Thermodynamic Analysis of the Single-Stranded DNA Binding Activity of the Archaeal Replication Protein A (RPA) from *Sulfolobus solfataricus*†

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ABSTRACT: The single-stranded DNA binding protein from *Sulfolobus solfataricus* (*Sso*-RPA) binds single-stranded DNA with dissociation constants in the range of 10−30 nM at room temperature. The affinity for DNA decreases at higher temperatures. At 85 °C, the optimal growth temperature of the crenarchaeot *S. solfataricus*, the dissociation constant is only about 1 μM. We analyzed the equilibrium between *Sso*-RPA and a fluorescently labeled 13 nucleotide oligonucleotide by fluorescence anisotropy measurements in the presence of four different salts and in the temperature range between 10 and 60 °C. In the presence of potassium chloride and choline chloride, three to four ions are released upon complexation, independent of the temperature. In contrast, in the presence of potassium fluoride and potassium glutamate, we observed a significant change of the number of ions released when the temperature was varied. The binding reaction is strongly exothermic with enthalpies of about −55 to −70 kJ/mol, depending upon the salt. van't Hoff analysis suggests that the binding enthalpy is temperature independent.

Most cellular DNA is double-stranded. During essential life processes such as DNA replication, repair, and recombination, single-stranded DNA (ssDNA)[1] is generated and processed. Abundant single-stranded DNA binding proteins bind rapidly to ssDNA and confer protection to the single-stranded regions of DNA. The single-stranded DNA binding proteins are engaged in a number of protein−protein interactions, and proteins involved in DNA repair, replication, and recombination can be recruited to stretches of ssDNA. Single-stranded DNA binding proteins are found in all cellular organisms, as well as in some viruses (*1*, *2*).

Eukaryotes have a heterotrimeric replication protein A (RPA) composed of subunits of about 70, 30, and 14 kDa. Human RPA was originally purified as an essential component for SV40 in vitro replication. Numerous protein−protein interaction partners have been identified and mapped to different parts of RPA (*1*, *3*).

Bacteria have a homotetrameric single-stranded DNA binding protein, abbreviated as SSB. *Escherichia coli* SSB, the best-studied SSB, binds single-stranded DNA in several binding modes depending on the salt concentration (*2*). Its C-terminal acidic tail participates in protein−protein interactions (*4*).

The archaeal single-stranded DNA binding proteins are less well studied. The Crenarchaeota, one of the two major branches of the archaea, appear to have monomeric single-stranded DNA binding proteins that are structurally similar to the eukaryotic RPA (*5*). However these proteins possess C-terminal flexible acidic tails reminiscent of bacterial SSBs. The Euryarchaeota, on the other hand, have a heterotrimeric RPA (*Pyrococcus furiosus*), a large monomeric RPA (*Methanococcus jannaschii*), or a dimeric RPA (*Methanosarcina acetivorans)* (*6*−*8*).

Despite their dissimilar amino acid sequences and their different quaternary structures and domain organizations, all SSBs/RPAs bind to ssDNA through OB (oligonucleotide/oligosaccharide binding) domains, compact structural units of about 70−150 amino acids in length (*9*). The OB fold consists of two three-stranded antiparallel β-sheets forming a "flattened" β-barrel. The OB domains are found in a variety of proteins; however, most of them bind to single-stranded DNA or RNA. Some OB domains also participate in protein−protein interactions (*10*). The OB fold is ubiquitous and appears to be an ancestral fold.

RPA from *Sulfolobus solfataricus*, a thermoacidophilic crenarchaeote, is a 148 amino acid long protein and contains a single N-terminal OB fold. The C-terminal amino acids 120−148 are rich in glycine, proline, and acidic residues and form a flexible tail, which is sensitive to limited proteolysis (*11*). A protein lacking the C-terminal tail retains its DNA-binding activity. *Sso*-RPA has DNA binding activity toward single-stranded circular DNA as well as oligodeoxynucleotides (*11*, *12*). The binding site of *Sso*-RPA is about five nucleotides as has been determined by fluorescence titrations (*5*). Single-strand DNA binding proteins can bind cooperatively to DNA. The limited binding data presented so far suggest that *Sso*-RPA binds to DNA without cooperativity.

The precise role of *Sso*-RPA in replication, repair, and recombination has not been defined yet. However, most interestingly, *Sso*-RPA binds to RNA polymerase through its acidic flexible C-terminal tail. An in vitro transcription

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[1] Abbreviations: ssDNA, single-stranded DNA; *Sso*-RPA, *Sulfolobus solfataricus* replication protein A; SSB, single-stranded DNA binding protein.

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assay demonstrated that *Sso*-RPA could enhance transcription either through promoter opening or through RNA polymerase recruitment or both (*13*).

The quaternary structure of *Sso*-RPA is unclear. The group of Kowalczykowski (*12*) reports that *Sso*-RPA elutes from a gel-filtration column as tetramer, dimer, and monomer, whereas White's group reports that *Sso*-RPA is monomeric in solution (*11*). This indicates that *Sso*-RPA, unlike *E. coli* SSB, does not form a stable homotetramer. The similarity of the amino acid sequence from *Sso*-RPA to eukaryotic RPAs is also reflected by the crystal structure of *Sso*-RPA. The structure of *Sso*-RPA is more similar to human RPA than to bacterial SSB proteins (*5*). *Sso*-RPA shares with its human homologue two conserved hydrophobic residues (W56 and F79 in *Sso*-RPA), which are involved in stacking interactions with the bound single-stranded DNA in the complex structure of human RPA (*14*). Alanine substitutions at these positions in the *Sulfolobus* protein weaken the affinity for DNA about 10−20-fold.

The binding of a protein to nucleic acids usually results in the net release of ions. Cations, which bind to the polyelectrolyte DNA, are released when the protein binds to the DNA. Likewise anions, which bind to the DNA-binding surface of the protein, are released. Since proteins can also bind cations, it is also possible that cations are released concomitant with the DNA binding event. The ion release upon binding makes a favorable entropic contribution to the equilibrium.

For *E. coli* SSB, it could be shown for the first time that the ion release can also make an enthalpic contribution (*15*). The amount of ion released depends on the temperature. In the presence of sodium chloride, the ion release decreases from 9.3 ions at 10 °C to 5.1 ions at 37 °C. Such a temperature-dependent effect is however not observed in the presence of sodium fluoride. The temperature dependence of the sodium chloride release is coupled to a salt concentration dependence of the binding enthalpy. The binding enthalpy decreases (becomes less favorable) when the sodium chloride concentration increases. The chloride release from the protein thus appears to make an endothermic contribution.

