Interactions of the Major Cold Shock Protein of *Bacillus subtilis* CspB with Single-stranded DNA Templates of Different Base Composition*

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CspB is a small acidic protein of *Bacillus subtilis*, the induction of which is increased dramatically in response to cold shock. Although the exact functional role of CspB is unknown, it has been demonstrated that this protein binds single-stranded deoxynucleic acids (ssDNA). We addressed the question of the base composition on the CspB binding to ssDNA by analyzing the thermodynamics of CspB interactions with model oligodeoxynucleotides. Combinations of four different techniques, fluorescence spectroscopy, gel shift mobility assays, isothermal titration calorimetry, and analytical ultracentrifugation, allowed us to show that: 1) CspB can preferentially bind poly-pyrimidine but not poly-purine ssDNA templates; 2) binding to T-based ssDNA template occurs with high affinity ($K_\text{d}$ for 42 nt) and is salt-independent, whereas binding of CspB to C-based ssDNA template is strongly salt-dependent (no binding is observed at 1 M NaCl), indicating large electrostatic component involved in the interactions; 3) upon binding each CspB covers a stretch of 6–7 thymine bases on T-based ssDNA; and 4) the binding of CspB to T-based ssDNA template is enthalpically driven, indicating the possible involvement of interactions between aromatic side chains on the protein with the thymine bases. The significance of these results with respect to the functional role of CspB in the bacterial cold shock response is discussed.

An important characteristic of living cells is their ability to survive under extreme conditions such as chemical stress and heat or cold shock. Although much knowledge has accumulated on the mechanism of the heat shock response, the cold shock response of living cells was discovered only a decade ago (1–3). When the temperature is shifted from 37 to 10 °C, bacterial cells express a specific subset of proteins (2). This subset in *Escherichia coli* consists of more than 14 different proteins involved in various cellular processes and includes NusA, IF2, polynucleotide phosphorylase, RecA, H-NS, GyrA, RfbA, and several other unidentified proteins (3, 4). The dominant fraction of this subset is the so-called major cold shock protein CspA, a protein of 70 amino acid residues (4). Its induction level increases dramatically (5) even under conditions that completely block protein synthesis (6). Using the cspA gene as a probe, several other homologous genes named cspC, cspD, cspE, cspF, cspG, cspH, and cspI have been identified; however, only some of these genes code for cold shock-inducible proteins (7–10). The set of cold shock proteins first discovered in *E. coli* have been found in other bacteria (3, 11, 12) and are highly homologous to the eukaryotic Y-box transcription factors (13–15).

The cold shock response in *Bacillus subtilis* is also accompanied by an increased levels in CspB, a small acidic protein highly homologous to CspA of *E. coli* (16). The biological function and induction mechanism of the CspB protein is not yet clear; however, preferential binding to particular single-stranded DNA (ssDNA) sequences has been demonstrated (17). The structure of CspB has been determined both in crystal (18) and in the solution (19) and consists of five β-strands organized into an antiparallel β-barrel. Six aromatic residues (Phe15, Phe17, Phe27, Phe30, Phe38, and Trp26) and five positively charged amino acid residues (His22, Lys25, Lys39, and Arg26) are located on the same side of the CspB molecule. Amino acid substitutions at these positions have pronounced effects on CspB binding to ssDNA as measured in gel retardation experiments (17). The ssDNA binding activity of CspB has been tested exclusively in gel retardation experiments. These results have established that there is preferential binding of the protein to the ssDNA containing Y-box sequence ATTGG (20). Subsequent experiments have shown that binding of CspB to ssDNA is not limited to ATTTG; however, no unique recognition sequences have been identified (17).

In this paper we studied the interactions of the *B. subtilis* major cold shock protein CspB with ssDNA templates addressing the question of whether these interactions depend on the base composition of the template. Using a combination of four different techniques (fluorescence spectroscopy, gel shift mobility assay, isothermal titration calorimetry, and analytical ultracentrifugation) we show that CspB has a preference for poly-pyrimidine ssDNA templates. CspB seems to bind both C- and T-based ssDNA templates; however, the underlying mechanisms of these interactions appear to be very different. Binding of CspB to C-based ssDNA is strongly salt-dependent, indicating the involvement of a large electrostatic component in the interactions. In contrast, interactions of CspB with T-based ssDNA templates are independent of salt concentration and are characterized by an apparent binding constant in the nanomolar range.

