Thermodynamic and Kinetic Characterization of the Binding of the TATA Binding Protein to the Adenovirus E4 Promoter[†,‡]

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ABSTRACT: A thermodynamic analysis of the binding of the TATA binding protein (TBP) from *Saccharomyces cerevisiae* to the adenovirus E4 promoter was conducted using quantitative DNase I "footprint" titration techniques. These studies were conducted to provide a foundation for studies of TBP structure−function relations and its assembly into transcription preinitiation complexes. The binding of TBP to the E4 promoter is well described by the Langmuir binding polynomial, suggesting that no linked equilibria contribute to the binding reaction under the conditions examined. Van't Hoff analysis yielded a nonlinear dependence on temperature with the TBP−E4 promoter interaction displaying maximal affinity at 30 °C. An unusually negative value of the apparent standard heat capacity change, $\Delta C_p^\circ = -3.5 \pm 0.5$ kcal/mol·K, was determined from these data. The dependence of the TBP−E4 promoter interaction on [KCl] indicates that 3.6 ± 0.3 K^+ ions are displaced upon complex formation. Within experimental error, no linkage of proton binding with the TBP−E4 promoter interaction is detectable between pH 5.9 and 8.7. Rates of association of TBP for the E4 promoter were obtained using a novel implementation of a quench−flow device and DNase I "footprinting" techniques. The value determined for the second-order rate constant at pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 30 °C ($k_a = (5.2 \pm 0.5) \times 10^5$ M^{-1} s^{-1}) confirms the results obtained by Hawley and co-workers [Hoopes, B. C., LeBlanc, J. F., & Hawley, D. K. (1992) *J. Biol. Chem. 267,* 11539−11547] and extends them through TBP concentrations of 636 nM. The Arrhenius plot of the rate of TBP association is also nonlinear, yielding $\Delta C_p^\circ = -3.2 \pm 0.3$ kcal/mol·K. Thus, the ΔC_p° associated with the TBP−E4 promoter interaction appears associated principally, if not exclusively, with the rate-limiting step of binding. The consequences of these results for the interpretation of structural and biochemical studies of TBP−promoter complex formation are discussed.

Crucial to the regulation of gene expression are the events governing the initiation of transcription. Eukaryotic transcription is performed by three polymerases, each of which transcribes a particular set of genes and whose promoters, in general, have distinct structural features. Polymerase-specific initiation requires polymerase recruitment to the promoter, which is achieved through the interaction of the appropriate polymerase with a nucleoprotein preinitiation complex, the proteins of which are called general transcription factors. The TATA-binding protein (TBP), which in higher eukaryotes is associated with "TBP-associated factors" (TAFs), is required in all three polymerase systems [reviewed by Sharp (1992), White & Jackson (1992), Hernandez (1993), and Willis (1993)]. For genes transcribed by RNA polymerase II, the majority of which contain a "TATA box" element, the assembly of the preinitiation complex commences with the binding of the TBP−TAF complex (TFIID) to the TATA box (Sawadogo & Roeder, 1985; Van Dyke et al., 1989).

Atomic resolution crystal diffraction structures of TBP isoform 2 (TBP2) from *Arabidopsis thaliana* and the C-terminal core of TBP from *Saccharomyces cerevisiae,* and of their complexes with TATA-containing DNA, have been solved (Nikolov et al., 1992; Chasman et al., 1993; Kim et al., 1993a,b; Kim & Burley, 1994; Nikolov & Burley, 1994). These structural studies confirmed earlier biochemical studies demonstrating that TBP binds in the minor groove of the TATA-containing DNA (Starr & Hawley, 1991; Lee et al., 1991) and bends the DNA (Horikoshi et al., 1992) but also revealed unprecedented structural changes within the protein-DNA complexes. In particular, sequence-specific DNA binding by TBP results in a small reorientation of the two domains of the bound protein, the introduction of "kinks" in the DNA within the TATA element, and unwinding of the DNA helix. The DNA unwinding results in a relatively shallow and wide minor groove which provides a complementary surface to the concave underside of TBP. The role of these conformational changes in the sequence specificity of binding and transcription initiation is as yet unknown.

The studies of Hawley and co-workers (Hoopes et al., 1992) established that the slower than diffusion-limited rate of association of TBP to a promoter proceeds by at least a two-step mechanism; an initial rapid equilibrium is followed by a slow rate-limiting step. The dissociation of TBP is likewise slow, with a half-life of ~1 h under conditions comparable to physiological (Schmidt et al., 1989; Hoopes et al., 1992). Important questions to be answered concerning

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the DNA-binding characteristics of TBP include understanding what aspects of the unusual structure determine these functional properties and whether they contribute to the protein−protein interactions essential to the biological function of TBP.

Despite the important role played by TBP in the initiation of gene transcription in eukaryotes, and the myriad studies of its DNA binding, there exists, to our knowledge, no comprehensive characterization of the thermodynamics of its association with promoter DNA. Achieving an understanding of the interactions of TBP with the other transcription factors that are critical to its biological function requires a firm foundation built upon the characteristics of TBP−promoter interactions. Thus, a thermodynamic and kinetic analysis of the binding of TBP from *S. cerevisiae* has been conducted that provides a foundation for studies of TBP structure−function and its assembly into transcription preinitiation complexes.

MATERIALS AND METHODS

TBP Expression and Purification. Plasmid pKA9 carrying the *S. cerevisiae* SPT 15 gene in a pET expression vector was a gift of K. Arndt and F. Winston (Arndt et al., 1992). A modification of their purification scheme was developed and is presented below. BL21 (DE3) cells containing the plasmids pLysS and pKA9 are grown at 37 °C in LB broth containing 25 μg/mL ampicillin and 30 μg/mL chloramphenicol. At a density of $A_{600} = 0.4$, the broth is transferred to 30 °C, TBP production is initiated by the addition of IPTG to 1 mM concentration and growth is continued at 30 °C for 2 h. The cells are harvested and the cell pellets are stored at −70 °C.