In this study, we analyze the equilibrium between *Sso*-RPA and a fluorescently labeled oligodeoxynucleotide using fluorescence anisotropy measurements. We analyzed the DNA binding of *Sso*-RPA in the presence of four salts and at two different temperatures. *Sso*-RPA is especially suited for this type of study since this monomeric protein binds with 1:1 stoichiometry to suitable short DNA substrates and since its thermophilic character allowed us to study the equilibrium in the temperature range from 10 to 60 °C. We find in this study that the amount of ion release of the binding reaction between *Sso*-RPA and DNA depends on the temperature and the anion as observed for *E. coli* SSB. The two proteins however deviate considerably with respect to the different anions.

MATERIALS AND METHODS

Expression and Purification of the Mutants. The protein was expressed in recombinant *E. coli* cells transformed with a pET expression vector (a gift from M. White, University of St. Andrews) and purified by cation-exchange chromatography as described previously (*11*). The eluate of the ion-exchange column was then precipitated with ammonium sulfate at 60% and 95% saturation. The final precipitate was dissolved in 100 mM $(NH_4)HCO_3$ and purified by size-exclusion chromatography (Sephacryl 300 HR) using 100 mM $(NH_4)HCO_3$ as running buffer. The eluate was lyophilized and redissolved at a concentration of 105 μM in 50 mM sodium phosphate, pH 7.5, and shock frozen in liquid nitrogen. The protein aliquots were stored at −70 °C until use. The N-terminus of the protein (MEEKV) was confirmed by Edman degradation, and the purity of the recombinant protein was demonstrated in a protein gel (Figure 1A).

Fluorescence Anisotropy Measurements. To determine the dissociation constant of the protein−DNA complexes, we used fluorescence anisotropy measurements. In preliminary experiments (see also Results section), we found that *Sso*-RPA binds the 13 base long substrate (5′-GTTAATC-CTACC−rhodamin B) better than shorter DNA substrates. We also found that the reporter fluorophore rhodamin B (in contrast to fluorescein) has only marginal effect on the DNA−protein equilibrium and that this substrate is bound with 1:1 stoichiometry by *Sso*-RPA. We therefore used this 13 base long oligodeoxynucleotide for all experiments. A Perkin-Elmer LS-50B fluorescence spectrometer equipped with a thermostatted cuvette holder was employed. The titrations were carried out in 20 mM sodium cacodylate (pH 7.5) and 0.05% Tween 20 in the presence of various salts and at different temperatures in a stirred 1 mL cuvette silanized with 2% (v/v) dichlorodimethylsilane in trichloroethane (Merck, Darmstadt, Germany). We performed the experiments in cacodylate buffer since the ionization constant of cacodylate is only marginally dependent on the temperature.

Typically 100−2000 nM protein was added to 30 nM rhodamin B-labeled oligodeoxynucleotide. The protein concentration was then serially decreased by replacing aliquots of the protein−DNA solution in the cuvette with buffer containing the DNA substrate. The sample was excited at 530 nm using a 15 nm slit and a vertical polarizing filter. The vertical and horizontal emission was monitored at 580 nm with a slit of 20 nm. At each titration point, the anisotropy was measured at least three times with 10 s integration time.

Stoichiometric titrations were also performed with a 9 nt long (5′-ATCCTACCT−fluorescein) and 25 nt long (5′-TTGCCATTGGAATCGGTCCAATGTA−fluorescein) single-stranded DNA substrate.

Data Analysis. The experimental data was analyzed with a 1:1 model for the equilibrium using the formula

$$\mathrm{AB} = \frac{(A_0 + B_0 + K)}{2} - \frac{\sqrt{(A_0 + B_0 + K) - 4A_0B_0}}{2} \quad (1)$$

In eq 1, A_0 and B_0 are the total concentrations of protein and DNA, respectively, AB is the concentration of the complex, and K is the dissociation constant of the complex.

When fluorescence anisotropy data are analyzed, an eventual intensity change upon complexation has to be taken into account. Since the fluorescence intensity of the fluorescein-labeled DNA increased 10% during a titration, we corrected our data by the fractional intensity (*16*).

For the experiments performed at a constant temperature but with varying salt concentration, we analyzed the data