**EXPERIMENTAL PROCEDURES**

**Purification of CspB and ssDNA Templates**—Protein purification was modified from that described previously (21). Briefly, CspB was purified...
purified from *E. coli* strain BL21 (DE3) (22), which contained the overexpression plasmid pCPSB3 carrying the gene of CspB under control of the T7 RNA polymerase promoter. Cells were grown to an optical density 0.8 optical units at 600 nm at 37 °C in 2× YT medium containing 100 µg/ml ampicillin. CspB production was induced by addition of isopropyl-β-D-thiogalactopyranoside (final addition of 0.1 m) and incubated for 5 h. Cells were harvested by centrifugation at 7,500 × g. The cell pellet was resuspended in 20 mM Tris, pH 7.5, 1 mM EDTA, 2 mM diithiothreitol, and passed twice through a French pressure cell. Cellular debris was removed by centrifugation at 40,000 × g at 4 °C. The supernatant was diluted with an equal volume of resuspension buffer and applied on Fast Flow Q-Sepharose column (2 × 10 cm). After washing, the bound protein was eluted in a linear salt gradient (0–0.5 M NaCl). The CspB-containing fractions were eluted at ~150 mM NaCl, dialyzed against water, and lyophilized. Lyophilized protein was dissolved in 50 mM Tris-HCl, pH 7.5, 100 mM KCl and applied on a Sephadex G-50 column (2.5 × 100 cm) equilibrated with the same buffer and eluted from the column. The CspB containing fractions were dialyzed extensively against water, lyophilized, and stored at -20 °C. The purity of the protein was higher that 95% as judged from Coomassie staining of SDS-polyacrylamide gels. The concentration of CspB was measured spectrophotometrically using the extinction coefficient ε280 = 5690 M⁻¹ cm⁻¹ (23). Corrections for light scattering were taken into account as described (24).

**Fluorescence Spectra of ssDNA Templates**—The ssDNA oligodeoxynucleotides were purchased from Biosynthesis Inc. (Lewisville, TX) and purified on a Mono-Q column (HR 10/10, Amersham Pharmacia Biotech) equilibrated in 10 mM Tris-HCl, pH 7.0, 1 mM EDTA, and 20% acetonitrile. The ssDNA bound to the column was eluted with a shallow salt gradient prepared from equilibration buffer and equilibration buffer containing 1 M NaCl. The fractions of interest, eluted at ~0.5 M NaCl, were dialyzed against water and lyophilized. Lyophilized ssDNA was dissolved in 50 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, dialyzed against the same buffer, aliquoted in small volumes, and stored at -20 °C. The ssDNA concentration was calculated from the absorbance at 260 nm.

The extinction coefficients for individual ssDNA were estimated using a FluoroMax spectrofluorometer with DM3000F software. Concentrations were performed using 23G and 23pG templates.

**Cellular Debris Removal**—After washing, the bound protein was eluted in a linear salt gradient (0–1 M NaCl) and incubated for 5 h. Cells were harvested by centrifugation at 40,000 × g. The supernatant was diluted with an equal volume of resuspension buffer and applied on Fast Flow Q-Sepharose column (2 × 10 cm). After washing, the bound protein was eluted in a linear salt gradient (0–0.5 M NaCl). The CspB-containing fractions were eluted at ~150 mM NaCl, dialyzed against water, and lyophilized. Lyophilized protein was dissolved in 50 mM Tris-HCl, pH 7.5, 100 mM KCl and applied on a Sephadex G-50 column (2.5 × 100 cm) equilibrated with the same buffer and eluted from the column. The CspB containing fractions were dialyzed extensively against water, lyophilized, and stored at -20 °C. The purity of the protein was higher that 95% as judged from Coomassie staining of SDS-polyacrylamide gels. The concentration of CspB was measured spectrophotometrically using the extinction coefficient ε280 = 5690 M⁻¹ cm⁻¹ (23). Corrections for light scattering were taken into account as described (24).

**Fluorescence Measurements**—Fluorescence intensity was measured using a FluoroMax spectrofluorometer with DM3000F software. Constant temperature during the experiments was maintained using a thermoelectrically connected holder connected to a circulated water bath. The CspB concentration was 0.3 mM (equilibrium titrations) or 14 mM (stochastic titrations) in 50 mM Tris-HCl pH 7.5 buffer, containing either 100 mM NaCl (normal experimental conditions) or 1 M NaCl (high salt buffer). Tryptophan fluorescence was excited at 287 nm (under equilibrium conditions) or 300 nm (under stoichiometric conditions), and the emission was recorded at 349 nm. The experiments were performed at 10 °C (when not otherwise specified), and the initial volume in the cuvette was 1.1 ml. The solution was gently stirred during the titration, and the measured intensity values were corrected for sample dilution. Inner filter effects were taken into account, and blanks were subtracted. Because it is known that poly(dG) can form oligomers, only a limited number of experiments were performed using 23G and 23pG templates.