All subsequent steps in the protocol are conducted at 4 °C. Aliquots of the frozen cells are thawed on ice and resuspended (3 mL/g of cells) in 25 mM Hepes-KOH, 20% glycerol, 1 mM EDTA, 1 mM DTT, pH 7.9 (buffer A) with 100 mM KCl containing aprotinin (1 μg/mL), chloromethyl ketone (20 μg/mL), leupeptin (1 μg/mL), trypsin inhibitor (100 μg/mL), and 100 mM PMSF (1 mM) protease inhibitors that are added to the buffer just prior to resuspension of the cells. The cells are lysed in a Biospec Bead-Beater using acid-washed dry glass beads (<106 μm, Sigma) with an outer jacket filled with salt-saturated ice−water with four 30 s bursts, 3 min apart. The suspension is centrifuged in a Sorvall SS-34 rotor at 18 000 rpm for 20 min. Chromosomal DNA is precipitated from the supernatant by slowly stirring in a protamine sulfate solution (6 mg/mL) to a final concentration of 0.3 mg/mL. The precipitate is removed by centrifugation as described above.

A 2.2 × 25 cm column packed with 50 mL of Pharmacia S-Sepharose Fast Flow resin is equilibrated in buffer A with 100 mM KCl. Following sample loading, the column is washed with the equilibration buffer at 4 mL/min until A_{280} returns to baseline. The TBP is eluted with an 80 mL gradient of buffer A from 100 to 235 mM KCl followed by a 240 mL gradient of 235−370 mM KCl; TBP elutes between 235 and 300 mM KCl. Column fractions are analyzed by SDS−PAGE; the TBP-containing fractions are pooled, concentrated to ~1.5 mL using an Amicon Centricon 3 concentrator, and dialyzed overnight against buffer A with 100 mM KCl.

A Pharmacia Mono Q column is equilibrated with buffer A with 100 mM KCl at 0.5 mL/min. The sample is loaded with a Superloop and the flowthrough collected; the TBP elutes entirely in the flowthrough. The column is regenerated using buffer A with 1 M KCl. The TBP-containing solution is dialyzed against buffer A with 300 mM KCl, concentrated if necessary in an Amicon Centricon 3 concentrator, divided into aliquots, and stored at −70° C. TBP concentrations were determined from the absorbance at 280 nm using an extinction coefficient of 12 700 M^{-1} cm^{-1} calculated from the extinction coefficients of the tryptophans (5500 M^{-1} cm^{-1}) and tyrosines (1200 M^{-1} cm^{-1}) present in the protein (Wetlaufer, 1962).

DNA Preparation and Purification. The plasmid pGo-E4T (a gift of Dr. Steve Triezenberg) contains the adenovirus E4-TATA region from −38 to +250 cloned into the *Ava*I and *Bam*HI restriction sites in the polylinker of pGEM-3 (Promega). The sequence of the DNA surrounding the TATA box is with the TATA box in boldface type. The

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   -38        12345678
5'-TTTTTAGTCCTATATATACTCGCTCTGCA-3'
3'-AAAAATCAGGATATATATGAGCGAGACGT-5'
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TATA box is one of four sequences identified as high-affinity TBP-binding sites by an *in vitro* selection assay (Wong & Bateman, 1994).

A DNA restriction fragment of 334 bp labeled at one end by ^{32}P (bottom strand) is obtained by digestion of pGo-E4T with *Hin*dIII, incorporation of ^{32}P nucleotides with the large fragment of DNA polymerase (Klenow), followed by *Eco*RI digestion and purification using published protocols (Brenowitz et al., 1993). The TATA box is located 40 bp from the labeled 3′ end of the restriction fragment. The concentration of ^{32}P-labeled DNA used (<10 pM) is significantly lower than the equilibrium dissociation constants of the DNA-binding reactions in all experiments.

Quantitative DNase I Footprint Titration Method. Equilibrium binding experiments are conducted using the quantitative DNase I footprint titration method (Brenowitz et al., 1986a,b, 1993; Brenowitz & Senear, 1989). All experiments are conducted in an assay buffer containing 25 mM Bis-tris, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM DTT, 50 μg/mL bovine serum albumin (BSA), and 1 μg/mL poly(dG-dC) at the indicated concentration of KCl, pH, and temperature. The temperature-dependence studies are conducted at 100 mM KCl and pH 7.4. The monovalent ion-dependence studies are conducted at pH 7.4 and 30 °C. Studies of the pH dependence of TBP-promoter binding are conducted at 100 mM KCl and 30 °C except that Tris-HCl is used at pH 7.7 and above. BSA is present in the binding buffer in order to minimize absorption of the TBP to the surfaces of the pipet tips, microfuge tubes, etc. (Brenowitz et al., 1986a). dG−dC is included as carrier DNA and has no effect on the binding of TBP to the promoter containing DNA at the concentrations employed (unpublished data).

TBP titrations of the DNA containing the E4 promoter are conducted in 100 μL volumes of assay buffer incubated at the indicated temperature in a regulated water bath for 45−60 min. The TBP−DNA samples are exposed to DNase I for 2 min with the reaction quenched by the addition of 20 μL of 50 mM EDTA. The products of the DNase I reaction are separated by denaturing electrophoresis on a 10% acrylamide gel. Electrophoretograms are visualized using phosphor storage plates and a PhosphorImager (Molecular Dynamics). Densitometric analysis of the digital images

according to the protocols developed by Ackers and coworkers (Brenowitz et al., 1986a) is implemented using the ImageQuant software (Brenowitz et al., 1993). The binding isotherms obtained from these images are analyzed using techniques of nonlinear least-squares analysis as described below.