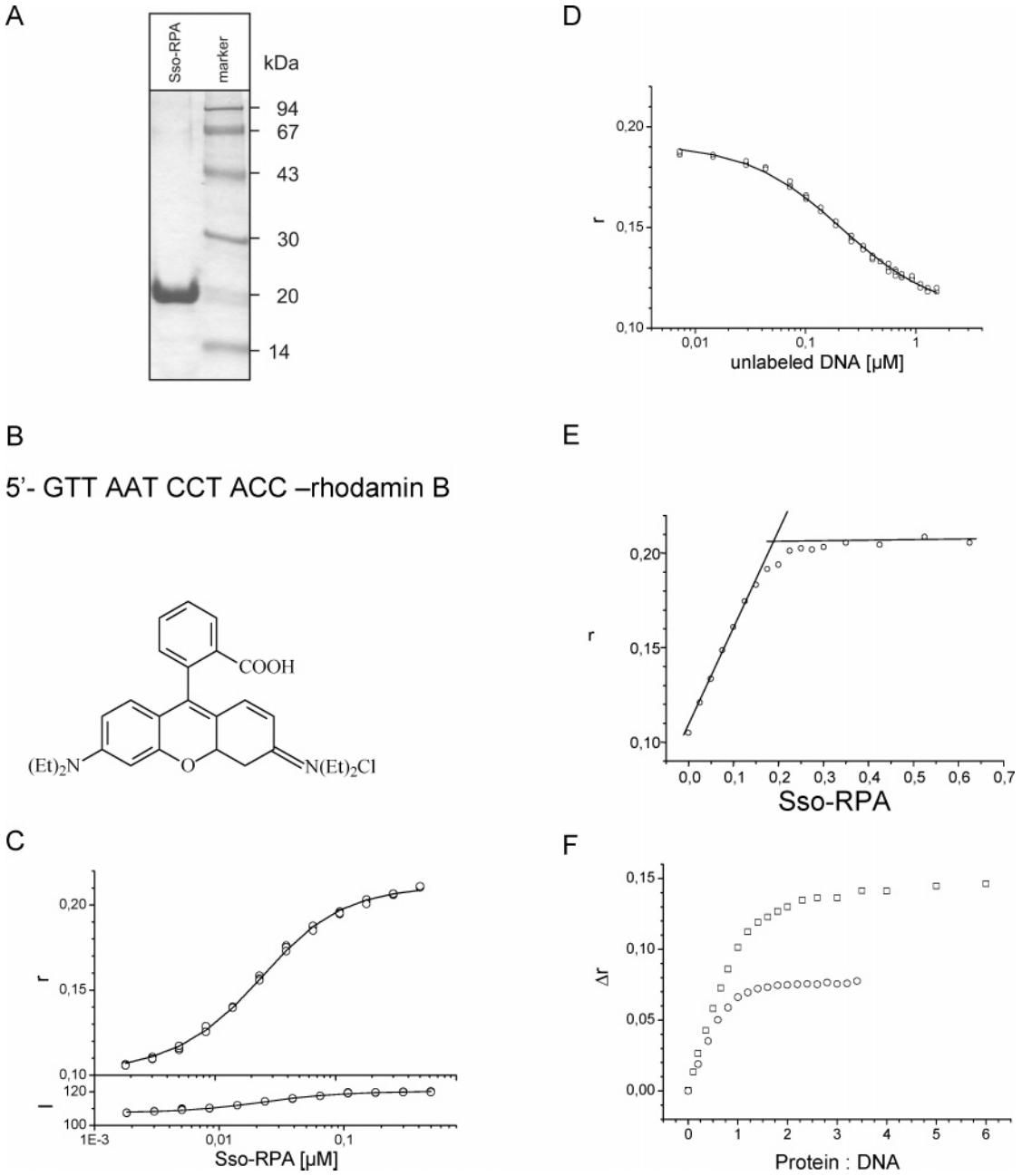

FIGURE 1: *Sso*-RPA binds with high affinity to a 13 nucleotide single-stranded DNA. Panel A shows a Coomassie-stained protein gel of purified *Sso*-RPA. Panel B shows the structure of the single-stranded substrate and the fluorophore rhodamin B used in this study. In panel C, 20 nM 13nt-DNA was titrated with 500−2 nM *Sso*-RPA by a reverse titration at 23 °C in a buffer containing 150 mM potassium chloride. Shown are the data points of the anisotropy (*r*, upper graph) and the intensity (*I*, lower graph). Both data sets were analyzed assuming a 1:1 stoichiometry. The dissociation constant determined from the anisotropy measurement is 16 nM taking the intensity increase of 13% into account (see Materials and Methods section for details). Panel D shows competitive titration of 20 nM labeled DNA with 100 nM *Sso*-RPA with 7−1500 nM unlabeled DNA. The analysis gives a dissociation constant of 24 nM for the complex between protein and unlabeled DNA. Panel E shows stoichiometric titration of 200 nM DNA with *Sso*-RPA in the presence of 100 mM potassium chloride. Stoichiometric equivalence is reached at about 190 nM protein. Panel F shows stoichiometric titration of 100 nM of a 9 nucleotide long DNA (○) and of a 24 nt long DNA (□). Plotted is the anisotropy change (Δr) against the protein−DNA stoichiometry.

with a global fit and treated the anisotropy of the free DNA and the anisotropy of the complex as global parameters. The obtained individual dissociation constants were then analyzed with the formula

$$\ln K = n \ln [\text{salt}] + \text{intercept} \qquad (2)$$

where [salt] is the molar salt concentration. The slope n is generally a positive value, and its value indicates the number of ions released upon complex formation (*17*).

The data from the experiments performed at varying temperatures were analyzed globally when we found that the reciprocal value of the anisotropy of the free DNA is linear to the absolute temperature. In this case, we restrained the anisotropy of the free and bound DNA with a linear relationship to the temperature. The resulting dissociation constants at the various temperatures were then plotted according to van't Hoff (ln K vs $1/T$).

A linear relationship in these plots is indicative of a temperature-independent ΔH and ΔS since the heat capacity

change ΔC_p of the respective reactions is zero or negligible. The binding data could then be analyzed with the formula $\ln K = \Delta H/(RT) - \Delta S/R$, ΔH and ΔS being the enthalpy and entropy of the association reaction.

To analyze the competition data, we used the program Dynafit (*18*). We assume that a single molecule of *Sso*-RPA can bind to either one molecule of labeled or one molecule of unlabeled DNA. The dissociation constant of the complex between protein and unlabeled DNA could be derived with the previously determined dissociation constant of the complex between *Sso*-RPA and the rhodamin B-labeled DNA.

RESULTS

Recombinant Sso-RPA Binds with High Affinity to Short Single-Stranded DNA. To study the DNA binding of *Sso*-RPA, we developed a fluorescence anisotropy based assay, which allows us to determine the dissociation constant of the DNA−protein equilibrium with good precision under a variety of conditions. Data analysis is greatly simplified when a 1:1 binding event is observed, and higher-order complexes between DNA and protein are not formed under the experimental conditions. We therefore tested oligonucleotides of different lengths and found that oligonucleotides of 9, 11, and 13 nucleotides are bound about equally well but that an oligonucleotide of only 7 nucleotides is bound considerably more weakly (data not shown). The 13 nucleotide long oligonucleotide is bound at 150 mM potassium chloride with a dissociation constant of 16 nM. The experimental data points determined by a titration covering more than 2 orders of magnitude of protein concentration could be very well analyzed using a single-site binding model (Figure 1C).

To exclude that the attached fluorescent dye influences the equilibrium, we tested the 13 nucleotide substrate in a competition experiment and found that unlabeled DNA is bound only marginally more weakly by the protein ($K = 24$ nM) indicating that the attached rhodamin B contributes little to the binding (Figure 1D). Next we determined the stoichiometry of the binding reaction. For the site titration, we lowered the salt concentration to 100 mM potassium chloride and used 200 nM DNA. Stoichiometric equivalence was reached at about 190 nM protein. The site size of *Sso*-RPA has been determined to be approximately five bases (*11*). It is therefore possible that additional protein molecules of *Sso*-RPA can be bound by this 13 nucleotide substrate. These binding events however are of weak affinity and do not affect the analysis of binding curves as in Figure 1C on the basis of a 1:1 stoichiometry. It should also be noted that the anisotropies of the free and bound DNA are nearly identical in the reverse titration (Figure 1C) and in the site titration (Figure 1E). For control purposes, we also determined the binding stoichiometry for a 9 and a 24 nt long DNA. The 9 nt long DNA clearly exhibits 1:1 stoichiometry whereas the 24 nt long DNA substrate seems to bind at least two molecules of *Sso*-RPA (Figure 1F). Given the low influence of the fluorescent dye on the equilibrium and the 1:1 stoichiometry, we conclude that the rhodamin B labeled 13 base long oligonucleotide is well suited to study the DNA binding properties of *Sso*-RPA.

