Site-Specific Cation Binding Mediates TATA Binding Protein−DNA Interaction from a Hyperthermophilic Archaeon

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ABSTRACT: *Pyrococcus woesei* (*Pw*) is a hyperthermophilic archaeal organism that exists under conditions of high salt and elevated temperature. In a previous study [O'Brien, R., DeDecker, B., Fleming, K., Sigler, P. B., and Ladbury, J. E., (1998) *J. Mol. Biol. 279*, 117−125], we showed that, despite the similarity of primary and secondary structure, the TATA box binding protein (TBP) from *Pw* binds thermodynamically in a fundamentally different way to its mesophilic counterparts. The affinity of the interaction increases as the salt concentration is increased. The formation of the protein−DNA complex involves the release of water and the uptake of ions, which were hypothesized to be cations. Here we test this hypothesis by selecting potential cation binding sites at negatively charged, acidic residues in the complex interface. These were substituted using site-directed mutagenesis of specific residues. Changes in the thermodynamic parameters on formation of the mutant protein−DNA complex were determined using isothermal titration calorimetry and compared to the wild type interaction. Removal of a glutamate residue from the binding site resulted in the uptake of one less cation on formation of the complex. This glutamate (E12) is directly involved in the binding of cations in the complex interface. Substitution of another acidic residue proximal to the DNA binding site (D101) had no effect on cation uptake, suggesting that the location of the amino acid on the protein surface is important in dictating the potential to coordinate cations. Removal of the cation binding site provided a more favorable entropy of binding; however, this effect is significantly reduced at higher salt concentrations. The removal of the cation binding site led to an increase in affinity with respect to the wild-type TBP at low salt concentrations.

The hyperthermophilic archaeon *Pyrococcus woesei* (*Pw*)[1] survives in conditions of elevated salt concentration and temperatures in excess of 100 °C (*1*). Despite this extreme environment, many of the proteins derived from this organism have high sequence and structural homology to mesophilic counterparts. Thus, survival in vastly different conditions is based on subtle adaptation of basic protein structure. This suggests an evolutionary advantage in maintaining a given protein structure and adapting it to be able to function in a dramatically different environment rather than evolve a protein de novo.

The TATA box-binding protein (TBP) is required as an initiator of the transcription machinery (*2*) in both archaeal and eukaryotic species (*3*, *4*). The *Pw* archaeal form binds upstream of transcription sites at promoters containing "box A motifs" possessing the consensus sequence TTTA(T or A)ANN (where N is any nucleotide) (*5*). This sequence is analogous to the eukaryotic TATA box (*6*). The structural homology of the *Pw*TBP (*7*) is high with respect to similar proteins from the mesophilic organisms *Arabidopsis thaliana* and *Saccharomyces cerevisiae* (*8*−*10*). Structural detail of the TBP from both archaeal and mesophilic organisms show that interaction with DNA occurs through binding in the minor groove (*11*−*16*). This effects a massive distortion of the oligonucleotide, resulting in the untwisting of the duplex by approximately a third of a helical turn (*17*).

Despite the similarities of structure of both free and bound forms of the *Pw* protein with mesophilic TBPs, the elevated salt concentrations in which the archaeal organism exists suggest that complex formation with DNA must be based on distinctly different criteria. The correlation between the observed binding constant (K_{obs}) for the interaction and the salt concentration can be modeled using the approach developed by Record and colleagues (*18*−*20*) shown in the integrated form of the equation defined by Ha et al. (*21*):

$$\log K_{obs,MX} = \log K_{REF,MX} - A\log[MX] + 0.016B[MX] \quad (1)$$

where K_{REF} is the observed binding constant at 1 M NaCl; A is the net number of ions released (a positive number) or taken up (a negative number) on forming the complex; [MX] is the univalent salt concentration; and B is the total number of water molecules released (a positive number) on forming the complex. From this equation, the change in numbers of cations and water molecules involved in binding can be estimated by determining the change in K_{obs} with varying salt concentration (*22*). In a previous study, we demonstrated that the dependence of K_{obs} on salt concentration for the *Pw*TBP−DNA interaction was opposite to that previously reported for other protein−DNA interactions (*23*), i.e.,

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[1] Abbreviations: TBP, TATA box binding protein; *Pw*, *Pyrococcus woesei*; ITC, isothermal titration calorimetry.

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increasing the salt concentration led to tighter binding. This suggested a very different mechanism of binding than previously observed in these types of interactions (*24*, *25*). Fitting binding data at different salt concentrations to the above equation led to the hypothesis that this effect could be attributed to a combination of the release of surface bound water molecules into bulk solvent and the net uptake of ions to mediate the protein−DNA interface (*23*). Because of the highly electronegative nature of the DNA, it was assumed that the ions were likely to be cations.