**Gel Shift Assays**—150 pmol of ssDNA were 5'-32P-end-labeled by incubating templates with T4 polynucleotide kinase (3 μM) in polynucleotide kinase buffer (New England Biolabs), and 0.03 mCi of [γ-32P]ATP (NEN Life Science Products) in a total volume of 60 μl at 37 °C for 1 h. The reaction was stopped by heat inactivation (30 min at 65 °C). Unincorporated [γ-32P]ATP was removed with QIAquick Nucleotide Removal Kit (Qiagen). The binding reaction was routinely performed by incubating 10 pmol of labeled ssDNA with different amounts of protein (total volume, 25 μl) at room temperature for 25 min in binding buffer (20 mM Tris, pH 8.6, 50 mM NaCl, and 5 mM MgCl2); however, incubation for shorter (10 min) or longer (60 min) periods of time did not produce appreciable change in the retardation patterns. 5 μl of dye solution (20% glycerol, 0.03% acridine orange) was added to the gel prior to gel electrophoresis. The electrophoresis was performed in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) buffer through a 22.5% acrylamide gel at 75 volts until the samples had entered the gel and then at 100 volts overnight. Autoradiograms were obtained by exposing gels to a Kodak MR-2 film for 0.5–2 h at room temperature.

**Isothermal Titration Calorimetry**—ITC experiments were performed using a titration microcalorimeter (MicroCal Inc., Northampton, MA). Protein and ssDNA were dialyzed simultaneously in 50 mM Tris, pH 7.5, 100 mM NaCl buffer. The protein concentration was 14 μM in a 1.34-m1 cell volume, and 4 μl of ssDNA were sequentially injected into the protein solution until no further heat effects were observed. As a control, identical concentrations of DNA were injected into the buffer to measure the heat of dilution. The heat of the reaction was obtained by integrating the peak after each injection of ssDNA ligand using Origin software provided by the manufacturer. Because the CspB-ssDNA binding is stoichiometric under our experimental conditions, ΔHrel was calculated by summing individual heat increments and dividing by the total number of moles of CspB present in the solution. Thus ΔHrel is expressed per mole of CspB molecules.

**Analytical Centrifugation Experiments**—Analytical centrifugation experiments were performed in a Beckman XL-A centrifuge. For each run three cells were used: one containing ssDNA only (2 μM for 23pT or 2.5 μM 23pC), another with CspB only (20-fold higher protein concentration than ssDNA), and a third containing CspB-ssDNA complex with the same concentrations of CspB and ssDNA as in the other cells. The CspB-ssDNA complex was formed by incubating CspB and ssDNA at room temperature for 25 min prior to each run. The runs were performed at 4 °C under different speed (20,000 or 25,000 rpm) for 10 h. Absorbances were recorded simultaneously at 260 and 280 nm during the runs. The partial molar volumes for CspB, ssDNA (23pT and 23pC), CspB-23pT complex, and CspB-23pC complex were 0.74, 0.55, 0.69, and 0.65 cm³/g, respectively, calculated as described (26–28). Analysis of the data was performed using the Beckman supplied script for Origin (MicroCal Inc.) software.

**Analysis of Binding Isotherms**—The binding isotherms obtained by monitoring the changes in the fluorescence intensity upon titration of CspB with ssDNA template was analyzed according to the following classical binding equation (29).

\[ Q = \frac{A}{2 \times \text{[CspB]_{total}}^n \times \text{[ssDNA]_{total}}} \]  

where A = [CspB]_{total} + n_{p} \times \text{[ssDNA]_{total}} + 1/K_{q,ss}^e$, Q is the quenching effect of CspB Trp fluorescence intensity after each addition of ssDNA, [CspB]_{total} is the maximum quenching obtained upon complete saturation of the protein with ssDNA, [ssDNA]_{total} and [ssDNA]_{adj} are the total concentrations of protein and ssDNA in solution, respectively, K_{q,ss}^e is the equilibrium association constant for the CspB-ssDNA interactions, and n_{p} is the number of molecules of CspB bound per molecule of ssDNA. One needs to keep in mind that for protein-DNA interactions when more than one molecule of protein binds to DNA, the K_{q,ss}^e obtained from Equation 1 represents an apparent equilibrium constant because this model does not consider the degeneracy of the binding site on nucleic acid. In the case of single binding site, this binding model is exact and has been successfully used for the analysis of protein-ssDNA interactions (30).