Salt Dependence of the DNA Protein Equilibrium. The negatively charged backbone of the nucleic acid and the protein are able to bind ions. Upon complexation of the DNA with the protein, ions may be released. This release makes an entropic contribution to the equilibrium, which can be formalized as the dilution entropy of the ions from unit activity to the activity of the ion in the bulk solution, that is, $RT \ln [\text{salt}]$:

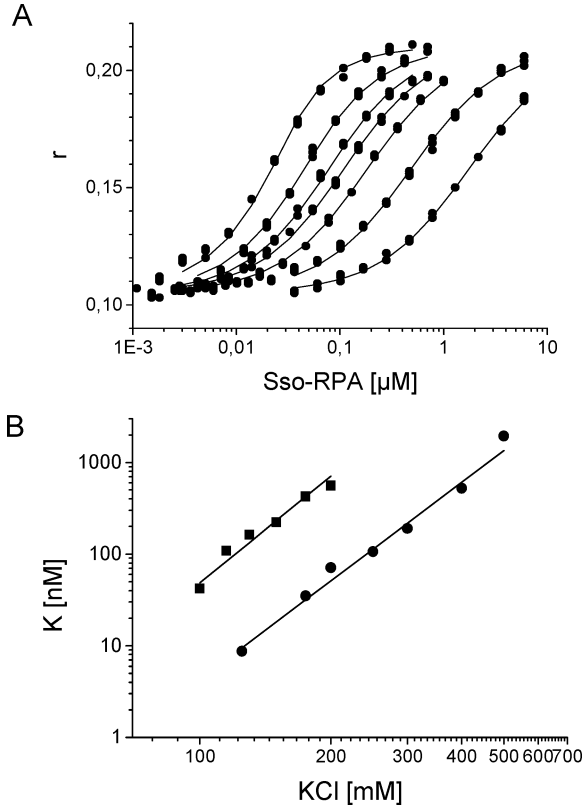

FIGURE 2: Influence of the potassium chloride concentration on the binding strength. Panel A shows isotherms at varying potassium chloride concentrations. In this experiment, 30 nM DNA was titrated with protein at 23 °C in the presence of 125, 175, 200, 250, 300, 400, and 500 mM potassium chloride (from left to right). The data were analyzed with the anisotropies of the free and bound DNA restrained as global parameters (see Materials and Methods section for details). Panel B shows a plot of the dissociation constants as a function of the potassium chloride concentration at 23 (●) and 52 °C (■). The data were analyzed with eq 2 and yielded $n = 3.6 \pm 0.03$ and 3.9 ± 0.02, respectively. The positive values indicate that ions are released when *Sso*-RPA binds DNA.

$$\Delta G = RT \ln K = \Delta G^\circ + nRT \ln [\text{salt}] \qquad (3)$$

K being the dissociation constant of the complex, ΔG° the free energy change at 1 M salt, n the number of ions released upon complexation, and [salt] the salt concentration (*17*).

According to eq 3, the number of ions released can be determined by measuring the dissociation constant at different salt concentration:

$$n = \frac{\partial \ln K}{\partial \ln [\text{salt}]} \qquad (4)$$

Figure 2A shows titrations of 30 nM DNA with *Sso*-RPA in the presence of 125−500 mM potassium chloride. The data set was globally analyzed and yielded a dissociation constant at each salt concentration. In double-logarithmic plots, the dissociation constants increase linearly with the salt concentration as expected from eq 4 (Figure 2B). The corre-