To test the above hypothesis, we attempted to determine the position(s) of ion integration in the protein−DNA interface. From structural determination, these cations are difficult to unequivocally assign; therefore, to identify potential sites of cation incorporation we adopted an approach based on site-directed mutagenesis. Since the coordination of cations in the interface would require interaction with negatively charged amino acids, we replaced selected acid residues in the TBP−DNA element interface. The effect of these TBP mutations could be observed by the change in salt dependence of binding when data were fit to eq 1. Since in eq 1 the effect of water release and cation binding have to be distinguished, we used osmotic stress experiments to verify the number of water molecules released on complex formation (ref *26* and references therein).

MATERIALS AND METHODS

PwTBP Expression and Purification. Full-length *Pw*TBP was expressed and purified as previously described. Protein concentrations were determined using an extinction coefficient of 11.05 mM^{-1} cm^{-1} at 280 nm, calculated from the individual extinction coefficients of aromatic residues and disulfides in the protein. Site-directed mutagenesis of the wild type (WT) *Pw*TBP was carried out using the QuikChange Site Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. Plasmids were sequenced (Oswel Sequencing Service) to verify the presence correct mutations and absence of random point mutations.

DNA Preparation. Binding experiments were carried out using 20mer DNA duplex synthesized by standard solid-phase methods (Oswel DNA Service). Concentrations of each strand were estimated using a calculated ϵ_{260} of 201.1 mM^{-1} for strand A (binding site underlined; CTGCACTTTAAAAAGACGTC) and 197.1 mM^{-1} for strand B (GACGTCTTTTTAAAGTCCAG). An equimolar mixture was annealed by slow cooling to room temperature. UV melting experiments showed that the DNA duplex had a single cooperative melting transition with a T_m of 63 °C in 100 mM NaCl, 20 mM NaH_2PO_4, pH 7.0, suggesting in the isothermal titration calorimetry (ITC) experiments carried out at 35 °C and over the range of conditions used that only a single species was present.

Isothermal Titration Calorimetry (ITC). All experiments were conducted on a VP ITC or an MCS ITC (Microcal Inc., Northampton, MA). Titrations were performed as described elsewhere (*27*, *28*). In a typical experiment 19, 15 μL injections of DNA (100 μM) were made into protein solution (10 μM) in the cell. The heats of dilution of the DNA into buffer and buffer into protein were determined in separate experiments and subtracted from the titration prior to the analysis. The data were analyzed using the ORIGIN software supplied with the calorimeter. Protein and DNA samples were dialyzed against the appropriate buffers prior to the experiments using 3500 MWCO dialysis membrane (Spectra/por, Spectrum Laboratories, Inc.). Titrations were performed in 10 mM MOPS (3-[*N*-morpholino]propanesulfonic acid), pH 7.0, and NaCl in the range 0.4 to 1.9 M. The pH of the interaction was checked before and after titrations since at the high salt concentrations used buffer pK_a values can be affected.

Osmotic Stress Experiments. Protein and DNA samples for the osmotic stress experiments were dialyzed using the same dialysis membrane into solutions containing 10 mM MOPS, pH 7.0, 0.7 M NaCl, and either betaine (SIGMA Chemicals) or sucrose (BDH Chemicals) added to the designated concentration (0, 0.15, 0.32, 0.53, 0.75, 0.88, 1.00, 1.14 molal and 0, 0.2, 0,38, 0.60, 0.65, 0.80, 0.90, 1.00 molal, respectively). The total osmotic potential for solutions were calculated using data provided by D. Rau (http://dir.nichd.nih.gov/Lpsb/docs/osmdata/osmdata.html). All experiments using osmolytes were performed using the MCS ITC (see above) with the temperature difference between the calorimetric cells and the water bath at 10 °C. Additional experiments were performed using the osmolytes triethylene glycol and glycerol; however, these showed no significant increase in K_{obs}. Poly(ethylene glycol) MW 400 (SIGMA Chemicals) was found to be unsuitable for osmotic stress experiments due to the very large heats of dilution and the resulting unstable calorimetric baseline. The osmotic data were analyzed as previously described (*29*) to estimate the number of water molecules released upon complex formation from the slope for the graph of the K_{obs} against osmolyte concentration (see below).

RESULTS AND DISCUSSION

ITC has been used to investigate the binding at 35 °C of *Pw*TBP to a 20mer DNA duplex, which contained the box-A recognition motif. The ITC data were fit using a model for a single site interaction. No effects due to nonspecific binding were observed, and in all cases the stoichiometry of the interaction was unity (mean for all experiments is 1.0 ± 0.1).

Cations are Incorporated into the Wild-Type PwTBP−DNA Interface. The effect of different salt types on the observed binding constant of wild-type TBP has previously been reported (*23*). Here titrations were performed using NaCl, since sodium ions had previously been shown to enhance high affinity complex formation. This meant that determination of K_{obs} for the *Pw*TBP−DNA interaction was possible over a wider range of salt concentration than previously reported.