In order to ensure that the errors in the binding measurements are propagated from the experimental data, the binding data are simultaneously ("globally") analyzed for the desired parameters [*cf.* Koblan et al. (1992), Senear and Bolen (1992), and Beechem (1992)]. For example, the value of $\Delta C_p°$ is determined by simultaneously analyzing the seven binding curves represented in Figure 3 using the coupled equations

$$K_i = \exp\left(\frac{\Delta C_p°}{R}\right)\left[\left(\frac{T_H}{T_i}\right) - \ln\left(\frac{T_s}{T_i}\right) - 1\right] \quad (1)$$

$$\bar{Y}_i = \frac{K_i^n[X]^n}{1 + K_i^n[X]^n} \quad (2)$$

$$p_i = p_{i,\text{lower}} + (p_{i,\text{upper}} - p_{i,\text{lower}})\bar{Y}_i \quad (3)$$

where $\Delta C_p°$ is the standard heat capacity change and the characteristic temperatures, T_H and T_S, occur where $\Delta H°$ and $\Delta S°$ equal zero, respectively (Ha et al., 1989), $\bar{Y}_i$ is the fractional saturation of the binding site on the DNA, K_i is the equilibrium binding constant, [*X*] is the ligand (protein) concentration, *n* is the Hill coefficient (which is equal to 1 for the case of the Langmuir binding polynomial), p_i is the "apparent saturation" calculated from the integrated optical density of the binding site and $p_{i,\text{lower}}$ and $p_{i,\text{upper}}$ are the upper and lower limits of the transition curve (Brenowitz et al., 1993). Each data set is weighted by the inverse of the square root of the variance of its individual fit. Each value of *i* corresponds to a separate experiment. Values of K_i are related to the Gibbs free energy by $\Delta G_i = -RT \ln K_i$.

Time-Resolved Rapid-Reaction Footprinting. A detailed description of the rapid-mixing techniques used to determine the progress curves for TBP-promoter binding will be presented elsewhere (Hsieh and Brenowitz, in preparation). In brief, a quench−flow apparatus (Kin-Tek) is used to mix TBP and [^{32}P]DNA. The samples are allowed to react for a defined time period, exposed to a brief pulse of DNase I, followed by quenching of the DNase I reaction with EDTA. The [^{32}P]DNA is then prepared for and analyzed by electrophoresis in exactly the same manner as the equilibrium experiments described above. The association reactions are conducted with TBP concentrations in excess of the [^{32}P]-DNA, allowing the pseudo-first-order approximation to be applied to the data analysis.

All solutions are prepared in the appropriate pH 7.4 buffer for each temperature (see above). The TBP stock solution and ^{32}P-labeled E4 DNA (see above) are diluted such that TBP concentration is twice the final concentration desired. [^{32}P]DNA (20 000−30 000 dpm) is diluted so that <30 pM is present in each reaction. All solutions are equilibrated in the reserve tank of the recirculating water bath connected to the quench−flow apparatus prior to the experiments. The apparatus itself is equilibrated for >30 min at the desired temperature.

Densitometric analysis of the digital images of the electrophoretograms yields p_i, the apparent saturation, as described above for the equilibrium titrations. $\bar{Y}_i$, the fractional saturation of the TATA box, is obtained by fitting the data to the coupled equations.

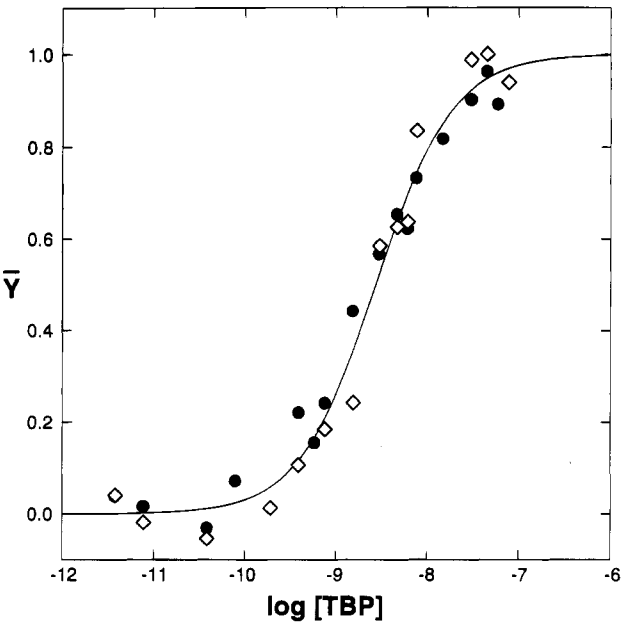

FIGURE 1: Equilibrium titration curves obtained for TBP binding to the adenovirus E4 promoter in assay buffer at pH 7.4 (●) and pH 6.9 (◇), 100 mM KCl, and 30 °C. The solid line indicates the best fit of eqs 2 and 3 to the two sets of titration data where $\Delta G = -11.8 \pm 0.1$ kcal/mol and $n = 1.0 \pm 0.1$.

$$\bar{Y}_i = e^{-k't} \quad (4)$$

$$p_i = p_{i,\text{lower}} + (p_{i,\text{upper}} - p_{i,\text{lower}})\bar{Y}_i \quad (5)$$

where k' is the pseudo-first order association constant, and p_i, $p_{i,\text{lower}}$ and $p_{i,\text{upper}}$ are as defined above. The pseudo-first-order association constant, k', is equal to k_a[TBP], where k_a is the second-order association constant. In all cases, the progress curves are adequately described by a single exponential (see Results). Multiple data sets are analyzed simultaneously as described above.

Numerical Analysis. All parameters were estimated using methods of nonlinear least-squares analysis. The best-fit parameter values corresponding to a minimum in the variance are determined using a variation of the Gauss−Newton procedure that searches the parameter space along a vector dependent on the parameter guesses (Johnson & Faunt, 1992). When convergence is reached, the parameter space is searched for the variance ratio predicted by an *F* statistic to determine the worst-case joint confidence intervals for the fitted parameters. The 65% confidence limits estimated in this manner correspond to approximately one standard deviation.