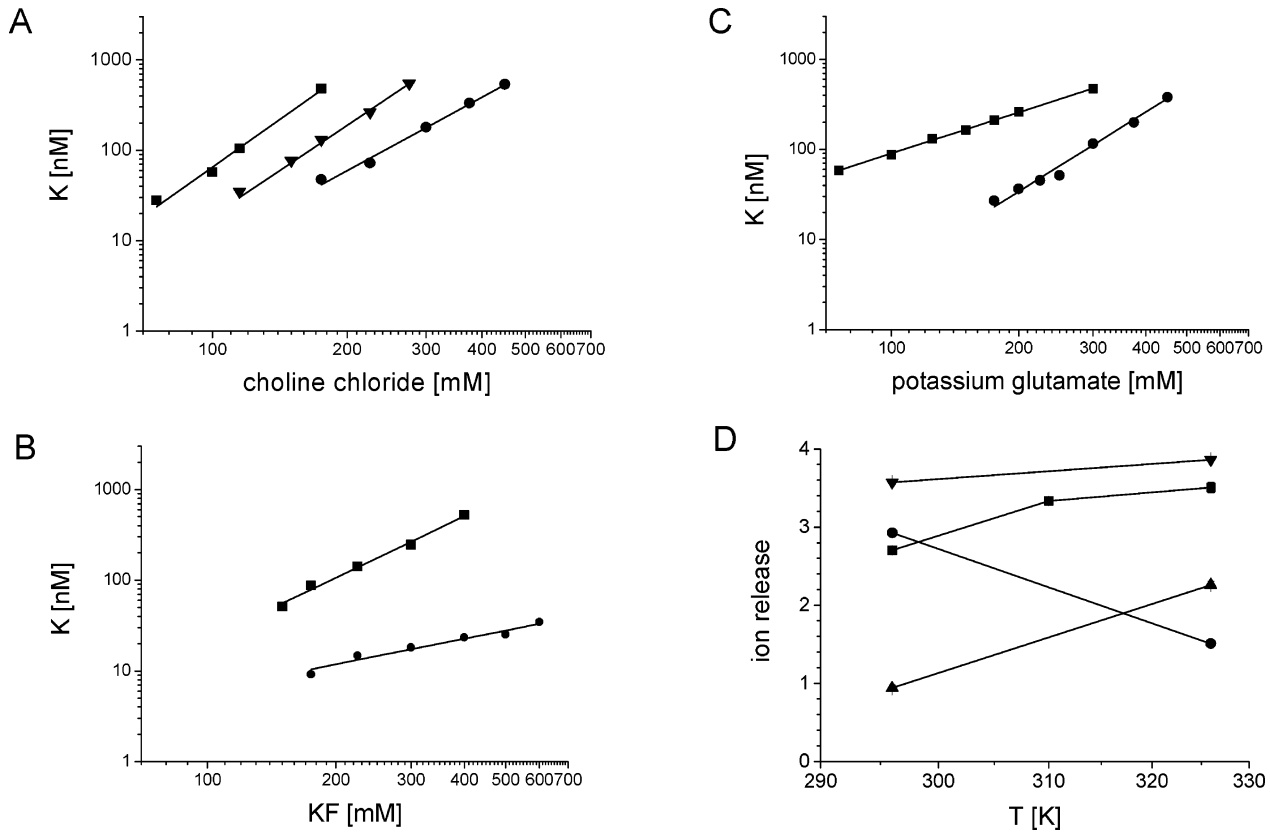

FIGURE 3: Influence of the salt concentration on the binding strength. The dissociation constants were determined for choline chloride (A), potassium fluoride (B) and potassium glutamate (C) at 23 (●), 37 (▼), and 52 °C (■). The derived values for the ion release are given as points in panel D. The amount of ion release is clearly temperature dependent for potassium fluoride (▲) and potassium glutamate (●). Potassium chloride (▼) and choline chloride (■) display only a weak temperature dependence. The abscissa is formatted as reciprocal.

sponding slopes indicate a release of 3.6 ions at 23 °C and 3.9 ions at 53 °C.

The experiments were repeated with different salts. We used salts with a larger cation (choline chloride) and with larger and smaller anions (potassium glutamate and potassium fluoride). Figure 3A−C shows the corresponding data for different temperatures (23, 37, and 53 °C). In Figure 3D, the slopes obtained for all four salts are shown as a function of temperature. For both chloride salts, only a weak temperature dependency is observed. In the presence of these two salts, approximately three to four ions are released. In contrast, the ion release in the presence of potassium glutamate and potassium fluoride is highly temperature dependent. Moreover the temperature dependence of both salts is opposite. In the presence of potassium fluoride, only about one ion is released at 23 °C; this value increases to about 2.3 ions at 53 °C. In contrast, in the presence of potassium glutamate, the number of released ions drops from approximately three at 23 °C to 1.5 at 53 °C.

The comparison of the different salts allows an estimation of which ions are actually involved in binding and release. Potassium chloride and choline chloride behave very similarly as about the same number of ions are released and there is only marginal temperature dependence. The small difference between the two salts originates probably from the cation. It appears that less choline ions are released than potassium ions. The choline ion has the same charge but is bulkier and might therefore be less well bound by the DNA or the protein.

At 23 and at 52 °C, more ions are released in the presence of potassium chloride as compared to potassium fluoride. We do not expect that DNA binds chloride and fluoride. These anions are thus probably bound by the protein and are released upon complexation. At both temperatures, fewer fluoride ions are bound/released as compared to chloride. The release of potassium fluoride increases at the higher temperature. In contrast, the ion release decreases with temperature in the presence of potassium glutamate.

Temperature Dependence of the DNA−Protein Equilibrium. The enthalpy and entropy of the DNA−protein binding reaction can be determined by studying the binding reaction at different temperatures. For this type of study, thermophilic proteins are preferable since it is possible to include experiments with higher temperatures than those with mesophilic proteins. Figure 4A shows the binding curves determined between 11 and 59 °C. The reciprocal anisotropy of the free DNA changes linearly with temperature. We assumed that such a linear relationship holds also for the protein−DNA complex and used these relationships to perform a global analysis, which yielded a dissociation constant for each temperature. The dissociation constants were plotted against the inverse temperature (Figure 4B). We observed a linear relationship in this van't Hoff plot, which suggests that the reaction enthalpy is temperature independent and that the heat capacity change, ΔC_p, of the binding reaction is close to zero. The heat capacity change for non-sequence-specific DNA-binding proteins is generally low compared to that for sequence-specific DNA-binding proteins. The latter proteins often bind to their cognate DNA with large negative ΔC_p values (*19*). The negative heat capacity change observed for sequence-specific binding is mainly explained by the burial of the nonpolar surface, which

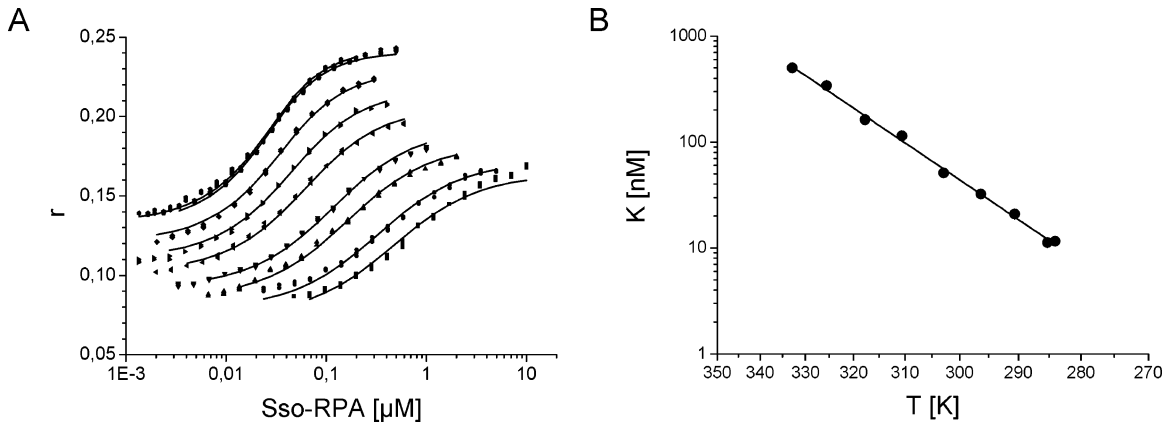

FIGURE 4: Temperature dependence of the binding strength. Panel A shows isotherms at nine temperatures between 284 and 332 K. In this experiment, 30 nM DNA was titrated with protein in the presence of 175 mM potassium chloride. The data were analyzed with the anisotropies of the free and bound DNA restrained as global parameters (see Materials and Methods section for details). Panel B shows van't Hoff analysis of the equilibrium data. The linear relationship indicates a temperature independent enthalpy (ΔH). The association reaction is exothermic with $\Delta H = -62 \pm 0.7$ kJ/mol. The abscissa is formatted as reciprocal.