Binding data for the wild-type *Pw*TBP over a range of NaCl concentrations are shown in Table 1. The data were fit to eq 1 using a nonlinear least squares algorithm with A, B, and K_{REF} as independent variables (Figure 1). From eq 1, it can be seen that the contribution to dependence of K_{obs} on salt concentration is nonlinear for the release of water molecules and linear for the incorporation of ions (*23*). For the wild-type interaction, the number of liberated water molecules derived from the fit is 39 ± 9; however, the fitting is rather insensitive to this variable. The net change in stoichiometry of ions upon complex formation of the wild-type TBP is -2.1 ± 0.1, corresponding to a net uptake of 2 ions on complex formation.

Table 1: Summary of Thermodynamic Parameters from ITC Experiments for the *Pw*TBP-d[CTGCACTTTAAAAAGACGTC/GACGTCTTTTTAAAGTGCAG] Interaction at pH 7.0 and 35 °C[a]

protein	[salt] M	[protein] (μM)	[DNA] (μM)	N	K ($\times 10^5$ M^{-1})	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	$T\Delta S$ (kJ mol^{-1})
WT	0.4	20	200	1.1	1.21 ± 0.5	110 ± 1.2	−30.0	140
WT	0.55	32	365	1.0	3.56 ± 0.8	107 ± 1.0	−32.8	140
WT	0.7	20	200	1.0	6.41 ± 0.8	108 ± 0.8	−34.3	143
WT	1.0	10	100	0.86	16.7 ± 1	111 ± 1.6	−36.7	149
WT	1.3	11	114	0.99	61.5 ± 3	110 ± 0.4	−40.1	150
WT	1.6	8	80	0.77	141 ± 8	111 ± 0.4	−42.3	153
WT	1.9	3.9	43	0.86	299 ± 34	111 ± 0.8	−43.9	155
E12A	0.2	12	120	1.3	2.99 ± 0.5	110 ± 0.8	−32.3	142
E12A	0.4	18	200	1.0	6.16 ± 0.2	111 ± 0.8	−34.2	145
E12A	0.7	12	120	1.0	23.4 ± 2	112 ± 0.8	−37.6	150
E12A	1.0	10	100	1.1	43.9 ± 2	113 ± 0.8	−39.2	152
E12A	1.6	8	80	1.0	135 ± 23	113 ± 1.6	−42.3	155
E12A	1.9	5	76	0.96	311 ± 36	111 ± 0.8	−44.4	156
D101A	0.4	18	200	1.1	1.15 ± 0.4	105 ± 2	−31.5	136
D101A	0.7	20	200	1.1	6.13 ± 0.3	110 ± 0.8	−34.1	145
D101A	1.0	12	120	0.96	20.9 ± 2	117 ± 1.6	−37.3	154
D101A	1.6	8	80	1.1	76.8 ± 9	115 ± 1.2	−40.6	156
D101A	1.9	5	43	0.79	288 ± 40	113 ± 1.2	−43.9	157
E12K	0.6	19	173	1.1	1.82 ± 0.6	119 ± 2.4	−31.0	150
E12K	0.7	12	133	1.1	3.20 ± 0.7	119 ± 0.8	−32.5	151
E12K	1.0	10	104	0.95	8.27 ± 0.2	118 ± 0.8	−34.9	153
E12K	1.6	9.5	99	0.87	36.1 ± 2	117 ± 1.2	−38.7	156
E12K	1.9	5	70	0.88	72.8 ± 5	118 ± 1.2	−40.5	158
Q103A	0.4	11	117	1.1	5.09 ± 0.3	116 ± 1.6	−33.7	150
Q103A	0.7	12	133	1.1	20.0 ± 0.9	114 ± 1.2	−37.2	151
Q103A	1.0	10	104	0.97	76.5 ± 6	116 ± 1.2	−40.6	157
Q103A	1.6	9	99	0.89	342 ± 33	116 ± 0.8	−44.4	160
Q103A	1.9	5	72	1.1	645 ± 52	117 ± 0.4	−46.0	163

[a] Data were fit using a model based on a single set of identical binding sites after heats of DNA dilution had been subtracted. The errors shown correspond to the deviation of the nonlinear least-squares fit to the data points on the titration curve. N is the stoichiometry of the interaction as determined by the fitting of the ITC binding isotherm (*27*, *28*).