RESULTS

An equilibrium binding curve for the TBP interaction with the E4 promoter is shown in Figure 1. The binding curves are best described with Hill coefficients of unity (solid line) within experimental error. Thus, the Langmuir binding polynomial (eq 2, $n = 1$) is sufficient to describe the TBP−E4 promoter interaction. The binding of TBP to the E4 promoter shows no dependence upon the solution pH, within experimental error, under these experimental conditions (Figure 2).

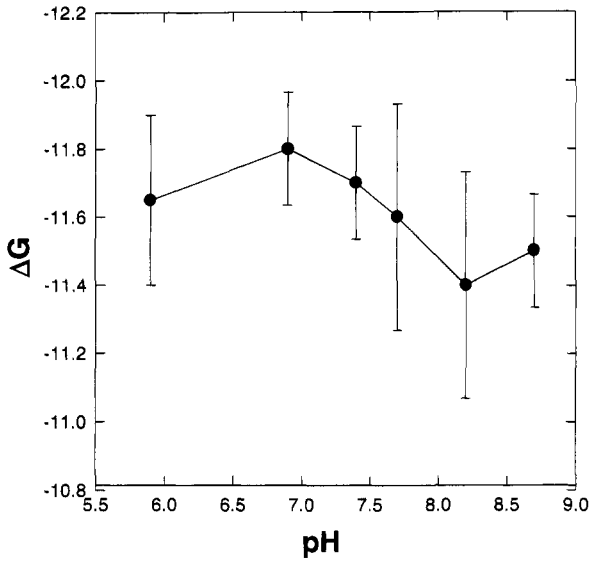

FIGURE 2: pH dependence of the TBP−E4 promoter interaction. All experiments were conducted in assay buffer plus 100 mM KCl at 30 °C as described in Materials and Methods.

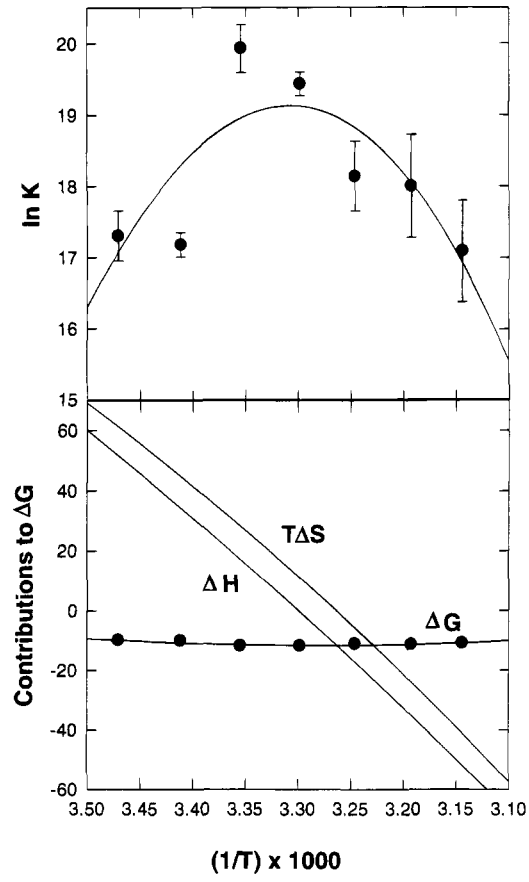

FIGURE 3: (A, top) van't Hoff analysis of the binding of TBP to the E4 promoter. Values of $\Delta C_p^\circ = -3.5 \pm 0.5$ kcal/mol·K, $T_H = 30.0 \pm 1.8$, and $T_S = 33.3 \pm 1.9$ are calculated from the simultaneous analysis of these data to eqs 1−3 as described in Materials and Methods. (B, bottom) Experimentally determined values of ΔG and values of ΔH and $T\Delta S$ calculated as described by Ha et al. (1989).

The temperature dependence of the TBP−E4 promoter interaction is distinctly nonlinear (Figure 3A) and reaches a maximum at the optimal temperature for growth of *S. cerevisiae* of 30 °C. Analysis of this temperature dependence as a change in the apparent standard heat capacity change, ΔC_p° [Ha et al. (1989) and references therein], yields an

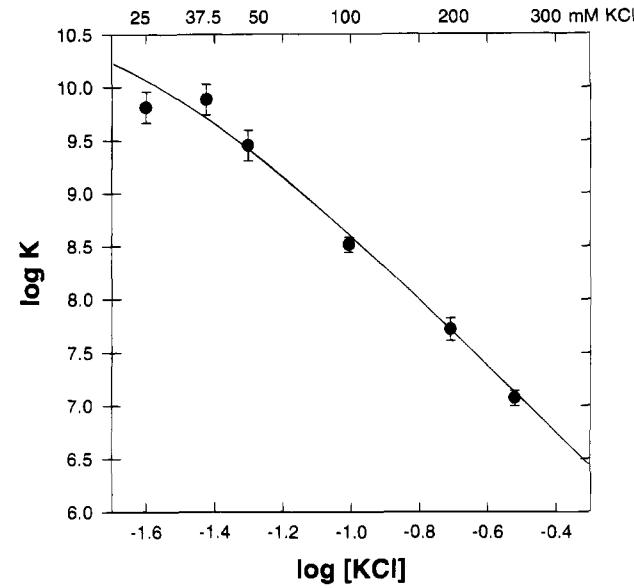

FIGURE 4: Dependence of TBP−promoter binding on KCl concentration. The solid line is best simultaneous fit of two data sets (see Materials and Methods) to the equations presented in Record et al. (1977) that explicitly account for the presence of divalent cations in the assay buffer. The data sets are not sufficient to yield unique values of m, K_o and K_o^{2+} when all three parameters are floated. Therefore m and K_o were floated with a range of fixed values of K_o^{2+}. The values of $m = 3.6 \pm 0.3$ and $K_o = (3.1 \pm 1.5) \times 10^5$ were determined at a value of log $K_o^{2+} = -0.88$, the limiting value at which no further improvement of the fit to the data was obtained. A value of $m = 3.4 \pm 0.3$ is calculated (neglecting the divalent cations) from the data above 50 mM KCl.