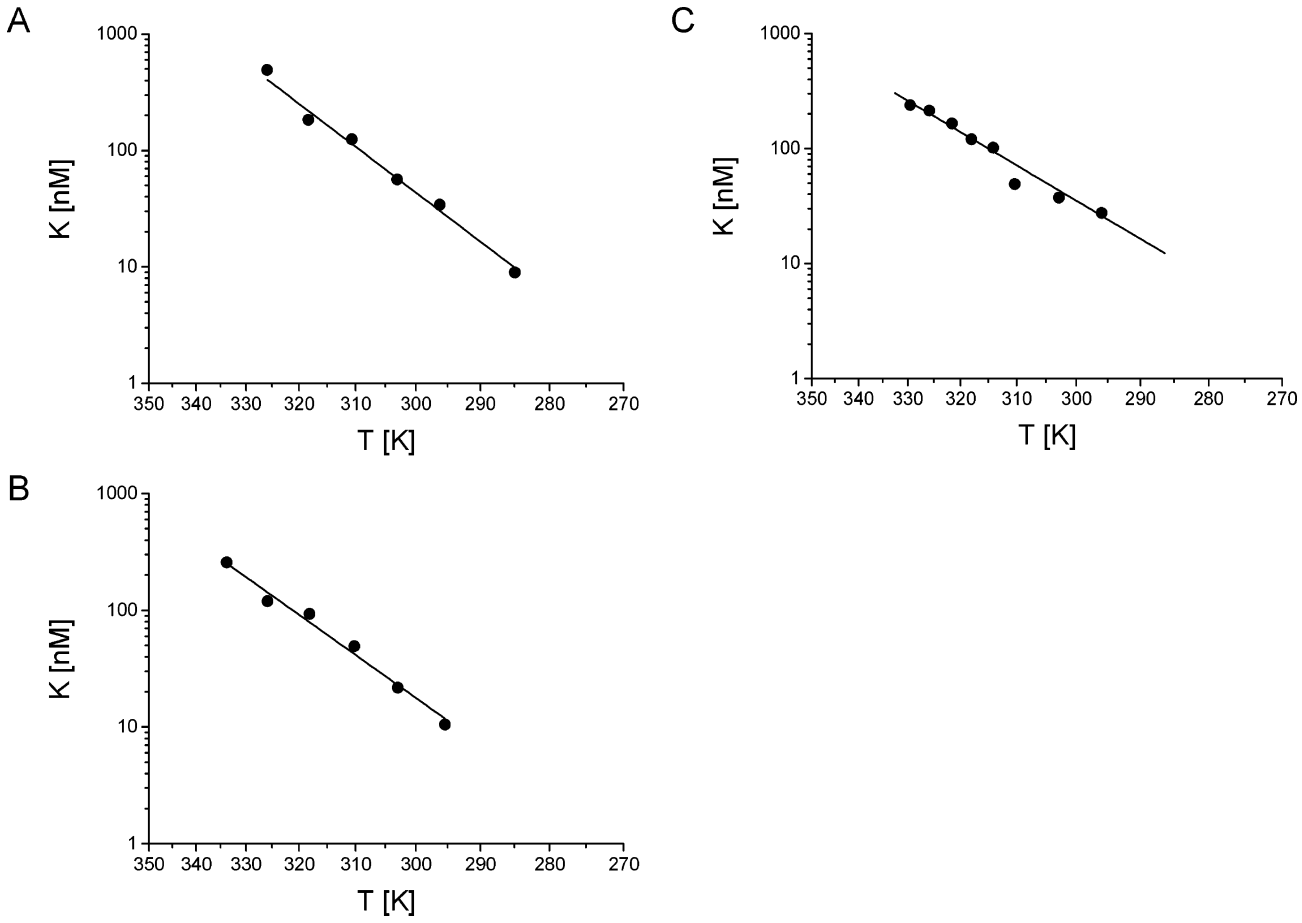

FIGURE 5: van't Hoff analysis of the association reaction under different salt conditions. The fluorescence titrations were carried out in the presence of 175 mM choline chloride (A), 225 mM potassium fluoride (B), and 175 mM potassium glutamate (C). For all salts, a linear relationship is observed. The enthalpies are -70 ± 1.5, -66 ± 1.5, and -55 ± 1.3 kJ/mol, respectively. The abscissas are formatted as reciprocal.

results from the good surface complementarity between the protein and the specific DNA substrate (*20*, *21*). Additional contributions to a negative heat capacity change may stem from protein folding upon binding, from base unstacking, and from vibrational tightening (*19*, *22*).

The association reaction in the presence of potassium chloride is exothermic with $\Delta H = -62$ kJ/mol. Similar values were also found for choline chloride (-70 kJ/mol), potassium glutamate (-66 kJ/mol), and potassium fluoride (-55 kJ/mol). For all salts, we found a linear relationship in the van't Hoff plots (Figure 5). The enthalpy of the association reaction has also been determined directly by isothermal calorimetry. Kerr et al. (*5*) found $\Delta H = -59$ kJ/mol and a dissociation constant of 360 nM in a buffer containing 100 mM potassium glutamate at 50 °C (*5*). They performed their study with a 21 base long oligodeoxynucleotide. Under the high concentrations necessary for the calorimetric measurements, four molecules of *Sso*-RPA bind

to this DNA substrate. The values reported above are corrected for a binding of a single protein molecule. Nevertheless their values agree quite well with our data, since we determined $\Delta H = -66$ kJ/mol at 175 mM potassium glutamate and 53 °C and a dissociation constant of 88 nM at 100 mM potassium glutamate. Our dissociation constant differs from their value by about factor of 4. This is certainly attributable to the weaker binding of the third and fourth *Sso*-RPA molecules on the already partly covered 21 bp DNA substrate.

Interdependence of the Salt and Temperature Effects on the Equilibrium. The linkage between ion release and temperature has been rigorously analyzed by the group of Lohman with *E. coli* SSB (*15*, *23*). They found that the net ion release in sodium chloride decreases considerably with temperature, whereas the ion release remains constant in the presence of sodium fluoride. Accordingly they found that the enthalpy change increases (becomes less negative) with increasing sodium chloride and choline chloride concentration. In the presence of sodium fluoride however, the enthalpy change appeared to be independent of the salt concentration.