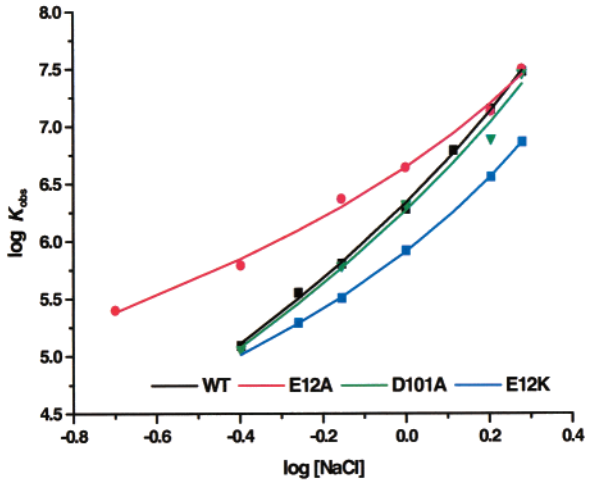

FIGURE 1: Plot of log K_{obs} against log[NaCl] for wild-type (WT) and mutant forms of *Pw*TBP. The data were fit to eq 1 using a nonlinear least squares algorithm where the net change in ions (in this case assumed to be cations), A, the net change in water molecules in complex formation, B, and K_{REF} were fit as independent variables.

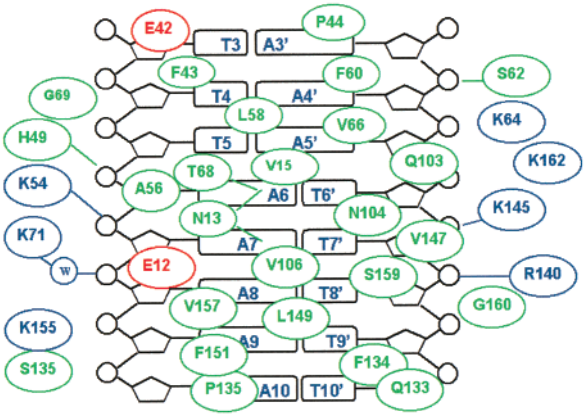

FIGURE 2: Schematic reproduced from ref *16* showing interactions in the *Pw*TBP-boxA/TATA element complex. Residues involved in van der Waals interactions are shown proximal to the region of contact DNA nucleotides. Solid lines indicate hydrogen bonds or salt bridge interactions. Basic residues are shown in blue, acidic residues are shown in red, and all other residues are shown in green. The interface possesses the pseudo 2-fold axis of symmetry.

Identification of Cation Binding Sites by Site-Directed Mutagenesis. Having determined that the interaction of wild-type *Pw*TBP involves the uptake of two ions into the binding site, we attempted to establish whether these could be attributed to specific cation binding sites in the interface. An approach based on site-directed mutagenesis of cation coordination sites was adopted. We assumed that uptake of cations into the complex binding site would involve interaction of the positive charge with both protein and DNA. The highly electronegative phosphate backbone of the oligonucleotide potentially provides multiple sites for cation coordination; however, for mediation of the interaction with the TBP suitable negatively charged sites integral to protein binding had to be identified. Recent crystallographic detail on the complex between *Bam*HI and DNA showed calcium ions interacting with aspartate or glutamate residues and the phosphate backbone of the DNA (*30*, *31*). X-ray crystallographic structural data (*7*, *16*) and comparison of *Pw* with mesophilic TBP primary sequences were used to select acidic residues close to the DNA backbone in different regions of the protein binding site. The E12 and D101 surface acid

