Comparison of the Effect of Water Release on the Interaction of the *Saccharomyces cerevisiae* TATA Binding Protein (TBP) with "TATA Box" Sequences Composed of Adenosine or Inosine

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ABSTRACT The formation of sequence-specific complexes of TATA binding protein (TBP) with the minor groove of DNA results in the burial of large nonpolar surfaces and the exclusion of water from these interfaces. The release of water is thus expected to provide a significant entropic driving force for formation of the transcription-preinitiated complexes mediated by the binding of TBP to specific sequences. In this article are described equilibrium-binding studies of *Saccharomyces cerevisiae* TBP to 14 bp oligonucleotides bearing either the tightly bound and efficiently transcribed adenovirus major late promoter (TATAAAAG) or its inosine-substituted derivative (TITIIIIG) as a function of neutral osmolyte concentration. These two DNA sequences present the same pattern of minor groove hydrogen-bond donors and acceptors to the protein. TBP-DNA complex formation was monitored by steady-state fluorescence resonance energy transfer measurements of the oligonucleotides end-labeled with fluorescein (donor) and TAMRA (acceptor). Correct interpretation of the results obtained with the inosine-substituted sequence required careful consideration of the optical properties of the dyes as a function of osmolyte concentration to demonstrate that the relative change in the end-to-end distances for TATAAAAG- and TITIIIIG-bearing oligonucleotides is the same upon TBP binding. Although the affinity of TBP is slightly greater for the adenosine compared with the inosine-substituted TATA sequence in the absence of osmolyte, the end-to-end distances of the bound DNA in complex with TBP, the enthalpic and electrostatic components of binding, are identical within experimental precision. However, ~18 additional molecules of water are released upon TBP binding the TATAAAAG as compared with the TITIIIIG sequence resulting in an entropic advantage to the binding of the natural promoter sequence. These results are considered with regard to differences in the flexibility and hydration of the two DNA sequences.

INTRODUCTION

The stability and sequence specificity of protein-DNA complexes is dependent on thermodynamic variables including temperature, pressure, pH, ion type, and ion concentration (Parsegian et al., 2000; Record, Jr. et al., 1985, 1998; Sidorova and Rau, 2001). Atomic resolution structures have been solved for a growing number of sequence-specific complexes of proteins with DNA, including many gene regulatory proteins. These structures show intimate association of site-specifically bound proteins with the DNA with water excluded from the macromolecular interface. The replacement of contacts with water by protein-DNA contacts is observed in some of the crystallographic studies (Pabo and Sauer, 1992).

The hydration shell that surrounds proteins and DNA in solution is perturbed upon formation of macromolecular complexes. The release of water bound to the interacting surfaces of the reactants into the bulk solution provides an entropic driving force for complex formation. Some of the complexes whose atomic resolution structures have been solved have also been the subjects of detailed thermodynamic investigations. These biochemical studies are uniformly consistent with the displacement of water from protein-DNA interfaces (Fried et al., 2002; Jen-Jacobson et al., 2000; Parsegian et al., 1995, 2000; Record, Jr. et al., 1998; Sidorova and Rau, 1996; Vossen et al., 1997).

A measure of water release upon complex formation can be obtained by osmotic stress measurements (Parsegian et al., 1995; Sidorova and Rau, 1996). This approach uses small neutral solutes (perturbing solutes; see Record, Jr. et al., 1998) that do not bind with high affinity to the reactants whose concentration affects the thermodynamic activity of the water (the osmotic effect) and the biopolymers (preferential interactions). Since preferential interactions are dependent upon the chemical composition of the solute, these effects can be differentiated by measurements conducted using multiple osmolytes. Although it has been suggested that the osmotic-stress technique provides only a lower boundary to the number of water molecules released due to steric exclusion (Courtenay et al., 2000), comparative studies provide a way to circumvent this limitation. For example, osmotic-stress analysis of the binding of a protein to specific and nonspecific sequences conducted under otherwise identical experimental conditions allows the effect of steric exclusion to be extracted from the two sets of data (Fried et al., 2002; Sidorova and Rau, 1996, 2001).