unusually negative value for a protein−DNA interaction of -3.5 ± 0.5 kcal/mol·K; the simultaneous analysis of all the binding curves with eqs 1−3 propagates the errors in the titration curves into the value of ΔC_p° (Materials and Methods). As shown in Figure 3B, ΔH and the ΔS vary linearly with temperature and change sign at ~30 °C. Over the range of the data, 15−45 °C, ΔH and $T\Delta S$ decrease from +52 to −53 kcal/mol and from +62 to −42 kcal/mol, respectively; the TBP−E4 promoter interaction is entropy driven at low temperatures and enthalpy driven at high temperatures.

The dependence of the binding of TBP to the E4 promoter on [KCl] is shown in Figure 4. The number of monovalent ions displaced from the DNA upon TBP binding, $m = 3.6 \pm 0.3$, was calculated as described by Record et al. (1977). The expected nonlinearity in d ln K/d ln [KCl] evident at low [KCl] is due to the presence of Mg^{2+} and Ca^{2+} in the binding buffer. [As with the ΔC_p° determination described above, the direct analysis of the binding curves propagates the errors in the titration data into the value of m (Materials and Methods).] This value is approximated within experimental error by the limiting slope at high [KCl]. The approximation is made in this analysis that the contribution of the two divalent ions is described by a single concentration-averaged binding constant (Senear & Batey, 1991).

A series of kinetic progress curves obtained at 30 °C using the quench−flow DNase I footprinting technique are shown in Figure 5 at 38−636 nM TBP concentrations. The data are adequately described by a single exponential; no evidence for additional phases in the progress curves was obtained at any TBP concentration analyzed. The second-order rate constants, k_a, determined for each of the curves are given in the legend of Figure 5. The values of k_a are identical, within experimental error, from 38 to 153 nM TBP. Small increases

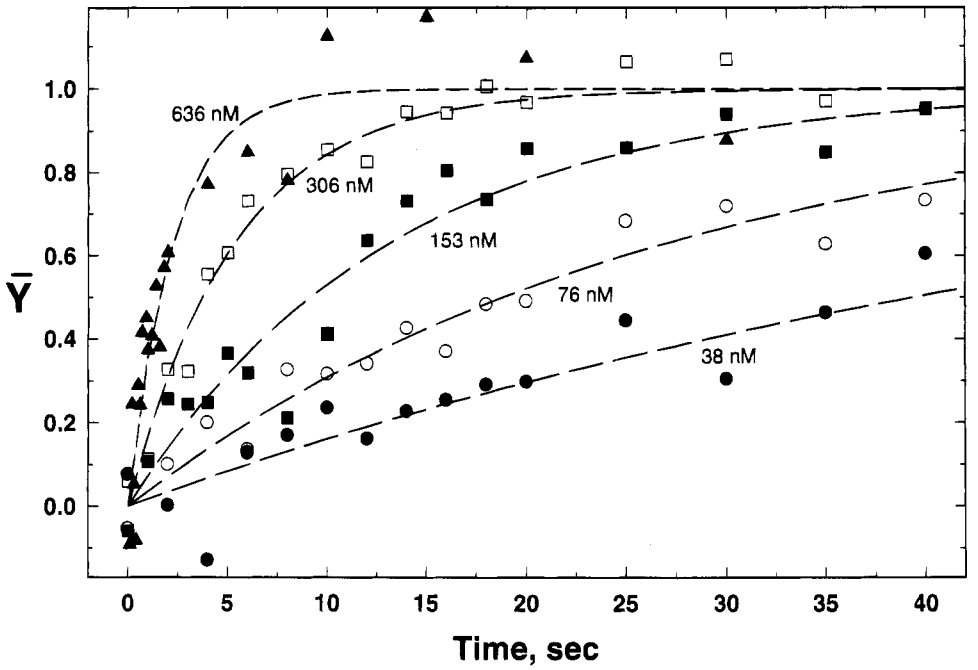

FIGURE 5: A family of progress curves of the TBP−E4 promoter interaction obtained using varying [TBP]. The solid lines are the best fits to eqs 4 and 5 as described in Materials and Methods: 636 nM, $k_a = (7.0 \pm 1.7) \times 10^5$ s^{-1} M^{-1}; 306 nM, $k_a = (6.1 \pm 0.6) \times 10^5$ s^{-1} M^{-1}; 153 nM, $k_a = (5.0 \pm 0.7) \times 10^5$ s^{-1} M^{-1}; 76 nM, $k_a = (4.8 \pm 0.5) \times 10^5$ s^{-1} M^{-1}; and 38 nM, $k_a = (4.6 \pm 0.8) \times 10^5$ s^{-1} M^{-1}.

in k_a are observed at the two highest TBP concentrations studied (see Discussion). Simultaneous analysis of the progress curves yields a value for k_a of $(5.2 \pm 0.5) \times 10^5$ M^{-1} s^{-1}. This value of k_a is in good agreement with that determined by Hoopes et al. (1991) based on data obtained with TBP concentrations from ~4 to 50 nM TBP to the major late promoter under similar, although not identical, experimental conditions. The values of k_a are also in good agreement with those obtained in a recent fluorescence study of yeast TBP binding the major late promoter (Perez-Howard et al., 1995). The correspondence of these data suggests that the E4 and major late promoters, which differ by T → A substitutions at positions 5 and 7 and a A → G substitution at position 8, have comparable thermodynamic properties. This result is not surprising since both sequences were selected as high-affinity TBP-binding sites by an *in vitro* selection assay (Wong & Bateman, 1994).