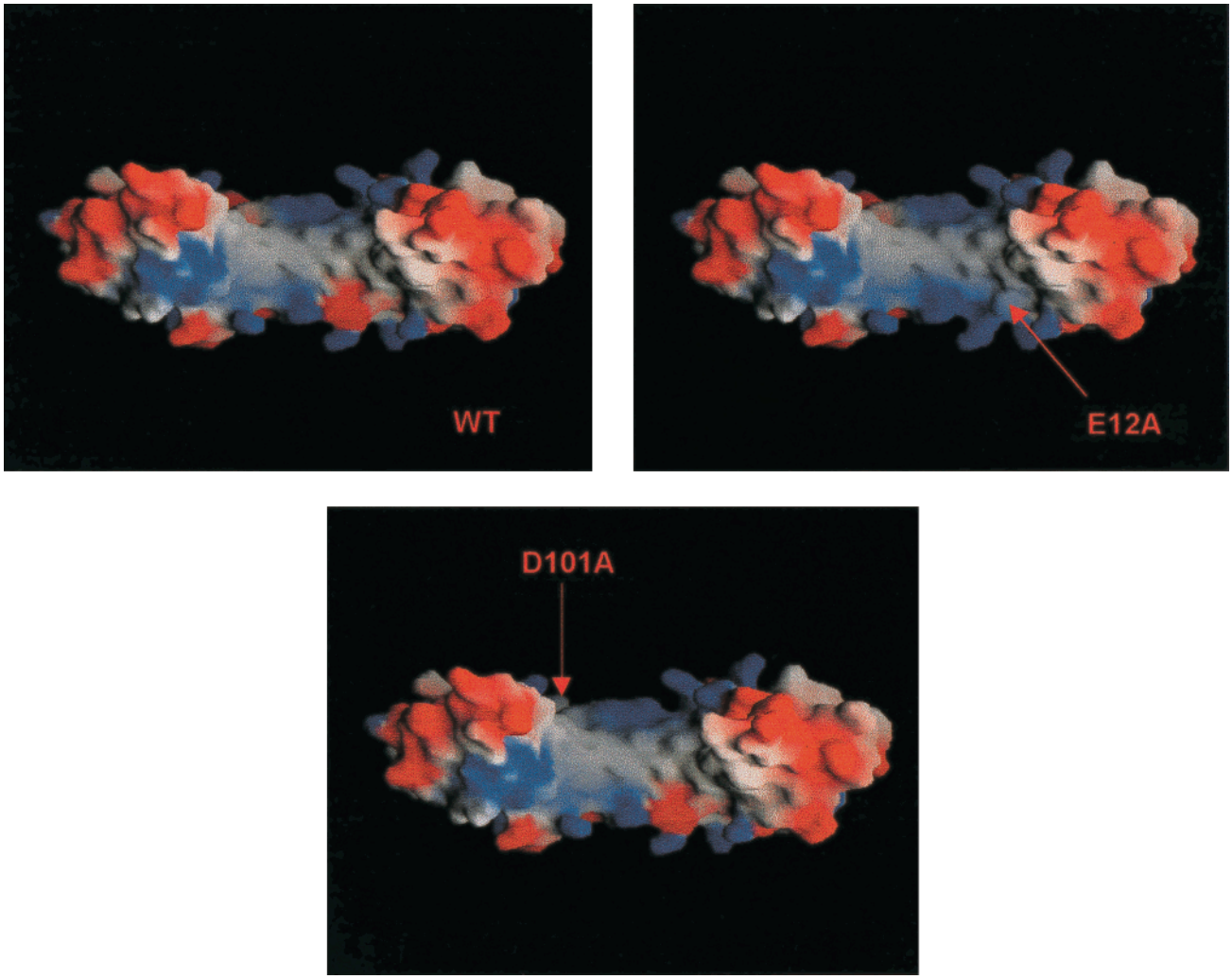

FIGURE 3: Surface potential diagrams to show the effect of point mutations E12A and D101A relative to the WT. Molecular surfaces and electrostatic potentials were rendered with GRASP (*32*) and calculated using 1 M salt and full charges.

residues were substituted for alanine (see Figure 2). The program GRASP (*32*) was used to model the effect of replacing acidic residues on the predicted electrostatic surface potential of the protein (see Figure 3). The change in potential for the E12A mutant TBP is localized to a position directly in the β-sheet "saddle" region (*8*) of the DNA binding site. The D101A mutation, however, is somewhat more distal from the site of DNA interaction (Figure 3). ITC binding experiments on mutant forms of *Pw*TBP and DNA were performed over a range of salt concentrations in an identical manner to those for the wild-type protein.

The K_{obs}−salt concentration dependence data for each mutant are shown in Figure 1 along with a nonlinear fit to eq 1. The data shows that for the E12A mutant there is a net ion uptake [A (eq 1) $= -1.2 \pm 0.3$; Table 2]. The substitution of this negative residue in the mutant proteins results in approximately one less cation being taken up in the complex with DNA with respect to wild-type *Pw*TBP indicating the involvement of E12 in cation coordination. The binding data for the E12A interaction shows a dramatic difference to that of the wild type at low salt concentration (discussed below). Although the dependence of K_{obs} on salt concentration varies from wild type for the E12A mutant protein, for the D101A mutation the binding data are essentially identical with respect to water expulsion and cation uptake (see Table 2).

Table 2: Table Shows the output from Fitting log K_{obs}−[NaCl] Data to Eq 1 Using a Nonlinear Least Squares Method[a]

	ions (A)	water (B)	K_{REF}	$\chi^2 \times 10^{-3}$
WT	-2.1 ± 0.1	39 ± 9	5.7 ± 0.2	1.7
E12A	-1.2 ± 0.3	33 ± 10	6.1 ± 0.2	5.3
D101A	-2.1 ± 1.1	35 ± 31	5.7 ± 0.6	17.1
E12K	-1.2 ± 0.2	42 ± 4	5.2 ± 0.1	0.1
Q103A	-2.3 ± 0.5	24 ± 14	6.5 ± 0.3	3.4

[a] A is the net change in the number of ions associated with the protein and DNA on going from unbound to complex states, B is the number of waters released, and K_{REF} is log K_{obs} at 1 M NaCl. Errors shown are based on the cumulative standard deviation of the fit.

The replacement of this negative charge has no effect on cation binding, and hence clearly this residue is not a coordination site for ions.

