Marked Stepwise Differences within a Common Kinetic Mechanism Characterize TATA-binding Protein Interactions with Two Consensus Promoters*

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Binding of the TATA-binding protein (TBP) to promoter DNA bearing the TATA sequence is an obligatory initial step in RNA polymerase II transcription initiation. The interactions of Saccharomyces cerevisiae TBP with the E4 (TATATATA) and adenovirus major late (TATAAAAG) promoters have been modeled via global analysis of kinetic and thermodynamic data obtained using fluorescence resonance energy transfer. A linear two-intermediate kinetic mechanism describes the reaction of both of these consensus strong promoters with TBP. Qualitative features common to both interactions include tightly bound TBP-DNA complexes with similar solution geometries, simultaneous DNA binding and bending, and the presence of intermediate TBP-DNA conformers at high mole fraction throughout most of the reaction and at equilibrium. Despite very similar energetic changes overall, the stepwise entropic and enthalpic compensations along the two pathways differ markedly following the initial binding/bending event. Furthermore, TBP-E4 dissociation ensues from both replacement and displacement processes, in contrast to replacement alone for TBP-adenovirus major late promoter. A model is proposed that explicitly correlates these similarities and differences with the sequence-specific structural properties inherent to each promoter. This detailed mechanistic comparison of two strong promoters interacting with TBP provides a foundation for subsequent comparison between consensus and variant promoter sequences reacting with TBP.

Assembly of the preinitiation complex, polymerase II recruitment, and subsequent gene transcription depend on the initial association of the TATA-binding protein (TBP)1 with TATA box-bearing promoter DNA (1–5). TBP binds productively to consensus (TATAA/AA/NN) and numerous variant TATA sequences, yielding binary complexes with the DNA bent in solution from 30° to 76° (6–8). These TBP-TATA complexes correspond ultimately to a broad range of transcription activities (9).

A strong correlation has been observed between the solution conformation of the TBP-TATA complex and the corresponding functional activity, including overall transcription efficiency and the details of the binary interaction. A very good correspondence is apparent between in vitro and in vivo transcription activity and the DNA solution bend angle in the binary complex (6), consistent with the TBP-TATA structure being a primary determinant of transcription activity. This relationship contrasts with a minimal correspondence of the TBP-DNA complex lifetime to bend angle and to transcription activity (6). The relationship among TATA sequence, DNA solution bend angle, and transcription efficiency are very well described by a two-state model in which the TBP-TATA complex exists in two solution conformations: a transcriptionally inactive form with only slightly bent DNA and a transcriptionally active form with the DNA bent −80° (6). The sequence-dependent probability for the complex to assume the transcriptionally active conformation would then be highly correlated with successful preinitiation complex formation and subsequent transcription.

The interaction between TBP and the consensus adenovirus major late promoter (AdMLP) (TATAAAAG) has been characterized using gel electrophoresis circular permutation analysis (10), DNase I footprinting (11, 12), and fluorescence resonance energy transfer (FRET) (13, 14). TBP-TATA association reactions proceed at rates significantly slower than diffusion limited (10–16). FRET studies reveal simultaneous binding and bending of AdMLP by TBP that is well described by a two-intermediate process with both intermediate conformers having bent DNA (14). One of these intermediate species is significantly populated and has been proposed as a TBP-DNA complex upon which the preinitiation complex is assembled (14).

The details of the TBP-AdMLP association kinetics, monitored in real time, are highly sensitive to single point mutations in the core TATA sequence (17). Furthermore, dramatic alterations in the solution geometry of a TBP-bound TATA variant (TATAAAG), induced by sequential addition of osmolyte, correspond to equally dramatic, successive changes in the TBP-DNA association binding curve (17). Differences in the mechanistic details of the TBP-promoter interaction must give rise to these kinetic changes and correlate with the sequence-dependent binary structures in solution.

Our goal is to further understand the functional consequences of variations in the TATA sequence-dependent solution conformation of the binary complex. A comprehensive kinetic and thermodynamic comparison of two consensus strong

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promoters reacting with TBP has thus been conducted, providing the foundation for subsequent studies utilizing variant TATA sequences. The interaction of the E4 core promoter sequence (TATATATA) with TBP was chosen for further analysis because previous characterization using DNase I footprinting revealed functional differences relative to the AdMLP sequence (11, 12).