The binding of TBP to a promoter has been modeled by Hawley and co-workers (Hoopes et al., 1992) as a two-step process

$$\text{TBP} + \text{DNA} \overset{K_1}{\leftrightarrow} \text{TBP–DNA}^* \overset{k_2}{\rightarrow} \text{TBP–DNA}$$

where the reaction K_1 is in rapid equilibrium, the reaction represented by k_2 is irreversible and $k_a = K_1 k_2$ (McClure, 1980). Double-reciprocal (τ) analysis of k_{obs} vs [TBP] is linear (Figure 6), yielding a negative intercept of -1.2 ± 0.6. The Y intercept of the τ analysis is equal to $1/k_2$ (McClure, 1980). Since a negative rate constant is meaningless, a lower limit of 0.4 s^{-1} for k_2 at 30 °C can be estimated from the k_{obs} obtained at the highest TBP concentration studied (636 nM). This rate is ~10-fold faster than the estimate of k_2 obtained by Hoopes et al. (1992) due, in part, to the higher concentrations of TBP used in these studies. An upper limit of 1.1×10^6 M^{-1} is calculated for K_1 compared with 3.3×10^8 M^{-1} ($K_d = 3.1$ nM) determined for the overall equilibrium binding constant at this temperature. The interpretation of these results will be considered more fully in the Discussion.

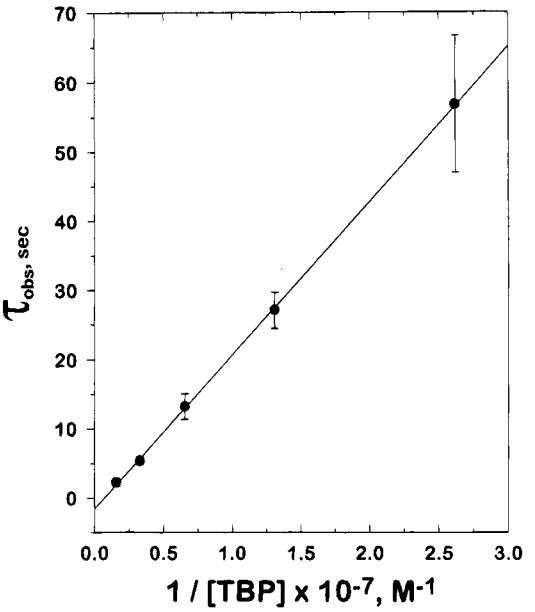

FIGURE 6: Double-reciprocal plot of the observed rate constants for the TBP−E4 promoter association reaction. τ_{obs} is $1/k_{obs}$. A value of $k_a = (4.7 \pm 0.5) \times 10^5$ M^{-1} s^{-1} is determined from the reciprocal of the slope of the line *compared with the value of* $(5.2 \pm 0.5) \times 10^5$ M^{-1} s^{-1} *determined from the direct analysis of the data (see text).* A value of $k_2 = -0.8 \pm 0.5$ was measured from the reciprocal of the Y-axis intercept of the line (see Discussion). The errors in the progress curves were propagated into these values as described in Materials and Methods.

The Arrhenius plot of the temperature dependence of the TBP−E4 promoter interaction is shown in Figure 7. The temperature dependence of TBP binding is clearly nonlinear, decreasing below and above ~30 °C. These data are described by $\Delta C_p^\circ = -3.2 \pm 0.3$ kcal/mol·K, identical within experimental error with the value determined from the van't Hoff analysis. Thus, the ΔC_p° of the TBP−E4 promoter interaction can be completely accounted for in the forward rate of reaction. The activation enthalpy of the binding reaction reaches maxima of −57 and 41 kcal /mol at high

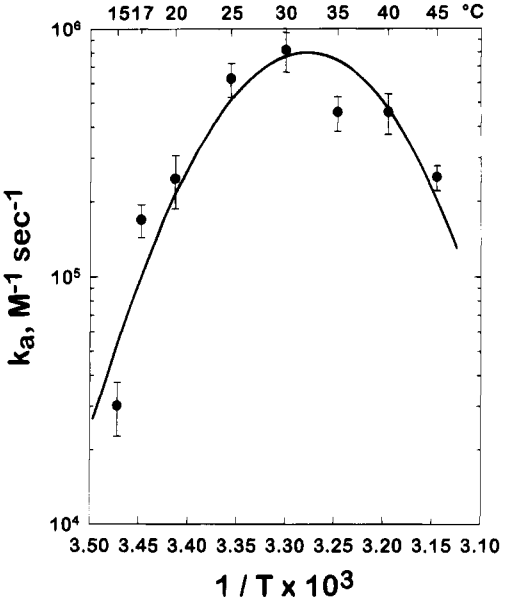

FIGURE 7: Arrhenius plot of the the temperature dependence of the TBP–E4 promoter interaction. Values of $\Delta C_p^\circ = -3.2 \pm 0.4$ kcal/mol·K, $T_H = 31.9 \pm 0.7$ °C, and $T_S = 34.5 \pm 1.0$ °C are calculated from the simultaneous analysis of the progress curves as described in Materials and Methods for the equilibrium titrations.

(45 °C) and low (15 °C) temperatures, respectively, and passes through zero at 30.5 °C.

DISCUSSION

Achieving an understanding of the role played by TBP in the assembly of transcription preinitiation complexes requires that its interaction with DNA, and with other transcription factors, be quantitatively characterized. Specific issues to be addressed include the determination of the role of the DNA conformational change induced by the TBP-promoter interaction in transcription initiation and the determination of the linked protein–protein and protein-DNA interactions that result in the recruitment of specific RNA polymerases. In addition, the characteristics of the TBP-promoter complex provide an excellent system with which to investigate several important questions regarding the nature of sequence-specific binding of proteins to DNA. The thermodynamic and kinetic studies presented in this paper provide a foundation for investigations into these questions.