Data analysis was also performed according to the model originally introduced by Epstein (31). This model is a particular case of the model of McGehee and von Hippel (32) for the short templates and takes into account the fact that for homogeneous template the binding site can be formed by any continuous segment with a given number of bases. According to Epstein (31), the fractional saturation of the lattice (θ) is defined as follows.

\[ \theta = n \times \frac{1}{M} \]  

where θ is the binding site size, M is the length of the ssDNA, and is the average number of bound ligands expressed as follows.

\[ k = \frac{g}{k + \frac{1}{g}} \sum_{k=0}^{g} \frac{1}{k+1} \text{[CspB]_{total}} \times \text{[ssDNA]_{total}} \]  

where k, g, and j represent the number of ligands bound, the maximum number of ligands that can bind to the lattice, and the number of adjacencies, respectively. K_{q,ss}^e and ω are the equilibrium association constant and the cooperativity parameter for the interaction, respectively. Finally, P_{ij} is the number of distinct ways that k-site ligands may bind to an M-site lattice with j adjacencies and is calculated as follows.
CspB-ssDNA Binding Monitored by the Trp Fluorescence Quenching—A number of aromatic (Trp, Phe, and His) residues of CspB have been proposed to be involved in the interactions with the Y-box motif, with higher preference to the (++)-strand-containing ATTGG core sequence than to the (−)-strand-containing CCAAT core sequence or the “nonspecific” sequence that contains neither the ATTGG nor the CCAAT sequence. The results presented in Fig. 1 agree with this conclusion, i.e. quenching CspB Trp fluorescence intensity occurs at lower concentrations of 43YB+ than for 43YB− or the control nonspecific 43NS template. Nevertheless, all three single-stranded templates quench the CspB Trp fluorescence, thus indicating that CspB interacts with all three of them. This is in agreement with the gel shift experiments in which just 2–3-fold higher concentration of CspB was needed to observe complete retardation of the YB− and NS templates as compared with the YB+ template (18, 19).

To simplify the system, we synthesized four oligodeoxynucleotides in which the core ATTGG sequence was positioned in the middle of a 23-mer with nine flanking A, T, C, or G nucleotides on both sides, i.e. XATTGGXY, where X = A, T, C, or G (see Table I for the nomenclature of the oligodeoxynucleotides). The quenching effects on the CspB Trp fluorescence intensity upon titration with 23T, 23G, 23C, or 23A at 25 °C are shown in Fig. 2A. Surprisingly, under our experimental solvent conditions (50 mM Tris, 100 mM NaCl, pH 7.5) there is a significant difference in the titration profiles. Changes in the fluorescence quenching reach saturation at 0.2 μM of 23T template. In the case of 23C ssDNA template saturation is reached at >1 μM, whereas 23A and 23G templates do not produce changes in the fluorescence quenching of CspB (Fig. 2A). In control experiments we measured the changes in the Trp fluorescence of CspB as a function of concentrations of A-, T-, G-, or C-based oligodeoxynucleotides that lacked the core ATTGG sequence, i.e. 23pA, 23pT, 23pG, and 23pC (Table I). We observe that, except for the 23C and 23pC, quenching of the protein Trp fluorescence is independent of the ATTGG core sequence (Fig. 2A). Notably, the total quenching of CspB Trp fluorescence by 23T and 23pT is much larger than for the other oligodeoxynucleotides and takes place at very low ssDNA concentration. The lower quenching effect observed for 23pC could be due to the stacking interactions of the C-bases at the pH of our experiment (7.5) (35). However, fluorescence experiments performed at pH 8.1, where no stacking is expected, did not differ from the titration curve obtained at pH 7.5 (data not shown).

The quenching of fluorescence intensity of Trp does occur because of the direct interactions of CspB with ssDNA templates. This can be demonstrated by measuring the changes in the fluorescence intensity of N-acetyl-tryptophan-amine upon addition of different amounts of 23T, 23A, and 23C (up to 0.5, 0.9, and 1.4 μM, respectively). Under the same experimental conditions titrations with these ssDNA templates did not lead to appreciable quenching of N-acetyl-tryptophan-amine fluorescence intensity (Fig. 2B). These results suggest that the quenching of the CspB intrinsic Trp fluorescence by ssDNA templates, as it was shown for other protein-DNA complexes (36), is entirely due to direct interactions and does not have contribution from simple collision of these molecules. Additional evidence for this conclusion follows from the gel shift experiments.