Further examination of the TBP-E4 interaction over a range of temperatures and TBP concentrations using FRET in conjunction with steady-state, stopped-flow, and time-resolved measurements yields detailed kinetic and energetic profiles. The interaction of TBP with AdMLP was previously characterized in an analogous manner (14). The complete set of kinetic and thermodynamic data describing that reaction has been further analyzed in this study to provide an unambiguous, direct comparison between the two strong promoters.

The overall interactions of both the E4 and the adenovirus major late promoters with TBP are well described by a linear two-intermediate model (14). This model is the simplest to which the data for each promoter correspond. Important differences in the stepwise progression of these two reactions can be related directly to differences in the sequence-induced structural features of the two promoters, yielding additional insight into the relationships among sequence, structure, and function.

**EXPERIMENTAL PROCEDURES**

**DNA, Protein, and Solution Conditions**—Fluorescently labeled 14-base DNA oligonucleotide probes, with 3'-fluorescein and/or 5'-TAMRA, and unlabeled complementary oligomers were synthesized and purified by Sigma-Genosys (The Woodlands, TX) as described (6, 14). The E4 coding strand was thus TAMRA-5'-GGGCTATATATAGG-3' fluoresein, with the duplex denoted as T*E4dup*. Full-length Saccharomyces cerevisiae TBP was prepared as described (11, 13). Studies were conducted at the indicated temperatures in 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 2.5 mM MgCl₂, 1 mM CaCl₂, and 1 mM dithiothreitol.

**Theory and Determination of TBP-bound E4 End to End Distance Distribution**—Detailed discussions of FRET and its application to the present study have been published (6, 13, 14, 18–24). Very briefly, FRET is the nonradiative transfer of excited state energy from a donor to an acceptor fluorophore, with the efficiency of such transfer exquisitely dependent on the distance between the two dyes. The binding of TBP to TATA-bearing DNA may thus be monitored via changes in the donor emission, because the TBP-induced DNA bending significantly decreases the inter-dye distance. The interaction of E4 with TBP has been monitored herein using FRET in conjunction with steady-state emission, stopped-flow, and time-resolved fluorescence decay measurements.

The solution geometry of TBP-bound E4 was investigated by extracting the probability distributions characterizing the 5' end to 3' end distances from time-resolved fluorescence decays, as described (6), for the E4 duplex free and TBP-bound. A model-dependent solution bend angle for the bound E4 duplex was then determined as described (6).

**Equimolar TBP-DNA Stopped-flow Measurements**—Numerous studies have demonstrated that TBP is monomeric under the conditions of our studies (14, 25–27). However, to test the proposal that TBP dimerization explains the observed TBP-DNA kinetics (15, 28), stopped-flow association binding measurements have been conducted using equimolar concentrations of TBP and DNA. A 2 μM TBP solution was flowed together with a 2 μM AdMLP solution, with T*AdMLPdup* comprising 2% of the total DNA (the reliability of using the double-labeled duplex as a trace probe has been demonstrated) (14). Simulations were conducted using the two-intermediate model that has been shown to characterize the TBP-AdMLP interaction together with the previously determined 20 °C rate constants (14), with and without an additional step for TBP self-association. The rate constant for TBP₅₅₅₅ → 2 TBPmonomer was assigned the published value of 7.7 × 10⁻⁸ s⁻¹ (29). This value and the reported monomer-dimer equilibrium constant, 0.02 μM (15), were used to determine the rate constant for the reverse reaction. The experimentally and spectrally corrected curves were then fitted to a linear two-intermediate model with distance-dependent rate of energy transfer between the two fluorosc labels and are interpreted to reflect the relative extent of DNA bending in each binary complex (6, 13, 14, 17).

**SBP-E4 Equilibrium Binding Isotherms and Association Kinetics**—Equilibrium constants for TBP binding to T*E4dup* were determined at 10, 15, 17.5, 20, 22.5, and 25 °C using steady-state emission spectra exactly as described (14, 20, 22).