The assembly of the TATA binding protein (TBP) to promoter DNA is required for the initiation of eukaryotic gene transcription by all three RNA polymerases. The biological function of TBP is clearly linked to its specific interaction with a myriad array of other transcription control proteins and, at least for RNA polymerase II, to its specific binding to particular sequences of DNA. Crystallographic

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studies have shown that TBP binds to the minor groove of DNA exposing a broad flat surface to the protein and dramatically bending the DNA (Burley and Roeder, 1996). The binding and bending of the bound DNA has been investigated in solution by gel permutation (Starr et al., 1995), gel phasing (Bareket-Samish et al., 2000), and fluorescence resonance energy transfer (FRET) (Parkhurst et al., 1996, 1999; Powell et al., 2001, 2002). In addition, time-resolved FRET studies conducted as a function of the TATA box sequence revealed dramatic differences in both the end-to-end distances and the distribution of distances for the TBP-bound DNA that are osmolyte-sensitive for lower affinity sequences (Wu et al., 2001a,b).

A comparative approach has been used in this study to explore the role of dehydration in the sequence-specific binding of the *Saccharomyces cerevisiae* TATA binding protein (TBP) to the high affinity sequence TATAAAAG and its inosine-substituted derivative (TITIIIIT). These sequences present an identical pattern of hydrogen-bond donors and acceptors in the minor groove that contribute to TBP binding (Lee et al., 1991; Starr and Hawley, 1991) and both sequences support the similar level of transcription activity (Lee et al., 1997). Despite this similarity, these two sequences have very different physical properties. Indeed, the inspiration for these experimental studies was a computational analysis of these two sequences and their interaction with TBP, particularly with regard to hydration and water release upon complex formation (Pastor et al., 1999). The sequence of adenosine is calculated to bind 19 more water molecules in the minor groove as compared with that of inosine. Thus, comparison of TBP binding to the two sequences is an ideal context within which to explore the entropic consequences of water release upon the TBP-DNA complex formation.

The accurate analysis of the steady-state FRET intensity changes was critical to the conduct of these studies due to the small number of water molecules released upon complex formation. In addition, careful consideration was given to the effect of osmolyte on the spectroscopic parameters of the fluorescent dyes attached to the oligonucleotides. A generally applicable formalism is presented for the calculation of the efficiency of energy transfer that accounts for the properties of a particular donor-acceptor pair.

MATERIALS AND METHODS

Protein, DNA, and other reagents

S. cerevisiae TBP was expressed in *Escherichia coli* and purified as previously described (Parkhurst et al., 1996; Petri et al., 1995). An extinction coefficient of $13.4 \times 10^3\ M^{-1} \times cm^{-1}$ was used to calculate the concentration of the protein. Triethylene glycol (TEG) and methyl glucoside (methyl-alpha-*d*-glucopyranoside; MG) were purchased from Sigma (St. Louis, MO) and used without additional purification. The osmolal concentration of TEG and MG were calculated from the dependence of their osmotic pressure on molal concentration (N. Sidorova and D. Rau, personal communication). Labeled and unlabeled DNA oligonucleotides were obtained from TriLink Biotechnologies (San Diego, CA). The concentration of oligonucleotides was determined using extinction coefficients calculated from the known basepair composition correcting for the absorbance of fluorescein or TAMRA at 260 nm.

TBP binding to 14-bp oligonucleotide duplexes double-labeled with fluorescein and TAMRA attached through 6-carbon linkers was measured by FRET (Parkhurst et al., 1996, 1999; Wu et al., 2001a,b). This fluorescence donor-acceptor pair has been utilized to maximize consistency with the published studies. Separately labeled top and bottom strands were annealed in this study to yield the duplexes

A-AdMLP
Fluorescein-CGC**TATAAAAG**GGC-3′ $F14_A$
3′-GCG**ATATTTTC**CCG-TAMRA $T14_A$
I-AdMLP
Fluorescein-CGC**TITIIIIG**GGC-3′ $F14_I$
3′-CGG**ACACCCCC**CCG-TAMRA $T14_I$.

A stability of $\Delta G = -17.2$ kcal/mol was calculated for the A-AdMLP duplex at the experimental ionic conditions and 37°C (SantaLucia, 1998), consistent with experimental results (Morrison and Stols, 1993). Whereas an I-C basepair has two hydrogen bonds similar to the A-T basepair, its stability is substantially stronger due its electronic distribution (Lankas et al., 2002). Thus, the stability of the I-AdMLP oligonucleotide duplex will be greater than that of the A-AdMLP. Unlabeled oligonucleotides of the same sequence were used as the complementary strand in studies conducted using either fluorescein or TAMRA single-labeled duplexes (Khrapunov et al., 2002). The high stability of these duplexes results in <0.1% single-strand oligonucleotides at the duplex concentration of 10 nM used in all of the TBP-binding experiments.