CspB-ssDNA Binding Monitored by Gel Shift Electrophoresis—Fig. 3 presents the results of the mobility shift assay on nondenaturing polyacrylamide gels for 23T, 23pT, and 23pC ssDNA templates. This assay allows separation of free ssDNA from the CspB-bound ssDNA by retardation of electrophoretic mobility. CspB-ssDNA complexes were formed at varying concentrations of CspB incubated with invariant amounts of 5′-32P-labeled ssDNA templates. Increase of the concentration of CspB was needed to observe complete retardation of the CspB-ssDNA complex (up to a 200:1 ratio of CspB to ssDNA).
band corresponding to free ssDNA (Fig. 3). Similar experiments using 23A as the template showed no appreciable ssDNA retardation even at a 300:1 ratio (data not shown).

Comparison of the gel retardation experiments with the results of the Trp fluorescence titration of CspB with ssDNA templates (Fig. 2) shows that there is a clear correlation between these two data sets. The degree of Trp quenching as a function of ssDNA concentrations correlates well with the CspB-ssDNA ratio required for the complete retardation in the gel shift assay, thus confirming that quenching of the CspB intrinsic Trp fluorescence by ssDNA results from the direct interactions of the ssDNA with the protein. It is also notable that the T-based ssDNA templates 23T and 23pT behave similarly and seem to have the highest affinity for CspB.

**ssDNA-CspB Binding Monitored by ITC**—Further characterization of the thermodynamics of formation CspB-ssDNA complexes was done using ITC. This technique measures directly the heat of the interactions at a given temperature. The change in the heat of the reaction as a function of ligand concentration contains all the information on the thermodynamic parameters of interactions in the system including the stoichiometry and binding constant (67). Because in many cases the heat of the reaction is relatively small, ITC experiments are generally performed at relatively high concentrations of protein or ligand. Fig. 4A presents the typical ITC recording of 15 injections (4 μM each) of 23pT (220 μM) into the cell containing 14 μM CspB at 25 °C. The reaction is exothermic, and each addition of 23pT leads to a lower amount of heat released until the reaction is saturated. After integration of each peak and normalization for the ligand (ssDNA) concentration (Fig. 4B) the titration profile shown on Fig. 4A is very steep, indicating that under these concentrations of CspB and 23pT binding is stoichiometric (strong). This enables an estimate of the stoichiometry of binding as the ssDNA to CspB ratio at which the break point on the titration profile occurs. From the profile for 23pT-CspB shown on Fig. 4B, it appears that the break point occurs at the ration of 23pT to CspB of ~0.3, i.e. the stoichiometry of binding is three molecules of CspB per one molecule of 23pT. This result was corroborated by titration of the same concentrations CspB with 23pT monitored by Trp fluorescence (Fig. 4C). The ITC and Trp fluorescence titration profiles show perfect overlap if converted into fractional saturation, indicating that both techniques follow the same process and that the stoichiometry of 23pT-CspB interactions is indeed three molecules of CspB bind one molecule of 23pT.

Additional evidence for a 3:1 stoichiometry of CspB-23T/23pT interactions was obtained by analytical ultracentrifugation. The analytical centrifugation experiments were performed simultaneously on 23pT template, CspB, and their mixture at a 20:1 ratio of CspB to 23pT (Fig. 5). The average apparent molecular mass of 23pT is 8.9 ± 0.5 kDa, which compares with the theoretical molecular mass of 7.1 kDa. The average apparent molecular mass for CspB under these conditions was found to be 8.8 ± 0.7 kDa, which is close to the expected molecular mass of 7.5 kDa calculated from the amino acid composition. Under the same solvent conditions (50 mM Tris, 100 mM NaCl, pH 7.5) the average apparent molecular mass of the species formed in a solution containing both CspB
and 23pT was 37 ± 7 kDa. Using the average apparent molecular masses of CspB and 23pT, this gives the stoichiometry of the formed complex of 3.2 molecules of CspB per one molecule of 23pT, identical to the results obtained by ITC and Trp fluorescence spectroscopy. Considering the length of ssDNA and the model-independent treatment of the data is required (36–38). Thus the relationship between the change in the spectroscopic signal and the extent of binding for CspB-ssDNA systems must be established prior to the analysis of such data. The fluorescence signal of quenching of Trp of CspB appears at least in a first approximation to reflect the extent of binding to ssDNA template. This can be expected because the spectroscopic signal is originated from a single fluorophore, Trp^a of CspB. However, the direct indication of this is an overlap of the normalized signals obtained by two very different methods: fluorescence spectroscopy and ITC (Fig. 4C). It is notable that the overlap of the normalized signals obtained by the two techniques is observed for both 23pT and 23pC ssDNA templates. For 23pT there is higher degree of quenching observed, and correspondingly there is higher heat associated with the interaction as measured by ITC. For 23pC it is just opposite: there is less fluorescence quenching observed, and the heat of the reaction is also lower. These observations provide a background for the validity of the analysis of the CspB-ssDNA interactions using fluorescence spectroscopy.