The interaction of TBP with AdMLP was characterized previously using fluorescence stopped-flow and steady-state emission measure-
19 points/curve and scaled from 0 to 0.419, the average overall amplitude change observed in steady-state FRET measurements. These 19 points emphasized the region from 45 to 55% saturation but extended to 98% saturation. Fitting to the shape of the curve rather than to a single $K_a$ value allows a priori for heterogeneity in analyses of these equilibria, although heterogeneity was neither detected nor expected for this model.

The theoretical association response functions were determined as described (14). Biphasic replacement response functions, reflecting the approach to equilibrium in the reverse direction, were determined in an analogous manner. The calculated equilibrium binding curves were constructed using $K_a$ values derived from the microscopic rate constants associated with the two-intermediate model (Equation 1).

$$K_a = \frac{k_1}{k_2} \left(1 + \frac{k_3}{k_4} \left(1 + \frac{k_5}{k_6}\right)\right)$$

(Eq. 2)

Evaluation of the overall quality of the fit was based on the weighted variance between the observed and theoretical points describing the shapes of the biphasic stopped-flow association ($\sigma_{wp}^2$) and replacement ($\sigma_{pl}^2$) curves and the equilibrium binding isotherms ($\sigma_{pl}^2$). The weighting factors of 0.041, 0.030, and 0.013 derived from the respective average errors in these data. To obtain $\sigma_{global}^2$, the three terms were further weighted to reflect the relative information content of each term, as follows.

$$\sigma_{global}^2 = \frac{1}{\sigma_{wp}^2} \cdot 9\sigma_{wp}^2 + 3\sigma_{pl}^2 + 4\sigma_{pl}^2$$

(Eq. 3)

An ideal global fit to a model then yields $\sigma_{global}^2 \leq 1$, with each term within its experimental error. Error estimates for the optimal parameters were obtained exactly as described (14).

The ensemble of TBP-E4 data was initially investigated, in a manner entirely analogous to that just described, using a one-intermediate model.

$$\text{TBP} + \text{DNA} \xrightarrow{k_1} \text{TBP-DNA} \xrightarrow{k_2} \text{I}_1 \xrightarrow{k_3} \text{I}_2 \xrightarrow{k_4} \text{TBP-DNA}_{\text{FINAL}}$$

(Eq. 4)

This analysis yielded values at 30°C for the rate constants, $k_1$–$k_4$, the corresponding activation enthalpies, and the relative quantum yields for TBP-bound DNA in the intermediate and final complexes.

The TBP-TATA interactions were further explored in terms of an

**FIG. 1.** Typical stopped-flow fluorescence decays, for 20 nM T*E4*duplex-F reacting with 400 nM TBP (after mixing) at 30°C. The first and fifth decay curves of a data set collected using the same material over a total time of 5 min are shown. The superimposition of these curves demonstrates the stability of our protein preparation over the time course of this reaction.

**FIG. 2.** Stopped-flow association kinetic curves for 20 nM T*E4*duplex-F reacting with 400 nM TBP at 15 (□), 20 (■), 25 (▲), and 30°C (○). Shown are the biexponential decays corresponding to the observed progress curves. The values observed for $\lambda_1$ are 0.29, 0.80, 1.5, and 3.6 s$^{-1}$, respectively. The faster binding and decreased biphasicity of TBP-E4 (●) association kinetics relative to those of TBP-AdMLP (×) are shown in the inset for 20°C with 400 nM TBP reacting with 20 nM labeled duplex.
extended one-intermediate model having two forms of free DNA duplex and two TBP-bound complexes.

\[
\begin{align*}
\text{DNA}_2 & \quad \overset{k_1}{\underset{k_2}{\rightleftharpoons}} \quad \text{DNA}_1 + \text{TBP} \\
& \quad \overset{k_3}{\underset{k_4}{\rightleftharpoons}} \quad \text{TBP-DNA}_{i\text{conformer}} \quad \overset{k_5}{\underset{k_6}{\rightleftharpoons}} \quad \text{TBP-DNA}_{\text{FINAL}} \quad \text{(Eq. 5)}
\end{align*}
\]

An equilibrium between two conformations of DNA, one bent and thus predisposed to TBP binding (DNA$_1$), characterizes the model (13, 30). Global analysis was as described for the two-intermediate model but with $k_3$ as a second-order rate constant and the quantum yields of DNA$_1$, $\lambda$DNA$_1$, and TBP-DNA$_{\text{FINAL}}$ determined relative to a quantum yield of 1 for DNA$_2$. Only values of $k_3$ and $k_5$ of approximately the same order of magnitude as the other rate constants were deemed acceptable in this analysis. For much larger values of $k_3$ and $k_5$, Equation 5 is equivalent to the one-intermediate model in terms of accommodating the data, with $k_3$ simply scaled by $k_5/k_6$.