Fluorescence and absorption spectroscopy

All experiments were performed in buffer containing 25 mM Tris, 100 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 50 μg/mL bovine serum albumin, and 2 mM DTT at pH 7.5 and 22°C. A Beckman Coulter DU 7400 spectrophotometer (Fullerton, CA) was used for the absorption measurements. Fluorescence measurements were made using a Jobin Yvon Fluoromax-3 spectrofluorometer (Edison, NJ). All spectra were corrected for the spectral sensitivity of the instrument. Measurement of fluorescence spectra and intensities were performed at the *magic angle* (corresponding to the angle of 55° between the vectors of polarization of the excitation and emission light) to minimize error due to fluorescence polarization changes (Lakowicz, 1999). The intensity of the Raman scattering band of water was used as the internal standard of fluorometer sensitivity. The anisotropy was calculated by

$$A\lambda = \frac{Iv - GIh}{Iv - 2GIh}, \tag{1}$$

where $A\lambda$ is the fluorescence anisotropy measured at a combination of the excitation and emission wavelengths with the excitation polarizer in the vertical position, Iv and Ih are the fluorescence intensities measured at the vertical and horizontal position of the emission polarizer, and G is the instrumental factor accounting for the bias of the detection system for vertically versus horizontally polarized light.

Fluorescence resonance energy transfer (FRET)

The efficiency of the energy transfer (E) depends on the distance between a donor and an acceptor according to

$$1/E = 1 + (R/R_o)^6, \tag{2}$$

where R is the donor-acceptor distance and R_o is the Förster radius (the distance between donor and acceptor at which $E = 0.5$). R_o is specific for a particular donor acceptor pair,

$$R_o = 9790(J \times \kappa^2 \times Q_d \times n^{-4})^{1/2}, \quad (3)$$

where J is the spectral overlap integral, κ is the orientation factor for dipole-dipole coupling, Q_d is the donor emission quantum yield, and n is the refractive index of the medium. Small changes in R produce large changes in E due to the sixth power of the distance. Since TBP induces a significant bend in the DNA upon binding, a robust experimental signal for binding studies is provided (Parkhurst et al., 1996). The 14-bp length of the oligonucleotide (47.6 Å) and the chosen donor-acceptor pair ($R_o \approx 50$–61 Å) permit the measurement of the FRET efficiency parameters if the donor-acceptor distance changes. Analysis schemes based either on donor or acceptor emission can be used to determine changes in E (Clegg, 1992). Enhanced acceptor fluorescence emission is monitored in the present experiments for the determination of FRET.

The strategy used in this study of creating a double-labeled duplex from singly-labeled complementary oligonucleotides differs from the double-labeled single strand annealed with the unlabeled complementary strand used in previous studies of TBP interactions (Parkhurst et al., 1996, and subsequent publications). Creation and purification of the double-labeled oligonucleotide is synthetically demanding. Other FRET methods are based on the hybridization of single-strand, singly-labeled oligonucleotides followed by purification of the double-labeled duplex (e.g., Clegg, 1992). The present approach is based on a titration of the donor-labeled oligonucleotide with the acceptor-labeled complement to determine the equimolar ratio of two strands of DNA within the resultant duplex. This approach does not require additional purification of the DNA probe, is not sensitive to errors in the stoichiometry of the two DNA strands, and is very accurate. As will be presented in Results, it was the unexpected fluorescence behavior of the inosine-substituted oligonucleotide that prompted an analysis of the properties of the single- and double-labeled duplexes in the absence of TBP and the development of this approach.

The fluorescence of singly versus doubly labeled oligonucleotides

The fluorescence emission spectra of the fluorescein and TAMRA singly-labeled oligonucleotides were measured before and after annealing with unlabeled complementary strands. An increase in the emission of fluorescein at 520 nm was observed for the adenosine-bearing oligonucleotide in contrast to the decrease measured for the inosine-bearing one (compare Fig. 1, *A* and *B*, *curves 1* and *3*). A decrease in the fluorescence at 580 nm upon duplex formation was observed for both of the TAMRA-labeled oligonucleotides (Fig. 1, *A* and *B*, *curves 4* and *5*). These results reflect a DNA duplex-dependent sequence-specific interaction of fluorescein with the inosine-containing oligonucleotide. Saturation of each of the two singly-labeled duplexes with TBP yielded no detectable changes in their emission spectra (Fig. 1 *A* and *B*, *curves 2* and *6*).