**Discussion**

The use of spectroscopic methods for the analysis of protein-DNA interactions requires the knowledge of the relationship between the change in the spectroscopic signal and the extent of binding (36). In many cases this information is not available and the model-independent treatment of the data is required (36–38). Thus the relationship between the change in the spectroscopic signal and the extent of binding for CspB-ssDNA systems must be established prior to the analysis of such data. The fluorescence signal of quenching of Trp of CspB appears at least in a first approximation to reflect the extent of binding to ssDNA template. This can be expected because the spectroscopic signal is originated from a single fluorophore, Trp^a of CspB. However, the direct indication of this is an overlap of the normalized signals obtained by two very different methods: fluorescence spectroscopy and ITC (Fig. 4C). It is notable that the overlap of the normalized signals obtained by the two techniques is observed for both 23pT and 23pC ssDNA templates. For 23pT there is higher degree of quenching observed, and correspondingly there is higher heat associated with the interaction as measured by ITC. For 23pC it is just opposite: there is less fluorescence quenching observed, and the heat of the reaction is also lower. These observations provide a background for the validity of the analysis of the CspB-ssDNA interactions using fluorescence spectroscopy.

**Thermodynamics of CspB Interactions with 23pT and 23pC—Thermodynamics of CspB interactions with ssDNA templates was assessed by fluorescence spectroscopy and by ITC. Because of the high concentrations of CspB and 23pT, the ITC experiments were performed with 23pT and 23pC ssDNA templates required for the ITC experiment and the corresponding stoichiometric character of binding of CspB to 23pT under these reaction conditions, only the enthalpy of interactions and not the equilibrium constant could be obtained directly. The calorimetric enthalpy of the interactions of one molecule of CspB with 23pT at 25 °C is estimated to be −110 ± 7 kJ/mol (Fig. 4).

An alternative way of determining the enthalpy of interactions is from the temperature dependence of the equilibrium
constant using the well known van’t Hoff relation shown in Equation 8.

\[ \Delta H_{\text{m}} = -R \cdot \frac{d \ln K_e}{d(1/T)} \]  

(Eq. 8)

Temperature dependence of the equilibrium constant can be estimated from the equilibrium binding isotherms obtained at different temperatures.

Fig. 6 shows the binding isotherms for 23pT and 23T with CspB as monitored by the quenching of CspB Trp fluorescence intensity at four different temperatures: 25, 31, 37, and 42 °C. No significant difference in titration profiles was observed for the 23pT or 23T templates at any temperature. Data were analyzed according to the classical binding formalism (Equation 1) or Epstein model (Equation 7), and the results of analysis are shown in Table II. It is notable that the association constants obtained from these two different models are comparable. This is particularly due to the very moderate cooperativity of the interactions, as measured by the parameter \( \omega \) in the Equation 3 of Epstein model. Increase in temperature has a profound effect on the association constant leading to a decrease of this parameter (Table II). The van’t Hoff analysis (Equation 8 and Fig. 6B) for the enthalpy of CspB-23pT interactions gives enthalpies (-119 ± 6 kJ/mol using independent binding site model or -104 ± 10 kJ/mol using Epstein model) that are in an excellent agreement with the calorimetrically determined binding enthalpy of -110 ± 7 kJ/mol. Knowing the equilibrium constants and the enthalpy changes for CspB-ssDNA interactions allow us to estimate the entropy changes in the system (Table II). For 23pC the entropy change upon CspB-23pC interactions is positive, indicating that complex formation is favored both enthalpically and entropically. It is notable, however, that the enthalpy and the entropy changes for 23pT template are both negative (Table II), thus the favorable enthalpy change upon binding overcompensates the unfavorable entropic (\(-T \Delta S\)) term leading to an enthalpically driven association between CspB and the 23pT/23T templates.