The observation that the binding of TBP to the E4 promoter is well described by the Langmuir (single-site) binding polynomial is evidence that linked equilibria are not involved in the reaction. TBP self-association does not appear to play a role in promoter binding under the experimental conditions investigated. This conclusion is supported by the precision of the titration data obtained using the footprint titration methods employed (Figure 1).

However, oligomerization of TBP in solution has been reported from anisotropy measurements of the fluorescence of the single tryptophan present in yeast TBP although the TBP-promoter complex has a 1:1 stoichiometry (Perez-Howard et al., 1995). [Comparable results have also been reported for *E. coli* expressed mouse TBP (Kato et al., 1994).] Perez-Howard et al. (1995) also noted that the TBP multimers dissociate at temperatures greater than 29 °C, suggesting a temperature dependence of the oligomerization reaction. Since the fluorescence experiments were conducted at TBP concentrations that exceed the highest concentrations used in the footprint titration studies, it is probable that these higher-order assembly reactions do not contribute to the TBP-promoter interaction over the concentration range studied, although the contribution of higher-order assembly reactions to the TBP-promoter interaction cannot be discounted (Senear et al., 1993; Wong & Lohman, 1995). One place where TBP self-association may have a significant effect is on the extrapolation to infinite protein concentration in the τ (double-reciprocal) analysis. Nonlinearity at high TBP concentrations could result from the self-association reaction that has been described that would account for the negative intercept of the τ plot (Figure 6).

The absence of linkage of proton uptake or release with binding shows that the pK_a values of the groups involved in the TBP–E4 promoter interaction are not within the experimental range investigated (Figure 2). This result can be rationalized by examination of the co-crystal structures of TBP (Kim et al., 1993a,b; Kim & Burley, 1994). Within the TBP–DNA interface, the exposed backbone carbonyl and amide groups do not present polar atoms for interaction with the nucleic acid; most of the amino acid side chains projecting into the TBP–DNA interface are nonpolar. While such an interpretation does not consider potential global effects of protonation reactions, the absence of a pH dependence of the TBP-promoter interaction simplifies the interpretation of structure–function studies utilizing the co-crystal structures obtained at pH 6.2 and 7.5, respectively (Kim et al., 1993a,b; Kim & Burley, 1994).

The dependence of TBP-promoter interaction on monovalent cations is consistent with a dominant role played by the bulk thermodynamic properties of the solution (Figure 4); site-specific binding of K^+ need not be invoked. Whether divalent cations, in particular, Mg^{2+}, play a role in the stabilization of the bent DNA conformation within the TBP-promoter complex will be the subject of future investigations. The moderate dependence of TBP-binding affinity on monovalent ions is consistent with ethylation interference studies (Lee et al., 1991) and the co-crystal structures (Kim et al., 1993a,b; Kim & Burley, 1994) indicating the interaction of about six phosphates with the protein.

The temperature dependence of TBP binding to the E4 promoter is characterized by an observed negative heat capacity change, ΔC_p° (Figure 3). Negative values of ΔC_p° have been reported for a number of [*cf.* Spolar and Record (1994), and Landbury et al. (1994)], but not all (Koblan & Ackers, 1992), sequence-specific binding proteins that bind in the major groove of DNA. Thus, while this result is not unexpected, its magnitude is unprecedented, and it is a demonstration that heat capacity changes may accompany the sequence-specific binding of proteins in the minor groove of DNA. Interpretation of this value of ΔC_p° for TBP–E4 DNA interaction requires a consideration of the molecular events that may constitute sources of the heat capacity change. The large magnitude of ΔC_p° for the TBP–E4 DNA interaction, the availability of a large number of yeast TBP mutants, and a wealth of structural data will allow the contributions of different interactions to ΔC_p° to be critically examined. Although these questions cannot yet be answered, a brief discussion of the issue is informative.

Sturtevant (1977) considered the change in the exposure of nonpolar groups (the hydrophobic effect) and the change in internal vibrational modes as being the major contributors to the temperature dependence of ΔS for those processes where ΔC_p° is large. The ΔC_p° accompanying the solvation

of nonpolar residues, for which ΔH and ΔS are temperature dependent and compensate each other, is proportional to the solvent-accessible surface area (Baldwin, 1986; Privalov & Gill, 1988; Murphy et al., 1990). In protein unfolding, solvent exposure accounts for more than 70% of the observed change in ΔC_p° and hydration of both polar and nonpolar groups also contributes to the observed ΔC_p. These contributions are of different signs and magnitudes; the $\Delta H^\circ_{transfer}$ of nonpolar groups increases with increasing temperature and dominates the $\Delta H^\circ_{transfer}$ of polar groups, which is essentially temperature independent° (Privalov & Makhatadze, 1990, 1992; Makhatadze & Privalov, 1993).

Following this line of reasoning, Ha et al. (1989) proposed that the hydrophobic effect provides the major driving force in DNA−protein specific interactions. However, for many sequence-specific DNA−protein interactions, the solvent-excluded nonpolar surface area, as calculated from the experimental ΔC_p° based upon the transfer of model compounds from polar to nonpolar solvents, is larger than the areas calculated from atomic resolution X-ray diffraction structures (Spolar & Record, 1994). Similarly, the ΔC_p° value determined for TBP also cannot be accounted for simply by burial of nonpolar surface area. Approximately 12 600 Å^2 of buried hydrophobic surface area is calculated from the experimentally determined value of ΔC_p° of -3.5 ± 0.5 kcal/mol·K (Spolar, 1989, 1992; Livingstone et al., 1991). This value is 5-fold greater than the 2093 (3124 Å^2 total) and 2331 Å^2 (3150 Å total) nonpolar surface areas buried observed in the structures of the TBP−DNA complexes (Kim et al., 1993a,b; Kim & Burley, 1994).