The quality of the fit to Equation 5 was assessed as described for the two-intermediate model, with a constraint on the population distribution of DNA$_2$ and DNA$_1$ based on the near temperature independence of the fluorescence spectrum of the free duplex. At each iterative step of the global analysis, the corresponding theoretical fluorescein emission, $F(T)_{\text{calc}}$, was determined for each temperature, $T$, using the following expression.

\[
F(T)_{\text{calc}} = \frac{[k_1(T)/(20^\circ \text{C})]^{1} [QY_{\text{DNA}_1} k_i(T) + 1]}{[k_1(T)/(20^\circ \text{C})]^{1} [QY_{\text{DNA}_1} k_i(20^\circ \text{C}) + 1]} \quad \text{(Eq. 6)}
\]

where $QY_{\text{DNA}_1}$ is the quantum yield of free, prebent DNA. $F(T)_{\text{calc}}$ values falling outside of the experimentally determined range resulted in penalization of $\sigma_{\text{global}}$ (Equation 3).

Global analyses of the collective TBP-AdMLP data to one-, extended one-, and two-intermediate models were conducted, incorporating these modifications, and the optimal parameters were compared with those obtained previously (14). The analysis included eleven stopped-flow association and four replacement kinetic curves and five equilibrium binding isotherms, the latter scaled to 0.440. In contrast to TBP-E4 dissociation, TBP-AdMLP dissociation was independent of the concentration of unlabeled AdMLP$_{\text{sat}}$. These latter experimental curves were therefore a direct measure of the replacement process and were used in the global analysis.
RESULTS

Thermal Stability of TBP—The half-times for inactivation of the free and DNA-bound S. cerevisiae TBP preparation used in these studies (11, 13) have been determined by several independent laboratories and methodologies to be ~1 and ~10 h, respectively, at 30 °C under the conditions of these studies (6). During the total collection time of 5 min required to obtain a set of five stopped-flow association curves at 30 °C, ≤5% of the protein would thus be subject to inactivation, which approximates the variability of replicate curves under a given condition. This conclusion is consistent with the observed superimposition of the first and fifth replicate curves (Fig. 1), the lack of an observed kinetic trend reflecting progressive TBP inactivation, and a total amplitude change identical to that observed in the steady state. The same three observations obtain for replicate curves collected at 10 °C over 40 min. These results contrast sharply with those of Jackson-Fisher et al. (28), who reported half-times for TBP inactivation of ~4 s and 9 min at 30 and 10 °C, respectively.

Equimolar TBP-DNA Binding Studies—That TBP is monomeric under the conditions of this study has been demonstrated by analytical ultracentrifugation (25, 26) and biochemical (14) studies. To further establish that the rate constants obtained from stopped-flow binding experiments reflect TBP-DNA binding and not TBP dimer → 2 TBP monomer, stopped-flow association kinetics were measured with no excess TBP, thus sampling the entire active protein population. These new stopped-flow results were compared with two simulated kinetic curves: one based on the two-intermediate model (Equation 1) and previously determined rate constants (14) and the other based on results obtained using a pull-down assay (15). The experimental kinetic trace was in good agreement with the curve simulated using the two-intermediate model, with the predicted full amplitude decrease of 41 ± 1% observed within ~8 s for both the measured and simulated curves. In contrast, the curve simulated from the model including the additional TBP dimer-monomer equilibrium differed dramatically from the experimental curve. The latter simulated curve showed an initial rapid phase with an amplitude change of only ~3% over 0.5 s, followed by a very slow decay to the full amplitude change, complete only after 50 min, 500 times longer than that observed experimentally. These results show clearly that the kinetic progress curves in this study correspond to protein-DNA association and not a slow, rate-limiting process of dimer dissociation, as has been incorrectly asserted (15, 28).