These enthalpically driven interactions might be rationalized in terms of burial of aromatic side chains of CspB and of the pyrimidine ring of thymine. Five aromatic residues of CspB (Trp\(^8\), Phe\(^17\), Phe\(^17\), Phe\(^27\), and Phe\(^30\)) have been implicated in the binding to nucleic acids (17), and our results suggest that these aromatic residues probably interact with 6–7 thymine bases. The estimates of the enthalpy changes upon complex formation between the five aromatic groups of CspB and 6–7 pyrimidine groups of T-based ssDNA template can be made using model compound data. One can imagine a two-step process: dehydration of these ring structures followed by the formation of interactions between them. The first step can be modeled as a transfer of a compound from water into the gas phase and the second as a condensation process (39–41). The enthalpy of transfer of benzene ring from the aqueous phase to the gas phase (dehydration) at 25 °C is 29 kJ/mol (42), somewhat close to the enthalpy of transfer for toluene (33 kJ/mol). So, for the first step an enthalpy value of 32 kJ/mol can be a reasonable estimate. For the second step, the enthalpies of condensation of model compounds such as benzene (−42 kJ/mol), toluene (−38 kJ/mol), or pyrimidine (−50 kJ/mol) suggest an average enthalpy on the order of −43 kJ/mol (43). Thus the net enthalpy changes for both steps can be estimated at −11 kJ/mol. Using the calorimetric enthalpy of interactions of CspB with 23pT (−110 kJ/mol) and assuming that all enthalpy change comes from the two-step transfer considered above, we can estimate that −10 (110 divided by 11) aromatic groups are required to give the observed enthalpy changes. This estimate compares favorably with our previous estimate of 11–12 groups (5 aromatic side chains of the CspB and 6–7 pyrimidine rings of 23pT) being involved in the interactions between CspB and

**Table II**

| Temperature | \( K_{\text{obs}} \times 10^6 \) M\(^{-1} \) | \( K_{\text{eq}} \times 10^6 \) M\(^{-1} \) | \( \omega \) | \( \Delta G \) (kJ/mol) | \( \Delta H \) (kJ/mol) | \( \Delta S \) (J/(K mol)) |
|------------|-----------------|-----------------|---|-----------------|-----------------|-----------------|
| 23pT       |                 |                 |   |                 |                 |                 |
| 25 °C      | 24.0 ± 0.9\(^b\) | 3.1 ± 0.7\(^b\) | 16.3 ± 0.4 | -42/−44\(^d\) | -110 ± 7\(^e\) | -228/−221\(^f\) |
| 31 °C      | 8.3 ± 0.6\(^b\) | 2.0 ± 0.7\(^b\) | 4.6 ± 0.5 | -39/−40\(^d\) |                 |                 |
| 37 °C      | 3.7 ± 0.1\(^b\) | 0.8 ± 0.1\(^b\) | 2.8 ± 0.5 | -37/−38\(^d\) |                 |                 |
| 42 °C      | 1.8 ± 0.2\(^b\) | 0.3 ± 0.1\(^b\) | 1.9 ± 0.6 | -36/−33\(^d\) |                 |                 |

\(^a\) Estimated from the nonlinear least square fit of the data to Equation 1 with the parameter \( n_x \) fixed to 3 for 23pT and 1 for 23pC.

\(^b\) Estimated from the nonlinear least square fit of the data to Equation 7 with the parameter \( n_x \) fixed to 7 for 23pT.

\(^c\) \( \Delta G = -RT \ln(K_{\text{obs}}) \).

\(^d\) \( \Delta G = -RT \ln(K_{\text{eq}}) \).

\(^e\) The enthalpy of ssDNA-CspB interactions obtained from ITC experiments.

\(^f\) Calculated as \( \Delta S = \frac{\Delta H - \Delta G}{T} \), estimated error ±20 J/(K mol).

\(^g\) N/A, not applicable.
T-based ssDNA template. An independent indication for the validity of such analysis comes from the results of thermodynamic studies of interactions between ssDNA templates and Trp-containing oligolysine peptides (44, 45). The authors found that a single Trp residue contributes 8–12 kJ/mol to the binding enthalpy, a value in good agreement with our estimate based on the thermodynamics of the two-step model compound transfer process.

Enthalpically driven protein-nucleic acid interactions have been observed, for example, for the sequence specific interactions of CI repressor with the bacteriophage lambda O_4 operator (46, 47) and in the formation of the MetJ-operator complex (48). However, inspection of the crystal structure of the complexes (49, 50) does not show stacking interactions between aromatic side chains of protein and DNA bases but does bury a number of aromatic groups. Enthalpically driven interactions between E. coli single-stranded binding protein to different single-stranded nucleic acids have also been demonstrated. For example, the enthalpy of interactions of single-stranded binding tetramer with dT(pT)_4 varies from −600 kJ/mol in 10 mM NaCl to −470 kJ/mol in 1 M NaCl (30, 51). These enthalpies, calculated per mole of single-stranded binding monomer, are comparable with that obtained for the CspB-23pT interactions (−110 kJ/mol).