A Basic Amino Acid Side Chain in Place of the Cation Reduces Affinity at High Salt Concentration. Removal of a cation coordination site by site-directed mutagenesis of glutamate for alanine potentially leaves an unsatisfied negative charge on the DNA and a cavity, which in the wild-type protein complex was occupied by the carboxyl group of the acid and the cation. To reduce the effect of this, the E12K mutant was made, which substituted a positively charged lysine side chain to replace the cation. The positive

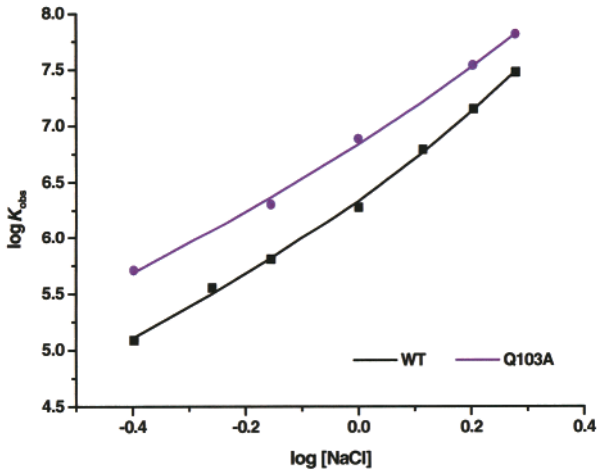

FIGURE 4: Plot of log K_{obs} against log[NaCl] for WT and Q103A mutant form of *Pw*TBP. The data were fit to eq 1 using a nonlinear least squares algorithm where the net change in ions (in this case assumed to be cations), *A*, the net change in water molecules in complex formation, *B*, and K_{REF} were fit as independent variables.

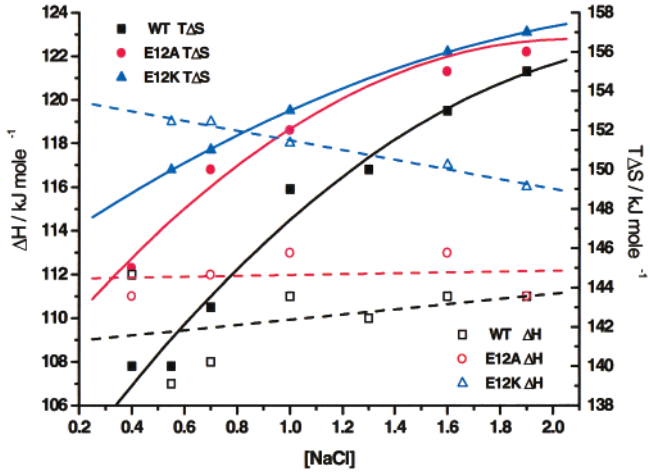

FIGURE 5: Plot to show the variation of thermodynamic parameters with NaCl concentration for wild type (black), E12A (red), and E12K (cyan) forms of *Pw*TBP. Δ*H* is shown by broken lines with open markers and *T*Δ*S* is shown by solid lines with filled markers.

charge of the ϵ-amine was provided to interact directly in place of the incorporated cation. The lysine side chain could extend to interact with the DNA, thus filling the cavity formed in the presence of the E12A mutation. Fitting the binding data from Table 1 to eq 1 demonstrates that the mutant protein incorporates one less cation than wild type ($A = 1.2 \pm 0.2$; Table 2). This is consistent with the data for the E12A mutant and confirms the role of the E12 in cation coordination. At high salt concentration, the K_{obs} for the E12K protein is significantly weaker than that for wild type (Table 1 and Figure 1). Furthermore, although the fitted data of the E12K mutant TBP has a similar slope and curvature to that of the E12A mutant, the binding is consistently weaker over the entire range of salt concentrations studied (discussed below).

Incorporation of Alanine into the Complex Interface Increases the Affinity. The E12A data at all salt concentrations shows a consistently tighter binding than E12K protein despite having similar numbers of cations and similar numbers of water molecules involved in binding. This is reflected in the lower value of K_{REF} (which is a fitted variable representing the binding constant at 1 M salt; Table 2) for E12K as compared to E12A. To isolate this from the effects of cation binding a mutant protein, which produced a similar effect in the absence of the change in cation content of the complex, was required. The binding site of TBP has an axis of 2-fold symmetry (Figure 2) such that the glutamine residue, Q103, is situated in a similar position with respect to the DNA bases as E12. Since Q103 is not negatively charged, and hence not involved in cation coordination, it provides a site for mutagenesis studies to assess the effect E12 mutations in the absence of the effects of cation removal/binding. Binding data at different salt concentrations for the Q103A mutant show similar involvement of water molecules and cations in forming the complex with DNA as the wild-type protein (Table 2). Therefore, the observed consistently higher binding of the Q103A mutant (Figure 4) can be attributed to the replacement of the polar glutamine side chain for the hydrophobic alanine in the absence of solvent effects. This is mirrored in the comparison of the E12A and E12K mutants. Interestingly, therefore, the substitution of an alanine residue for a polar or charged side chain in the 12, or analogous 103, position has a significantly favorable effect on binding. This is likely to be the result of changes in the steric properties of the substituted side chains.