Biphasic kinetics have been observed for TBP-DNA binding in our laboratory using FRET stopped-flow and elsewhere using [TBP] = 400 nM and 15 °C (A), replacement curves, 25 °C (B), and equilibrium binding isotherms, 17.5 °C (C). Overall σ_b, σ_r, and σ_o values of 1.03, 1.04, and 0.705, respectively, reflect theoretical fits to observed curves within experimental noise and were associated with globally randomized residuals. This close correspondence between the theoretical and experimental data sets is particularly notable considering the additional stringency introduced by noise-free experimental curves and absolute TBP activities.

TABLE I

Globally derived TBP-E4 kinetic and thermodynamic parameter values corresponding to the two-intermediate model (Equation 1)

The values shown are for 25 °C and 1 M standard state for each step, i, along the reaction pathway. Numbers in parentheses represent the upper and lower error bounds corresponding to the 68% confidence region, determined as described previously (14): k_i is a second order rate constant with units of μM⁻¹ s⁻¹; k_o-h_o are first order rate constants. Quantum yield ratios for I₁, I₂, and the final TBP-E4 complex are 0.605 (0.598, 0.612), 0.502 (0.496, 0.529), and 0.550 (0.547, 0.559), respectively. The relative quantum yield reflects the extent of DNA bending (14). Because I₁ and I₂, as well as the final complex, are present at equilibrium for infinite TBP, the measured ΔHᵢ° = Σ_i Φ_i H_i, where i indexes the three bound species, Φ is mole fraction, and H is the molar enthalpy referenced to H₀ = 0 for free DNA. For 25 °C, this apparent ΔH₀° = 35.6 (30.4, 38.5) kcal mol⁻¹. Refined TBP-AdMLP analysis yielded parameters statistically similar to the original values with the following exceptions: k₀ = 0.0070 (0.0062, 0.0073), k₄ = 0.293 (0.225, 0.344), k₅ = 2.32 (1.89, 2.89), k₆ = 0.0137 (0.0110, 0.0181), ΔH₄₋₃° = 24.5 (22.9, 26.9), ΔH₅₋₄° = 14.7 (12.0, 16.9). Because the concentration of the second AdMLP intermediate is relatively low and the data contain the least information about this species under the conditions of the measurements, it is not surprising that parameter deviations are predominantly associated with processes centered on I₄. Despite subtle parameter differences due to the increased stringency of the refined analysis, the overall characteristics of the TBP-AdMLP interaction, such as thermodynamic patterns and species concentrations, are retained.

| i   | k_i     | ΔH_i°   | ΔS_i°   | ΔO_i°  |
|-----|---------|---------|---------|--------|
| 1   | 2.84    | 30.7    | 73.9    | 8.65   |
| 2   | 0.0243  | 15.3    | -14.7   | -47.2  |
| 3   | 0.146   | 26.6    | -28.5   | -47.2  |
| 4   | 1.09    | 8.90    | -28.5   | -47.2  |
| 5   | 0.0407  | 5.28    | -61.4   | 19.3   |
| 6   | 0.00245 | 2.72    | -61.4   | 21.0   |
Fluorescence Stopped-flow Measurements—The temperature and protein concentration dependence of the TBP-T*E4_dual*F binding and bending interaction were monitored in real time using fluorescence stopped-flow and FRET. A total of 53 kinetic curves were collected, including replicate curves at each of nine conditions of temperature (10–30 °C) and TBP concentration (200–800 nM), yielding nine averaged curves. Each of these curves was very well described by biexponential decay, excluding photobleaching (13, 14), with an overall change in amplitude of 42.4 ± 0.8%, identical within error to the independently measured steady-state change. The binding of TBP to T*AdMLP_dual*F had been previously measured to obtain 11 analogous stopped-flow progress curves with an amplitude change of 44.0 ± 1.4% (14).

The association of TBP with the E4 promoter is biphasic over the experimental temperature range of these studies; the biphasicity increases with decreasing temperature and TBP concentration (Fig. 2) (14). The association of TBP with the E4 promoter is notably faster and less biphasic than with AdMLP (Fig. 2, inset). For neither interaction is a pattern based on temperature or TBP concentration apparent.