In contrast, when fluorescence of the doubly-labeled TATAAAAG duplex is measured, a new shoulder at 580 nm in the fluorescence spectrum appears due to resonance energy migration (Fig. 2; see also Parkhurst et al., 1996). The total emission (Fig. 2, *curve 1*) at 580 nm is composed of two components—donor (fluorescein) emission due to direct excitation, and the emission of the acceptor (TAMRA). To resolve the portion of the overall fluorescence caused by the acceptor, the emission spectrum of the donor (Fig. 1 *A*, *curve 2*) was normalized to the emission spectrum of the double-labeled duplex at 520 nm, where only the donor emits (Fig. 2, *curve 2*). The difference spectrum that results from the subtraction of spectrum 2 from 1 yields spectrum 3 (Fig. 2). The 580-nm fluorescence emission in difference spectrum 3 (denoted $dF_{490/580}$) consists of two components—direct TAMRA excitation and FRET. This parameter $dF_{490/580}$ is used for all

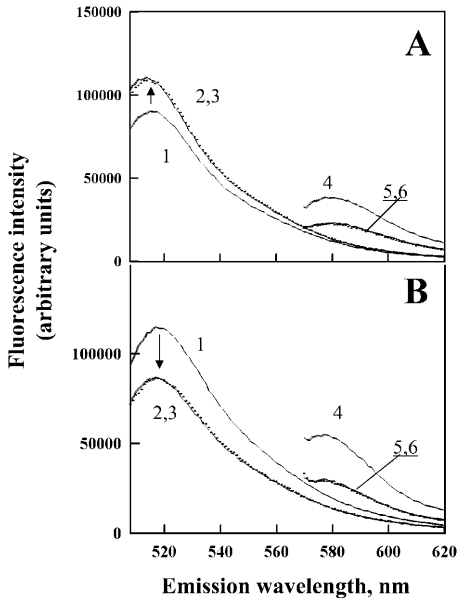


FIGURE 1 The fluorescence emission spectra of the single-strand and duplex oligonucleotides at 10 nM bearing the TATAAAAG (*A*) or TITIIIIG (*B*) sequence labeled with either fluorescein (F14) or TAMRA (T14) under the conditions described in Materials and Methods. Upon excitation at 490 nm: spectrum 1, F14 single-strand; spectrum 2, F14-duplex; and spectrum 3 (·····), F14-duplex and 700 nM TBP. Upon excitation at 555 nm: spectrum 4, T14 single-strand; spectrum 5, T14 duplex; and spectrum 6 (·····), T14 duplex + 700 nm TBP. The arrows indicate the direction of the fluorescence change of F14 upon duplex formation.

analyses of FRET efficiency. The perfect match of this difference spectrum (3) with the normalized spectrum of TAMRA-labeled duplex (5) demonstrates the absence of interactions except FRET between the donor and acceptor within the duplex (Fig. 2). Thus, measurement of the

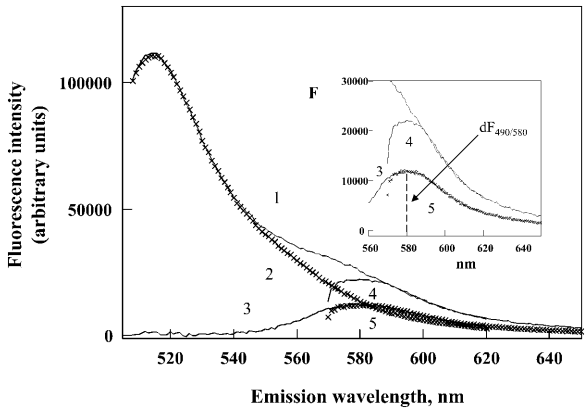


FIGURE 2 The fluorescence emission spectra obtained after excitation at 490 nm of duplexes bearing the TATAAAAG sequence double-labeled with fluorescein and TAMRA. Spectrum 1, double-labeled duplex. Spectrum 2 (××××××), F14-duplex (see *spectrum 2*, Fig. 1) normalized to spectrum 1 at 520 nm. Spectrum 3, difference spectrum of 1 − 2. Spectrum 4, T14-duplex (see *spectrum 5*, Fig. 1). Spectrum 5 (××××××); spectrum 4 normalized to 3 at 580 nm. (*Inset*) Spectra 3, 4, and 5 shown with higher resolution. The parameter $dF_{490/580}$ is fluorescence intensity of TAMRA at excitation 490 nm and designated as $F_{490}(\Sigma)$ in Methods.

fluorescence of the double-labeled duplex DNA at 580 nm provides a measure of the FRET efficiency, E, with this pair of donors and acceptors.

