from both prokaryotes and eukaryotes show distinct preferences for binding single-stranded homopolynucleotides of different base composition. In general, these proteins bind with highest affinity to poly(dT) and poly(dC) and with much lower affinity to poly(dA). Although the basis for these preferences is not well understood, the lower affinity of these SSB proteins for poly(dA) must be due, at least in part, to the higher extent of base stacking in the polypurines as suggested previously (Newport et al., 1981; Krauss et al., 1981; Bulsink et al., 1985). If the bases are partially unstacked in the protein–DNA complex, then increased base stacking in the free DNA will inhibit binding.

There is evidence that other SSB proteins cause at least partial unstacking of the bases upon binding either poly(A) or poly(dA). Newport et al.(1981) showed that a large hyperchromism is observed at 260 nm upon binding of T4 gene 32 protein to poly(dA), whereas little hyperchromism is observed upon binding poly(dC) or poly(dT). This increased hyperchromicity is attributed to unstacking of the poly(dA) bases upon formation of the complex. Using Raman spectroscopy, Otto et al. (1988) observed increased hyperchromicities upon binding of either M13 gene V or T4 gene 32 proteins to poly(dA) but not to poly(A). As mentioned above, Kuil et al. (1990) have shown using electric field induced birefringence that the bases in poly(dA) become partially unstacked upon binding *E. coli* SSB protein, and similar observations have been made for T4 gene 32 protein (Scheerhagen et al., 1985; van Amerongen et al., 1988). If coupling of base unstacking to protein–DNA binding provides the explanation for the nonlinear van't Hoff plots observed for the *E. coli* SSB tetramer–$dA(pA)_n$ binding, then nonlinear van't Hoff plots with $\Delta C_P < 0$ should also be observed for binding of poly(dA) to the T4 gene 32 protein, the f1 gene V protein, and possibly other SSB proteins. Unfortunately, systematic studies of the equilibrium constants for binding to poly(dA) or poly(A) over a wide temperature range are lacking for these proteins, and nonlinear van't Hoff plots have not been reported, although the existing studies are consistent with this possibility as discussed below.

Studies of the temperature dependence of K_{obs} have been reported for both the T4 gene 32 protein (Kowalczykowski et al., 1981) and the phage M13 gene V protein (Alma et al., 1983; Bulsink et al., 1985). Alma et al. (1983) determined that $\Delta H°_{obs}$ is near zero for fd gene V binding to poly(dA) over the range 5–25 °C; $\Delta H°_{obs} = 2$ kcal/mol (0.175 M NaCl, pH 7.0), and $\Delta H°_{obs} = -2$ kcal/mol (0.21 M NaCl, pH 7.0), whereas Bulsink et al. (1985) showed that $\Delta H°_{obs} = -17$ kcal/mol for the same protein binding to poly(dU) (0.70 M NaCl, pH 6.9). As Bulsink et al. (1985) suggest, these values of $\Delta H°_{obs}$ may differ due to the different extents of base stacking within poly(dA) vs poly(dU) and the requirement that the bases be unstacked upon binding gene V protein. However, since these experiments were performed at different salt concentrations, some of this difference may also reflect a dependence of $\Delta H°_{obs}$ on salt concentration, especially since we observe such effects with *E. coli* SSB protein (Overman, 1989; Lohman & Mascotti, 1992a). However, these results are qualitatively consistent with our observations, which show a small, positive $\Delta H°_{obs}$ in the range 5–25 °C for the SSB–$dA(pA)_n$ interaction, and therefore it is likely that a $\Delta C°_{P,obs} < 0$ does accompany gene V protein binding to poly(dA).

A value of $\Delta H°_{obs} = -22 \pm 2$ kcal/mol was reported for the cooperative binding of T4 gene 32 protein to the fluorescent polyribonucleotide poly(ϵA), based on studies of the temperature dependence of the equilibrium constant, $K\omega$, in the range 18–38 °C (Kowalczykowski et al., 1981). The binding of poly(ϵA) to T4 gene 32 protein (as well as to *E. coli* SSB protein) results in an enhancement of poly(ϵA) fluorescence that is believed to be due to partial unstacking of the ethenoadenine bases. This negative $\Delta H°_{obs}$ is similar to that reported here for *E. coli* SSB tetramer binding to $dA(pA)_n$ in this temperature range, and therefore it is also possible that a nonlinear van't Hoff plot would be observed if these studies were extended to lower temperatures.

Implications for the Interpretation of Temperature Dependencies of Other Protein–Nucleic Acid Interactions. The results presented here demonstrate that a nonspecific protein–DNA interaction, the binding of *E. coli* SSB tetramer to the single-stranded oligodeoxynucleotide $dA(pA)_n$, is accompanied by an *apparent* negative heat capacity change, although the binding of the same protein to $dT(pT)_n$ or $dC(pC)_n$ displays no apparent $\Delta C°_{P,obs}$. Therefore, although it has been suggested that such a large negative $\Delta C°_{P,obs}$ may be a distinctive feature of site-specific protein–DNA interactions (Spolar & Record, 1994), this appears not to be the case, at least for proteins binding to single-stranded nucleic acids. We conclude that the large negative $\Delta C°_{P,obs}$ accompanying the SSB–$d(pA)_n$ interaction is not due primarily to burial of water-accessible surface area (i.e., the hydrophobic effect). Rather it appears to result from the coupling of adenine base unstacking to protein–DNA binding, where both processes occur with $\Delta C_{P,obs} \approx 0$.

Interpretations of a large negative $\Delta C°_P$ as resulting from changes in accessible surface area and the hydrophobic effect (Ha et al., 1989; Spolar & Record, 1994) have generally assumed that the structures of the reactants and the products do not change appreciably with temperature (i.e., $\Delta C°_{P,obs}$ is temperature independent). This assumption has also generally been made for the analysis of protein unfolding (Becktel & Schellman, 1987; Baldwin, 1986). However, the base unstacking of poly(dA) provides a clear example of a temperature-dependent macromolecular structural change that is coupled to SSB binding. Although similar conformational changes are less likely to occur in duplex nucleic acids, they are likely to occur within proteins. Spolar and Record (1994) have proposed that local protein folding transitions are coupled to the binding of many gene regulatory proteins to specific DNA sites. These conformational transitions were assumed to be temperature independent and thus to contribute equally at all temperatures. However, if such local folding transitions

are themselves temperature dependent, then their relative contributions to the apparent $\Delta C°_{P,obs}$ will also change with temperature in a manner similar to the SSB–d(pA)$_n$ interaction. Although the linkage of such temperature-dependent protein conformational transitions cannot fully explain the large negative $\Delta C°_{P,obs}$ observed for site-specific protein–DNA interactions, the extent to which they contribute needs to be assessed.

The coupling to protein binding of temperature-dependent conformational changes within nucleic acids may also be important for many protein–RNA interactions. The binding of proteins to single-stranded RNA or possibly to the loop regions within RNA hairpins may show an apparent $\Delta C°_{P,obs}$ due to a coupled RNA conformational change, such as base unstacking. In fact, such an apparent negative $\Delta C°_{P,obs}$ has been observed for the binding of an RNA hairpin to the N-terminal domain of the human U1A protein, which is believed to interact with the nucleotides within the RNA loop (K. Hall, personal communication).

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