Cation Coordination is Entropically Unfavorable at Low Salt Concentration. In all cases, the thermodynamic parameters for the various TBP interactions (shown in Table 1) are characterized by an unfavorable enthalpy and a favorable entropy term. The binding of E12A is tighter than the wild-type interaction over almost the entire range of salt concentrations studied. However, the difference in K_{obs} gets less as the salt concentration is increased. This effect can be largely attributed to the *T*Δ*S* which is significantly more favorable for the E12A mutant at low salt concentration but equivalent to wild type at high salt concentration (the Δ*H* for the both E12A and wild type are approximately equivalent throughout; Figure 5). This is consistent with the finding that the E12A mutant incorporates less cations into the interface which would have a significant entropic advantage in binding only at low salt concentrations. Comparing the thermodynamic parameters for the interactions of E12K and wild-type TBP shows that the mutant protein has a more favorable *T*Δ*S*, which is less emphatic at high salt (Figure 5). This is consistent with the mutant protein not restricting an additional cation in the complex interface as described for E12A. E12K, however, has a much less favorable Δ*H* as compared to wild type and E12A. This is observed over the entire salt concentration range studied suggesting that it is not dependent on cation effects. This is likely to reflect that the lysine is sterically inept at replacing the glutamate in the binding site, an effect that would be independent of salt concentration.

The Number of Water Molecules Released on Complex Formation Derived from Fitting Are Consistent with Those Determined by Osmotic Stress Experiments. Table 2 shows the ITC binding data fit using eq 1 with the number of cations and water molecules included, or released, as independent variables. The number of water molecules released as derived from these fitted data are similar for the wild-type and mutant forms of *Pw*TBP. The average error on fitted value for the number of water molecules released is approximately 40%, and thus the fits are not highly sensitive to this variable.

Table 3: Summary of Thermodynamic Parameters from ITC Experiments for the WT *Pw*TBP−DNA Interaction at Different Concentrations of Osmolyte at pH 7.0, 35 °C, and 0.7 M NaCl[a]

osmolyte	[osmolyte] M	total osmolality	[protein] (μM)	[DNA] (μM)	N	K ($\times 10^5\,M^{-1}$)	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	$T\Delta S$ (kJ mol^{-1})
		1.20	20	206	1.0	7.24 ± 0.8	108 ± 0.8	−34.6	143
betaine	0.15	1.35	10	100	1.3	11.6 ± 1.4	108 ± 0.8	−35.8	144
betaine	0.32	1.53	10	116	1.1	16.6 ± 2.0	107 ± 2.0	−36.7	144
betaine	0.53	1.77	10	107	1.1	28.5 ± 2.0	107 ± 0.8	−38.1	145
betaine	0.75	2.00	10	100	1.1	50.6 ± 4.0	102 ± 0.8	−39.5	142
betaine	0.88	2.10	10	97.0	0.99	65.7 ± 9.0	95.8 ± 1.2	−40.2	135
betaine	1.00	2.32	10	100	1.1	96.1 ± 5	93.7 ± 0.8	−41.2	135
betaine	1.14	2.55	12	100	1.1	128 ± 17	95.8 ± 0.4	−41.8	140
		1.20	20	200	1.0	7.24 ± 0.8	108 ± 0.8	−34.6	143
sucrose	0.20	1.41	18	180	1.1	8.32 ± 0.6	107 ± 1.2	−34.9	142
sucrose	0.38	1.61	10	100	1.2	11.1 ± 0.7	104 ± 1.2	−35.6	140
sucrose	0.60	1.77	10	100	1.2	14.1 ± 1.4	101 ± 1.2	−36.3	138
sucrose	0.65	1.90	10	100	0.97	18.5 ± 1.2	96.2 ± 1.2	−40.0	133
sucrose	0.80	2.05	10	100	1.1	21.6 ± 1.8	97.9 ± 0.8	−37.4	135
sucrose	0.90	2.19	10	100	0.97	26.3 ± 2.0	99.2 ± 0.8	−37.9	137
sucrose	1.00	2.33	10	100	1.1	36.9 ± 2.6	96.7 ± 0.8	−38.7	136

[a] The errors shown correspond to the deviation of the nonlinear least-squares fit to the data points on the titration curve. N is the stoichiometry of the interaction as determined by the fitting of the ITC binding isotherm (*27*, *28*).