Equilibrium Binding Constants—Seven equilibrium binding isotherms for the TBP-T*E4_dual*F interaction were obtained from 10–30 °C, shown for 20 °C in Fig. 3. These data were well described by a linear van’t Hoff plot, yielding $K_a$ values of 43.3 ± 6.4 μM$^{-1}$ at 10 °C, 93.7 ± 9.4 μM$^{-1}$ at 15 °C, 136.5 ± 11.6 μM$^{-1}$ at 17.5 °C, 197.7 ± 15.7 μM$^{-1}$ at 20 °C, 284.5 ± 24.2 μM$^{-1}$ at 22.5 °C, 406.8 ± 40.3 μM$^{-1}$ at 25 °C, and 817.3 ± 115.2 μM$^{-1}$ at 30 °C. The affinity of TBP for E4 is approximately two to seven times higher than for AdMLP (Fig. 3, inset) (14). Essentially the same relative affinities are observed with the E4 and AdMLP TATA boxes in the contexts of the native promoters (12).

Dissociation Kinetic Measurements—The time course of the release of labeled DNA from TBP-bound complex was monitored using steady-state emission changes following the addition of large excesses of unlabeled DNA. Three concentrations of the latter were used at each temperature to yield one related set of dissociation curves. All TBP-AdMLP and TBP-E4 dissociation kinetic curves collected at 15, 20, 25, and 30 °C were well described by biexponential decay. Notably, TBP-E4 dissociation kinetics, unlike TBP-AdMLP, showed a measurable
dependence on the concentration of unlabeled DNA, revealing contributions to this process from both replacement and displacement.

Inclusion of both replacement and displacement processes in TBP-E4 global analysis would have necessitated additional fitted parameters. The curve corresponding to the replacement process, which is independent of the concentration of unlabeled DNA, was therefore extracted from the set of decays obtained at each temperature using two different procedures (see "Experimental Procedures"). The extracted replacement curves were essentially independent of the method used. The pure replacement curve at 20°C is shown in Fig. 4 together with the set of DNA concentration-dependent dissociation curves from which it was extracted.

The replacement kinetics for both TBP-E4 and TBP-AdMLP are biphasic, although the relative amplitudes of the fast and slow phases differ significantly between the two promoters (Fig. 4, inset). The values of \( \lambda_{\text{rate}} \) for E4 replacement kinetics are approximately two to eight times slower than that for AdMLP. In contrast, the rate constants determined for the dominant slow phases of E4 and AdMLP, 0.0018 ± 0.0002 and 0.0015 ± 0.0004 s\(^{-1}\), respectively, are identical within error and are essentially temperature-independent. These values, and their invariance with temperature, are in excellent agreement with those previously reported for the overall dissociation of TBP from E4 (11, 12) and AdMLP (12, 14).

Global Analysis Using Linear One- and Two-intermediate Models—The collective TBP-AdMLP kinetic and thermodynamic data have been shown unequivocally to be inconsistent with the simplest one-intermediate model (Equation 4) (14). This two-step mechanism likewise could not accommodate the ensemble of TBP-E4 data. Although good correspondence to Equation 4 was obtained using only the four replacement and six equilibrium binding curves, inclusion of the nine stopped-flow binding curves resulted in average weighted residuals approximately four times the experimental error.

Both the TBP-E4 and TBP-AdMLP data were subsequently analyzed using the next simplest, two-intermediate model, Equation 1. The ensemble of TBP-E4 kinetic and energetic data is well described by this mechanism, with \( \sigma_{\text{global}} = 0.981 \) and the weighted average residuals within experimental error for the association, replacement, and equilibrium binding curves (Fig. 5). The optimal kinetic and thermodynamic values corresponding to this model are shown in Table I. As for AdMLP (14), E4 bending occurs simultaneously with TBP binding in the first reaction step, as evidenced by nearly equivalent quantum yield values for the intermediate and final conformers.

Global analysis of collective TBP-AdMLP data using Equation 1 was performed previously with dissociation described using an overall steady-state relaxation expression (14). The more stringent analysis described herein yielded a similar quality fit, with the average weighted residual nearly identical to experiment error. Similar parameter values were also obtained (Table I). The differences were associated primarily with \( I_2 \), the second intermediate, present throughout the reaction at very low concentration and for which parameters derived from both methods of analysis were the least well determined. The defining characteristics of the TBP-AdMLP interaction, such as the thermodynamic profile, are unchanged.