The procedure used in this study involves the annealing of singly-labeled oligonucleotides to make a doubly-labeled duplex. A titration is used to establish a proper ratio of annealed donor/acceptor-labeled oligonucleotides. The equilibrium between oligonucleotides bearing a donor (D) and an acceptor (A) existing in the solution along titration is shown in Scheme 1. Fig. 3 shows the results of a titration of the fluorescein-labeled oligonucleotide (F14) with the T14 complement over the concentration range that spans zones I–IV (Scheme 1) in which TAMRA emission at 580 nm was monitored after excitation at either 490 or 555 nm. Excitation at 490 nm results in FRET and intrinsic TAMRA fluorescence ($dF_{490/580}$) whereas excitation at 555 nm results in only TAMRA emission. However, $dF_{490/580}$ is not a unique measure of E during the course of a titration since it includes contributions from both FRET and the increasing concentration of TAMRA. Titration of fluorescein-labeled single-strand oligonucleotide with the TAMRA-labeled complement is performed to extract the FRET component from $dF_{490/580}$ (Fig. 3).

In Fig. 3 *A*, line 1 depicts the TAMRA fluorescence change upon excitation of 490 nm (*zone II* in Scheme 1) reflecting changes due to both FRET and the direct excitation of TAMRA within the duplex. Line 2 depicts the TAMRA fluorescence change due to increasing concentration of the free acceptor strand (*zone IV* in Scheme 1). The stoichiometric T14/F14 duplex (*zone III* in Scheme 1) occurs at the intersection of lines 1 and 2. Fig. 3 *B* depicts the TAMRA fluorescence change due as duplex (line 3) or mixture of duplex with single-strand (line 4) upon direct excitation at 555 nm, conditions under which FRET does not contribute to the observed fluorescence. As above, the intersection of these two lines indicates the formation of the stoichiometric T14/F14 duplex.