To confirm that the number of water molecules released derived from data fitting to eq 1 was appropriate, it was necessary to independently estimate this number. To do this, the affinity of complex formation was determined under different conditions of osmotic stress. Known quantities of neutral solutes (or osmolytes), which are not directly involved in the binding interaction, were added to increase the chemical potential of water in the solution, thus favoring the release of associated water molecules (*26*, *33*, *34*). The number of water molecules can be determined from the relationship between the K_{obs} and the concentration of osmolyte (*29*, *33*) (see eq 2). where B is the change in

$$\frac{\mathrm{d}\log(K_{obs})}{\mathrm{d}[\mathrm{solute}]_{osmolal}} = -\frac{2.303B}{55.5} \qquad (2)$$

number of water molecules on going from the unbound to the bound state (i.e., number of water molecules released; a positive number). K_{obs} values were determined using ITC experiments performed at a fixed concentration of NaCl (0.7 M), with increasing amounts of osmolyte (either sucrose or betaine) as shown in Table 3. A plot of log K_{obs} against concentration of osmolyte (osmolal) is shown in Figure 6. From these data, we estimate the net number of water molecules released upon complex formation in sucrose and betaine is 16 ± 1 and 24 ± 2, respectively. Experiments performed using both glycerol and triethylene glycol as osmolytes did not produce significant changes in K_{obs}. In previously studies involving a wide range of osmolytes, varying numbers of released waters from interactions were reported depending on the osmolyte used (*29*, *35*, *36*). This is likely to result from none of the osmolytes used being completely excluded from the interacting molecular surfaces. A recent investigation of the hydration of small proteins showed that, of the small sample of different osmolytes tested, betaine was the most and glycerol was the least excluded (*37*). Our experiments are in agreement with this order of osmolytes in their ability to determine the number of water molecules released from the protein−DNA surface. Furthermore, it was found that even betaine was not completely excluded from the protein surface, resulting in an underestimate of the number of hydrating water molecules. This is also consistent with the observation in this work that the osmotic stress experiments give a slightly lower number of released water molecules on complex formation than determined from the fitting of our binding data to eq 1.

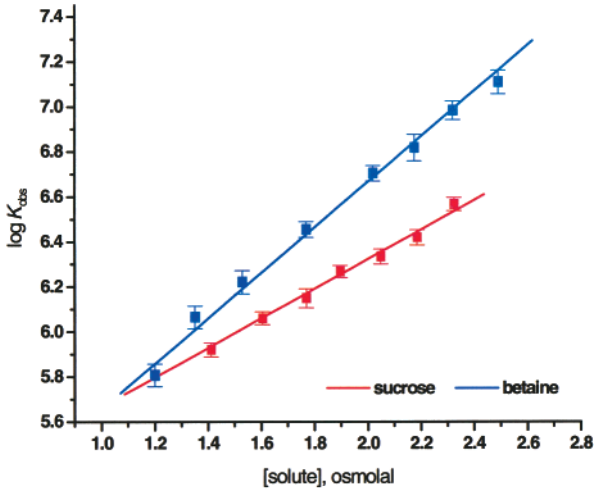

FIGURE 6: Plot of log K_{obs} against $[\mathrm{solute}]_{osmolal}$ for wild-type *Pw*TBP. The betaine data (shown in blue) corresponds to a net release of 24 waters upon complex formation. The data for sucrose (shown in red) corresponds to a net release of 16 waters.

CONCLUSION

Given the drastically different effects of variation in salt concentration on formation of the *Pw*TBP−DNA complex as compared to mesophilic counterparts, our aim has been to provide thermodynamic data to test the hypothesis that mediation of this interaction is, at least in part, through cation bridges. To this effect, we used site-directed mutagenesis to change the salt dependence of the interaction by selectively removing cation binding sites. In so doing, we demonstrated that some of these mutants are able convert the behavior of *Pw*TBP toward that of their mesophilic counterparts. Replacement of glutamate residue at position 12 in the protein shows changes in the slope of the dependence of K_{obs} on salt concentration, consistent with the reduction in the net

uptake of cations as compared to wild type. Moreover, mutations at the E12 position result in tighter binding at low salt concentrations, making this protein more mesophile-like than wild-type *Pw*TBP. The importance of the E12 mutation can be shown in primary sequence alignment of halophilic and thermophilic species. These species all have a highly conserved glutamate residue in the 12 position; however, this is not found in mesophilic protein where it is generally replaced by glutamine. Thus, although there is continued debate on this issue (*38*), assuming that both archaeal and mesophilic species evolved from an extremophilic last common ancestor, a major step in the adaptation of a TBP protein to ambient conditions would require the substitution of acid groups such as E12. Mutations that change the cation binding behavior of the complex are insufficient to convert the *Pw*TBP to a completely mesophilic nature. This is because mesophilic behavior requires the expulsion of ions from the interface to give it the negative dependence of K_{obs} on salt concentration. This would involve a completely different mutation strategy.

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