The calorimetric determination of the reaction enthalpy is error-prone in the case of *Sso*-RPA since higher complexes might form at the high protein and DNA concentrations that are necessary for an isothermal titration calorimetry. However assuming that the binding reaction maintains a heat capacity change around zero in the temperature and salt concentration range of this study, the enthalpy change at different salt concentrations can be extracted from the salt dependence of the equilibrium at the two temperatures. With $\partial \ln K/\partial \ln$ [salt] depending linearly on $1/T$ and $\partial \ln K/\partial \ln[1/T]$ depending linearly on ln [salt] (*15*) integration yields

$$\ln K = \text{slope}_0 \ln [\text{salt}] + \Delta H_0/R(1/T) + i \ln [\text{salt}] (1/T) + \ln K_0 \quad (5)$$

with a new interaction factor i, which quantifies the linkage between ion release and temperature. For $i = 0$, the ion release is temperature independent and the reaction enthalpy is independent of salt concentration. For positive values of i, the ion release decreases with increasing temperature and the enthalpy change increases with salt concentration. Thus for negative values of i, the ion releases increases with temperature and the enthalpy change decreases with increasing salt concentration. The interaction factor i equals the slope of a plot of ion release vs $1/T$, that is, $i = \partial/\partial T^{-1}(\partial \ln K/\partial \ln$ [salt]) and is also related to the slope of a plot ΔH vs ln [salt]:

$$\frac{\partial \Delta H}{\partial \ln [\text{salt}]} = Ri \quad (6)$$

Based on the salt and temperature dependence of the dissociation constants, the values for the interaction factor were determined for all four salts. We found for potassium chloride $i = 30 \pm 182$ K; thus, the interaction factor for this salt could be zero. A rather low value was also determined for choline chloride with $i = -592 \pm 144$ K. In contrast, we found a highly positive value for potassium glutamate, $i = 3540 \pm 130$ K, and a highly negative value for potassium fluoride, $i = -3430 \pm 210$ K. Thus the four salts reflect the different extent of linkage between ion release and the thermodynamics of the binding reaction: no linkage (potassium chloride), weak linkage (choline chloride), and strong but opposite linkage (potassium fluoride and potassium glutamate).

Table 1: Global Analysis of the Thermodynamics of the *Sso*-RPA DNA Binding Reaction[a]

	298 K		358 K			
	K [nM]	ΔG [kJ/mol]	K [nM]	ΔG [kJ/mol]	ΔH [kJ/mol]	ΔS [J/(K·mol)]
choline chloride	26	−43.2	2500	−38.3	−67	−81
potassium fluoride	9,2	−45.8	381	−44.0	−55	−31
potassium glutamate	18	−44.2	1130	−40.7	−61	−57
potassium chloride	23	−43.6	1790	−39.4	−65	−70

[a] The equilibrium data from all data sets were analyzed with eq 5, which implies that enthalpy and entropy are temperature independent. The data originating from the titrations with varying salt concentration and varying temperatures agreed well with regression coefficients $r^2 > 0.95$ for every complete salt data set. At room temperature (25 °C) and the physiological growth temperature of *Sulfolobus* (85 °C), the binding is strongest in the presence of potassium fluoride followed by potassium glutamate. The data were calculated for a salt concentration of 150 mM. We estimate that the dissociation constants have a relative error of about 50% corresponding to an error of about 2 kJ/mol for ΔG. For ΔH, we estimate an error of 5 kJ/mol and for ΔS an error of 10 J/(K·mol).

We have also used eq 5 to calculate the dissociation constant K, the enthalpy ΔH, and the entropy ΔS at a salt concentration of 150 mM and at 298 and 358 K, the growth temperature of *Sulfolobus* (Table 1). The binding affinity is considerably higher at room temperature than at the physiological growth temperature of *Sulfolobus*. The binding of proteins to single-stranded DNA appears to be inherently exothermic, and therefore, also thermophilic proteins display a weaker binding at higher temperatures, $\partial \ln K/\partial T^{-1} = \Delta H/R < 0$. In the physiological context however, the binding affinity seems to suffice although mesophilic proteins, such as human RPA and *E. coli* SSB, have a lower dissociation constant and high cooperativity leading to coating of single-stranded DNA at substantially lower, that is, nanomolar, concentration of protein at their physiological temperature. Among the four salts studied, potassium fluoride supports the highest binding affinity followed by potassium glutamate. A strong "glutamate effect" as seen for other DNA binding proteins (*24*) is not apparent for *Sso*-RPA.

DISCUSSION

Sso-RPA Binds Single-Stranded DNA. We used heterologously expressed *Sso*-RPA to study its DNA binding. The recombinant protein exhibits the same DNA binding properties as the native purified protein (*11*). In agreement with others, we find that *Sso*-RPA elutes as a monomer from a gel-filtration column. We quantified the DNA binding activity of *Sso*-RPA with a highly sensitive fluorescence depolarization assay. This assay allowed us to determine the binding affinity with 10−30 nM DNA in the presence of four different salts and in the temperature range from 284 to 332 K. As the reporter molecule, a rhodamine dye is attached at the 3′-end of the DNA; the binding of *Sso*-RPA

to the DNA is possible without steric interference from the dye. We do not observe an appreciable fluorescence intensity change nor a change of the emission spectra of the dye. Both effects would be indicative of binding of the protein to the rhodamine dye. Moreover a competitive titration confirms that the labeled and unlabeled DNA are bound with about the same affinity. Taken together, these observations suggest that the rhodamine dye, which is attached at the 3′ end of the DNA, makes only a minor contribution to the binding. The length of the DNA, 13 nucleotides, is larger than the binding site of *Sso*-RPA, which has been determined to be five bases (*11*), allowing a single molecule of protein to bind to the middle of the DNA without binding to the ends or to the dye. To exclude that more than one protein molecule binds to the DNA substrate, we carried out a site titration. This experiment confirms the 1:1 stoichiometry.

Salt Dependence of the Binding. The amount of net ion release can be determined by studying the DNA−protein equilibrium at different salt concentrations. However this analysis does not allow determination of the contributions of anions and cations individually. By use of several different ions, it is however possible to draw some conclusions about the nature of the ion release.

Our data suggest that the amount of ions released and its temperature dependence is related to the anions. The salts potassium chloride, potassium fluoride, and potassium glutamate differ in their anions and result in different ion release properties (Figure 3D). In contrast, a change of the cation, that is, from choline chloride to potassium chloride, only results in a small difference in ion release. The anion related effect is probably due to the different interactions of the anions with the protein since anions do not bind to DNA.

The temperature dependence of the ion release can be contributed by a temperature dependent cation release from the DNA and by a temperature dependent ion release from the protein. With cationic peptides, the release of cations from DNA has been studied in detail. The cation release has been shown to be independent of temperature (*25*). Therefore the temperature dependence of the ion release that we observe for the *Sso*-RPA DNA equilibrium is mainly caused by the temperature dependence of the anion binding/release on behalf of the protein.