Spolar and Record (1994) demonstrated for several systems that the "deficit" in the burial of nonpolar surface area could be accounted for by coupled protein-folding reactions. Comparison of the structures of TBP in solution and complexed with DNA reveals a small change in the orientation of the two domains but no significant global changes in the protein conformation (Nikolov, et al., 1992; Nikolov & Burley, 1994; Chasman et al., 1993; Kim et al., 1993a,b; Kim & Burley, 1994). Although there exists no atomic resolution structural information on the N-terminal domain of yeast TBP, a large red shift in the emission spectra of the fluorescence of the single tryptophan of yeast TBP that is within the N-terminal domain occurs upon promoter binding (Perez-Howard et al., 1995). These authors suggest that the amino terminal region is tucked within a hydrophobic pocket of a TBP−multimer and becomes more solvent exposed upon dissociation of the multimers. However, this *increase* in the solvent exposure of the tryptophan does not provide an explanation for the additional *buried* hydrophobic surface area calculated from the experimentally determined value of ΔC_p°.

There exists some uncertainty in the literature regarding the influence of its N-terminal domain on promoter binding by yeast TBP. Lieberman et al. (1991) reported increased promoter affinity and decreased temperature dependence of the C-terminal tryptic peptide (TBPΔ48) of yeast TBP. Parvin et al. (1995) demonstrated that the association rate and the affinity of the C-terminal domain of yeast TBP expressed from a truncated gene (TBPΔ61) is comparable to the wild-type protein for a promoter on a linear restriction fragment. Only small differences in the DNA-binding properties of yeast TBP and TBPΔ57 were observed by Kuddus and Schmidt (1993). In contrast, Perez-Howard etal. (1995) reported a 45-fold decrease in the second-order rate constant for the association of the C-terminal chymotrypic peptide (TBPΔ40) of yeast TBP with promoter. A critical analysis of truncated yeast TBP molecules and TBP molecules naturally lacking an N-terminal domain will be required to determine the role of the N-terminal domain in modulating promoter binding by TBP and its role, if any, in the mechanism underlying the temperature dependence of binding.

An alternative view of the source of the "deficit" of ΔC_p° has been proposed by Ladbury et al. (1994) based upon calorimetric studies of the *E. coli trp* repressor in comparison with the co-crystal structure of trp repressor and DNA. These authors suggested a decrease in the degrees of freedom of the complementary polar hydrated surfaces at the protein-DNA interface is a contributor to ΔC_p°. A decrease of degrees of freedom of mobile elements is viewed as a consequence of the stereocomplementarity of a macromolecular interface. In the case of the TBP−DNA complex, it seems unlikely that these effects are of sufficient magnitude to completely account for the ~ -3.0 kcal/mol·K "deficit" in ΔC_p° observed for the TBP−E4 promoter interaction.

Another potential source of the large and negative observed ΔC_p° is the changes in the conformation of the DNA. The distortion of the DNA from the B form that occurs within the TATA box results in DNA that is unwound, severely bent, and doubly kinked, resulting in the widening of the minor groove and the creation of a surface that is complementary to the TBP−DNA-binding interface. Coupling of the enthalpies of protein binding and the unstacking of the bases may make a significant contribution to the observed ΔC_p°. Ferrari and Lohman (1994) proposed that the observed ΔC_p° of the nonspecific interaction between the *E. coli* SSB protein and poly(dA) is the result of the unstacking of the DNA bases and the binding of the protein to the fully unstacked DNA. Although the individual equilibria of this binding reaction have ΔC_p° values equal to zero, the different values of ΔH° that characterize the two equilibria can give rise to an apparent ΔC_p° in a coupled system (Gill et al., 1985; Wyman and Gill, 1990). Careful evaluation of the enthalpies of the component equilibria of the TBP-promoter interaction will be required to evaluate this possibility.

There are intriguing parallels between the mechanism of promoter binding by *E. coli* RNA polymerase and TBP. The parallels include the slower than diffusion limited, multistep binding mechanism (McClure, 1980; Hoopes et al., 1992), conformational changes in the DNA induced by protein binding (Suh et al., 1993; Kim et al., 1993a,b), a large negative ΔC_p° characterizing the formation of a promoter-specific complex [Roe et al. (1985), this paper, and Figure 3], and a negative ΔC_p° characterizing the association rate [Roe et al. (1985), this paper, and Figure 7]. A thermodynamic relationship that may underlie these similarities may be found in studies suggesting that unpairing of the DNA duplex must be favored by bending or torsional stress (Kahn & Crothers, 1992; Kahn et al., 1994). Thus, the binding characteristics of RNA polymerase and TBP may be uniquely suited for the initiation of gene transcription. The activation enthalpy of the TBP binding reaction passes through zero at 30 °C, a fact that can account for the large magnitude of the lower limit of k_2 estimated at this temperature. If the parallels with RNA polymerase hold true (Roe et al., 1985), then k_2 of TBP will also vary with temperature. If so, this variable could prove a valuable probe into the mechanism of TBP

binding. Studies are in progress to answer this question.

The identity, within experimental error, of the values of $\Delta C_p°$ obtained for the equilibrium and kinetic measurements suggests that the source of the observed $\Delta C_p°$ is the rate-limiting step in the reaction. Since only a small reorientation of the protein domains accompanies promoter−complex formation, we believe it likely that the observed $\Delta C_p°$ results from coupling of the unwinding of the DNA helix and base unstacking. Additional interactions likely to contribute significantly to the thermodynamics of complex formation are the intercalation of phenylalanine side chains in the DNA "kinks" between bases 1 and 2 and bases 7 and 8 (Kim et al., 1993a,b; Kim & Burley, 1994). Systematic analysis of the contributions of the functional groups contributing to binding on both the DNA and the protein will be required to dissect the energetic contributions and the kinetic steps of the binding reaction.

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