**Difference in the Mechanism of CspB Interactions with 23pT and 23pC**—Thus all four methods, analytical centrifugation, gel shift assays, ITC, and Trp fluorescence spectroscopy, show that there are significant differences in the interactions between CspB and the ssDNA templates 23pC and 23pT. CspB binding to the T-based templates has four distinctive features. First, the equilibrium dissociation constants for T-based template are at least several times lower than those for CspB binding to the 23pC template (Table II). Second, the stoichiometry of binding of CspB is different for 23pT than for 23pC. Three molecules of CspB bind to one molecule of 23pT, which indicates that about seven nucleotides are involved in the interaction with one CspB molecule. Geometrical considerations based on the structure of CspB (18, 19) and on the length of the seven nucleotide long stretch of ssDNA are consistent with this estimate. In contrast, only one molecule of CspB binds to 23pC. Third, the enthalpy of interactions of CspB with 23pT is five times that observed for the interactions of CspB with 23pC (−110 kJ/mol versus −25 kJ/mol). Fourth, the entropy of interactions of CspB with 23pT is negative, whereas the entropy of interactions of CspB with 23pC is positive. All these results suggest that distinct mechanisms govern the interactions of CspB with 23pC and 23pT/23pT templates.

Protein-DNA interactions in general and protein-ssDNA interactions in particular can be mediated through the formation of contacts between the basic side chains on a protein with the negatively charged phosphodiester backbone of DNA and/or through the contacts between side chains on a protein with the sugar moity and base. In the first case, the interactions will have a significant electrostatic component. In the second case, other types of noncovalent forces such as hydrogen bonding, van der Waals’ interactions, and hydrophobic effect will be involved. To distinguish between these two mechanisms of interactions we analyzed the effect of ionic strength on the CspB-ssDNA binding isotherms. Increased ionic strength weakens the charge-charge interactions, thus making electrostatically formed complexes less stable (52–54). Fig. 7 compares the results CspB titration with T- and C-based oligodeoxyribonucleotides in the presence of 100 mM and 1 mM NaCl monitored by quenching of CspB Trp fluorescence. Addition of 1 mM NaCl to the solution did not affect the binding isotherm for T-based template. The profiles in the presence of low (100 mM NaCl) and high (1 M NaCl) salt are superimposable not only at 25 °C but also at 37 °C, a condition of lower binding affinity. However, in the presence of 1 mM NaCl binding of CspB to the C-based template is virtually abolished (Fig. 7). This provides an indication that the interactions of CspB with C-based templates are mainly mediated by electrostatic interactions of basic side chains on the protein with the phosphodiester backbone of ssDNA template. On the other hand interactions of CspB with the T-based template occur mainly via the interactions of protein with the bases and to a lesser extent with the phosphodiester backbone of ssDNA.

**Concluding Remarks**—The observation that CspB can bind preferentially T-rich stretches of ssDNA with an affinity on the order of 10^3 M^-1 or higher at temperatures below 25 °C might suggest a possible role for in vitro function of CspE. There are two prevalent occurrences of T-rich regions identified in DNA. First, T-rich sequences occur at factor-independent transcription termination signals (see for reviews Refs. 55–59). These sites are characterized by a GC-rich region of dyad symmetry followed by a run of 6–8 T residues (59). The possibility of CspB involvement in transcription termination at low temperatures can be rationalized in the terms of possible CspB interactions with the T-run on the coding strand of DNA, which will force dissociation of RNA polymerase. Second, T-rich runs are frequently located downstream from promoter sequences as part of the sequences contained within the unusually long 5'-untranslated region of cold shock proteins (17). The importance of these 5'-untranslated region in stabilizing the mRNA of the major cold shock proteins of E. coli has been clearly demonstrated (60–65). Possible role of the Cap family of proteins in the transcriptional initiation was recently demonstrated for CspB. Using photocross-linking ribonucleotide analogs Hanna and Liu (66) showed that CspE (~66% sequence identity to CspB) is cross-linked to the nascent (but not full-length) mRNA in an in vitro transcription reaction. These possible functional roles for CspB follow directly from the observed high binding affinity of this protein to the T-rich ssDNA templates. Direct demonstration of the role of these in vitro observed properties...
for the cellular function of Csp family of proteins awaits experimental validation.

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