The relative fraction of each species along the association pathway was determined using the microscopic rate constants at low and high temperatures for E4 and AdMLP (Fig. 6). For the latter, the profile was essentially unchanged from that in the initial analysis (14). The species populations vary with TBP concentration and temperature for both the TBP-E4 and TBP-AdMLP reactions, with \( I_1 \) present at high concentration for a significant portion of the reaction time for all conditions examined. Notably, \( I_1 \) persists at equilibrium for both TBP-promoter complexes, with this conformation even more predominant for E4 than for AdMLP. At equilibrium and 400 nM TBP, \( I_1 \) comprises 18.2% of all bound E4 species at 30°C and, remarkably, is the dominant bound form at 15°C at 53.1%.

Thermodynamic Profiles for the TBP-E4 and TBP-AdMLP Reaction Pathways—The thermodynamic profiles for TBP-E4
FIG. 8. Raw kinetic data obtained at 15 °C for overall TBP-T*E4dpx*F dissociation using 5.56 μm unlabeled DNA (○) and the corresponding pure replacement curve (×). Theoretical fits derive from analysis of the set of DNA concentration-dependent curves at a given temperature using a dissociation model based on Equation 1, the optimal rate constant values, and displacement from either I₁ (solid line) or TBP-E4FINAL (dashed line). For the latter model, transformation of the pure replacement curve to capture the fast phase of the observed dissociation required a rapid rate of displacement from TBP-E4FINAL. However, because the TBP-E4FINAL species gives rise to the slow replacement phase of dissociation, its rapid removal eradicates that phase, producing a severe lack of fit. Conversely, a slow rate constant (<k₁) for displacement from TBP-E4FINAL transforms the replacement curve to mimic the slow dissociation phase but results in an equally severe misfit of the fast phase. In contrast, displacement from I₁ simply increases the already rapid rate of I₁ dissociation while retaining the slow dissociation phase deriving from depopulation of the final species.

and TBP-AdMLP association are compared stepwise in Fig. 7 for the two-intermediate model. Overall free energy changes of −10.7 (−10.8, −10.6) and −11.5 (−11.6, −11.3) kcal mol⁻¹ are obtained for the TBP-AdMLP and TBP-E4 interactions, respectively. Both reactions are entropy-driven under the experimental conditions with overall ∆S° values obtained for AdMLP and E4 of 105.3 (90.1, 117.2) and 157.9 (140.2, 167.6) cal K⁻¹ mol⁻¹, respectively. Activation enthalpies for the initial step of 35.4 (34.6, 35.9) kcal mol⁻¹ for AdMLP and 30.7 (29.9, 31.4) kcal mol⁻¹ for E4 present the largest energetic barrier to these reactions.

Global Analysis Using an Extended One-intermediate Model—The collective TBP-DNA kinetic and thermodynamic data were also fit, for both promoters, to an extended one-intermediate model (Equation 5) characterized by two forms of free DNA duplex and two TBP-bound complexes. This model is comparable in complexity to the linear two-intermediate model (Equation 1). The TBP-E4 data corresponded within experimental error to this model. The results of this analysis yielded several qualitative features consistent with those from the two-intermediate model: the relative quantum yields indicate that the DNA in the intermediate species is bent to essentially the same extent as in the final complex and a high intermediate population is present throughout the reaction and persists at equilibrium (8% at 30 °C and 32% at 15 °C with [TBP] = 400 nM). However, the TBP-AdMLP kinetic and thermodynamic data could not be accommodated by Equation 5, with average weighted residuals up to six times the experimental error. An extended one-intermediate model (Equation 5), having two conformations of free DNA, accommodated the E4 but not the AdMLP data. Ultimately, the entire ensemble of association, relaxation, and equilibrium binding measures of the AdMLP and E4 interactions with TBP were well accommodated by Equation 1, a linear two-intermediate model.