To our knowledge, the temperature dependence of ion release has only been analyzed in detail for the homotetrameric *E. coli* SSB (*15*). For this protein, the ion release was studied in the presence of sodium chloride and sodium fluoride in the temperature range from 10 to 37 °C (*15*). In the presence of sodium chloride, a decrease of ion release from nine ions at 37 °C to five ions at 10 °C was observed. When the ion release was plotted against the inverse of the absolute temperature, a slope of −13 300 K was determined for poly(U) and −7100 K for dA_{70} (*15*). These slopes are equal to the negative value of the interaction parameter i that we introduced to describe the relationship between temperature and ion release (see eq 5). In contrast, the ion release in the presence of sodium fluoride appeared to be constant in the temperature range studied.

For *Sso*-RPA, we found that potassium chloride and choline chloride display only a weak temperature dependency with value of i below 1000 K. In contrast, potassium glutamate with $i = 3540 \pm 130$ K displays the same behavior as sodium chloride with *E. coli* SSB, a decrease of ion release at increasing temperatures. In the presence of potassium fluoride, we observe for *Sso*-RPA an increase of the ion release with increasing temperature ($i = -3430 \pm 210$ K). The amount of change is comparable between the bacterial and the archaeal protein since the *E. coli* ion release data refers to the binding of a tetramer, whereas the *Sso*-RPA data relates to the binding of a monomer.

The single-stranded DNA-binding proteins from *E. coli* and *S. solfataricus* differ with respect to the temperature dependency of the release of anions. The differences between the enzymes might be partially explained by their different quaternary structure. *E. coli* SSB binds DNA in different binding modes depending upon the ion concentration suggesting that there is an intimate interplay between ion concentration and DNA-binding activity. It is therefore likely that the binding of the *E. coli* protein to DNA involves the release (and binding) of ions not related to the primary binding of the DNA but rather to interactions between the subunits. The latter effects are not expected for the *Sulfolobus* protein since it binds as a monomer to the DNA substrate. Clearly the different salt "behaviors" point to an individuality of these proteins, which has not yet been fully addressed.

Thermodynamics of DNA Binding. The binding of *Sso*-RPA to the DNA is dominated by its large favorable enthalpy change. At 175 mM salt, the enthalpy change is −55 to −70 kJ/mol (Figure 5). The reaction enthalpy seems to be constant over the investigated temperature range indicating that the heat capacity change in the investigated temperature range of 290−332 K is rather small. A temperature independent heat capacity change of 0.5 kJ/(mol·K) would lead to a change of the reaction enthalpy of 20 kJ/mol, which would result in a nonlinear van't Hoff plot.

The reaction enthalpy determined by us agrees well with the value determined by White's group through isothermal calorimetry, which was −59 kJ/mol for the binding of one molecule of *Sso*-RPA (*5*). We found that *Sso*-RPA binds more strongly than reported in this study. This difference can be explained by the different buffer and DNA substrate used and particularly by the completely different experimental approach. Accordingly Kerr et al. find the entropy change at about −140 J/(K·mol) at 100 mM potassium glutamate compared to about − 57 J/(K·mol) calculated from our titration data (150 mM potassium glutamate, Table 1).

The binding of homotetrameric *E. coli* SSB protein to DNA is strongly exothermic with enthalpies up to − 600 kJ/mol (*23*). Stacking interactions of aromatic amino acids with the nucleobases, protonation of the protein, and ionic interactions between basic amino acids and phosphates from the DNA backbone contribute to the large negative enthalpy of the binding reaction. For *Sso*-RPA, the mutation of two conserved aromatic residues (W56A and F79) to alanine has been shown to strongly increase the enthalpy of the binding reaction from −59 to −33 and −41 kJ/mol, respectively (*5*). It therefore appears that these side chains are involved in the binding of DNA in a contact that makes an exothermic contribution, such as stacking interactions.

The salt dependence of the binding enthalpy has so far been studied only for a few proteins, for example, *E. coli* SSB (*23*), integration host factor (IHF) from *E. coli* (*26*), and the DNA binding domain from the protooncogene *c*-Myb (*27*).

For IHF, the thermodynamics of the binding reaction to double-stranded DNA was analyzed in the presence of different potassium chloride concentrations. For potassium chloride concentrations above 100 mM, an increase of the reaction enthalpy was observed with increasing salt concentrations; for example, at 40 °C the reaction enthalpy increases from −240 kJ/mol at 100 mM potassium chloride to −45 kJ/mol at 350 mM potassium chloride (*26*). Reanalysis of their data yields an interaction factor *i* (eq 6) of about 19 000 K for the binding reaction between the dimeric IHF protein and a 34 bp DNA. Because in this case the heat capacity change was salt dependent, the interaction factor *i* was also temperature dependent and decreased to about 4600 K at 20 °C.

Similar observations were found for the DNA binding domain of *c*-Myb (*27*). When the potassium chloride concentration was raised from 200 to 400 mM, the reaction enthalpy increased from −52 to −38 kJ/mol, and the heat capacity change increased from −2.6 to −1.8 kJ/(mol·K). These data corresponds to an interaction factor of 2400 K at the temperature of 20 °C.

In the case of *E. coli* SSB, the enthalpy increases with increasing choline chloride and sodium bromide concentration. The reaction enthalpy increased from −237 kJ/mol at 180 mM sodium chloride to −141 kJ/mol at 420 mM sodium chloride for the binding of poly(U). The increase was linear in a plot of reaction enthalpy vs the logarithm of the salt concentration and corresponds to an interaction factor of 13 600 K (eq 6). However, at low salt concentration (below 0.1 M), the enthalpy remains fairly constant and starts to increase at higher salt concentrations. Thus it appears that the linkage between salt concentration and enthalpy is more complex than the simple linear relationship that we used in eq 5. We have not determined the enthalpy directly by calorimetry. Instead we have used van't Hoff analysis and assumed that the binding enthalpy is independent of temperature. With these suppositions, we suggest that potassium glutamate and potassium fluoride have opposing effects on the enthalpy: an increase of salt concentration lowers the enthalpy in case of potassium fluoride and increases it in case of potassium glutamate. The structural basis of these effects can however not be elucidated with the available data. For *E. coli* SSB, it has been shown in a detailed study that preferential exothermic binding of chloride to SSB along with some coupled protonation might be responsible for the observed linkage between salt concentration and enthalpy (*23*). Further, more detailed studies on *Sso*-RPA and on other DNA binding proteins are required to fully understand the thermodynamics of DNA binding and to dissect the enthalpic and entropic contributions from ion binding, protonation, folding, and conformational changes to the overall binding reaction. These contributions must then be related to the known structures of the protein and the protein−DNA complexes to fully appreciate the forces and mechanism of specific and unspecific DNA binding by proteins.

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