That these extensive data sets were well described by the same model is not surprising. Both E4 and AdMLP form tightly bound complexes with TBP (36), display high transcription efficiencies (9), have very similar co-crystal structures (31–35) and have comparable TBP-bound solution structures. The similarity of these structural and functional characteristics is reflected by common qualitative features along the two-intermediate pathway. The values of the relative quantum yields reflect simultaneous DNA binding and bending for both promoters (13, 14). Additionally, I₁ is present at high mole fraction throughout both interactions and persists at equilibrium. We therefore propose that for E4, as with AdMLP, both the I₁ and final conformers may bind transcription factor IIB in the subsequent step of RNA polymerase II preinitiation complex assembly (14).

Thermodynamically, the two promoters react with TBP very similarly in the initial binding/bending step, the formation of I₁ from free TBP and DNA (Fig. 7). This initial event proceeds for both sequences through a large energetic barrier together with the largest stepwise increase in entropy. After the first step, however, the thermodynamic profiles of the two reactions are...
very different. The second step, \( I_1 \rightarrow I_2 \), is exothermic and accompanied by a large decrease in entropy for AdMLP. In contrast, this step for E4 is endothermic and has a large increase in entropy. The final step, \( I_2 \rightarrow \text{TPB-DNA}_{\text{FINAL}} \), is thermodynamically similar to the first for AdMLP, highly endothermic and entropically favorable, whereas this step for E4 has only slight endothermic and entropic changes.

Kinetically, the consequences of the stepwise differences are most clearly evidenced by differences in the time evolution of each species along the two pathways (Fig. 6). For E4, \( I_1 \) is present at higher mole fraction throughout most of the reaction and at equilibrium under all conditions than for AdMLP. In addition, the mole ratio of \( I_2-I_1 \) is two and a half times greater for E4. Notably, maximum displacement occurs at 15 °C, where \( I_1 \) is the dominant equilibrium species for E4. These data suggested that the measurable displacement (versus replacement) of E4 by competitor DNA might be attributable to the high relative abundance of the intermediate species.

To identify the predominant species from which displacement occurs, the observed dissociation kinetics were thus compared with theoretical curves incorporating both replacement and displacement. The latter were generated using Equation 1, the six rate constant values at a given temperature, and an additional pathway for displacement from either \( I_1 \) or TPB-E4\(_{\text{FINAL}}\). The displacement rate constant that best described the set of three DNA concentration-dependent curves at a given temperature was determined. The model assuming displacement from only \( I_2 \) accommodates the dissociation data within error at all temperatures with globally randomized residuals. In contrast, displacement from only TPB-E4\(_{\text{FINAL}}\) results in large errors (residuals were one and a half to three times the error) and the systematic deviation of the calculated from the observed dissociation curves. These two theoretical curves are shown in Fig. 8 for 15 °C together with the observed dissociation data and the pure replacement curve. This analysis points to a dominant role of the \( I_2 \) intermediate in the displacement reaction, although it does not rule out a contribution of the final complex to this process. The facilitated removal of TPB from the E4 promoter by regulatory proteins may similarly proceed from this intermediate conformer rather than from TPB-E4\(_{\text{FINAL}}\). These data afford an independent line of evidence demonstrating the presence of intermediate species at equilibrium. They further reveal a new and novel biological consequence of structural variations in TBP-TATA binary complexes.

That both replacement and displacement processes were observed for TPB-E4 dissociation kinetics suggests caution when deriving a dissociation rate constant directly from a measured “replacement” process. The displacement process is revealed through the use of multiple competitor DNA concentrations in dissociation experiments (37), and its presence or absence is apparent only in retrospect. This concern is of particular importance for measurements aimed at capturing the fast reaction phase. For other protein-DNA complexes with nanomolar affinities, a dependence of dissociation kinetics on competitor DNA concentration may indicate the presence of multiple species at equilibrium.

E4 and AdMLP ultimately form structurally similar binary complexes with TBP. What, then, is the origin of these stepwise similarities and differences? Unique and well characterized structural features are associated with each of these sequences. The uniform deformability of repeating TA base steps, as in the E4 sequence, has been established (38–45). In contrast, A tracts have been understood to be rigid (31, 46–48) but with flexibility introduced at the junction between the continuous run of As and mixed sequence DNA (49, 50). Very recent bio-

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Marked Stepwise Differences within a Common Kinetic Mechanism Characterize TATA-binding Protein Interactions with Two Consensus Promoters

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