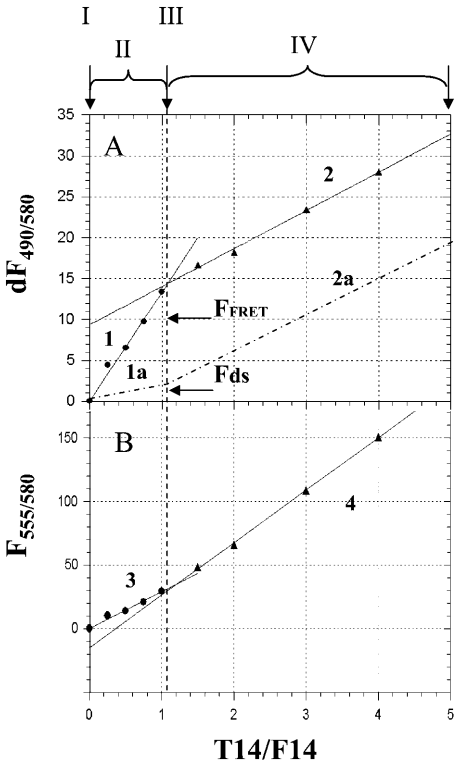


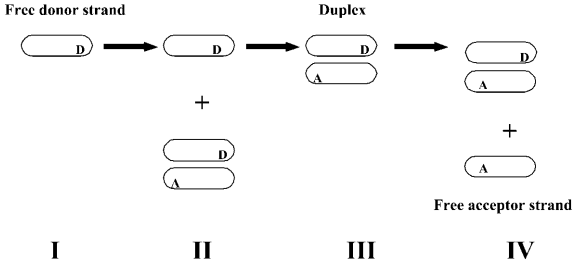
FIGURE 3 Titration of the single-strand oligonucleotide bearing the TATAAAAG sequence labeled with fluorescein (F14) by its complementary single-strand oligonucleotide labeled with TAMRA (T14). (*A*) $dF_{490/580}$ (see Fig. 2 and text) indicates 490-nm excitation and 580-nm emission wavelengths. (*B*) $F_{555/580}$ indicates 555-nm excitation and 580-nm emission. $F_{555/580}$ is a change in fluorescence of TAMRA due to direct excitation that does not depend on FRET (see text for details). Lines 1 and 3 depict a change in fluorescence intensity for the double-strand probe whereas lines 2 and 4 depict the change for the labeled single-strand DNA in presence of constant concentration of the duplex. Dashed lines 1a and 2a are the same as 1 and 2, assuming the absence of FRET. F_{FRET} and F_{ds} are the portions of fluorescence caused by FRET, and by direct excitation of fluorescence of the acceptor within the duplex, respectively. Concentration of F14 was 10 nM in all samples. T14/F14 is the molar ratio of T14 to F14. I, II, III, and IV correspond to the zones shown in Scheme 1 in the text.

Determination of the relative portion of TAMRA fluorescence due to FRET

The titration data shown in Fig. 3 provides an approach by which the fluorescence energy transfer, E, can be determined with high accuracy from the changes in the fluorescence intensity as a function of T14 concentration over the zones depicted in Scheme 1. At the intersection of lines 1 and 2 in Fig. 3 *A*, where the donor and acceptor are equimolar, the observed fluorescence upon excitation at 490 nm ($F_{490}(\Sigma)$) is composed of contributions from FRET (F_{FRET}) and direct TAMRA excitation within the duplex ($F_{490}(ds)$) which can be written as $F_{490}(\Sigma) = F_{\mathrm{FRET}} + F_{490}(ds)$. Thus, the relative portion of TAMRA fluorescence due to FRET can be written as

$$R_{\mathrm{ET}} \equiv \frac{F_{\mathrm{FRET}}}{F_{490}(\Sigma)} = 1 - \frac{F_{490}(ds)}{F_{490}(\Sigma)}. \qquad (4)$$

To determine R_{ET}, the values of F_{FRET} and $F_{490}(ds)$ must be extracted from data such as shown in Fig. 3. This is done by separately considering the fluorescence below the equivalence point of the titration (i.e., the intersection of lines 1 and 2, and of lines 3 and 4), where F14 is in excess of duplex, and above the equivalence point where T14 is in excess of duplex. Transformation of Eq. 4 yields



SCHEME 1

$$R_{\mathrm{ET}} = 1 - \frac{S_2 \times S_3}{S_1 \times S_4}, \qquad (5)$$

where S_1, S_2, S_3, and S_4 are the slopes of lines 1–4, respectively, in Fig. 3. (The derivation of Eq. 5 is presented in Online Supplement 1.) It is critical that FRET studies be conducted under conditions such that the donor and acceptor are either precisely equimolar or that the donor is in excess.

It can be shown (see Online Supplement 2) that R_{ET} is simply the efficiency of energy transfer (E) with the proportionality coefficient, F_{abs}, specific for a donor-acceptor pair,

$$E = R_{\mathrm{ET}} \times F_{\mathrm{abs}}. \qquad (6)$$

Thus, R_{ET} can be used instead of E in FRET analysis of relative structural changes of macromolecules providing experimental advantages due to the high accuracy with which R_{ET} can be measured and absence of a requirement for absorption measurements.

Measurement of FRET in TBP-DNA binding titrations

Titrations such as shown in Fig. 3 were conducted for each set of individually single-labeled and the double-labeled duplex stock solutions prepared for protein binding studies. The concentration at which the labeled complementary oligonucleotides was equimolar, the value of R_{ET} for protein-free DNA, and the ratio S_2/S_4 required for the determination of R_{ET} for TBP-bound DNA were determined from that titrations. A ratio of 0.95 of TAMRA to fluorescein-labeled single-strand oligonucleotide was used to anneal double-labeled duplexes in TBP-binding experiments to insure that excess TAMRA-labeled single-strand DNA was not present. All of the data required for the calculation of R_{ET} is obtained for each concentration of TBP at pairs of excitation/emission wavelengths of 490/520, 490/580, and 555/580 nm. R_{ET} was calculated (Eq. 5) from the change in TAMRA fluorescence accompanying TBP binding as a function of TBP concentration and is the parameter used to measure TBP binding. (The details of this procedure are presented in Online Supplement 3.)

TBP-DNA titrations

S. cerevisiae TBP is monomeric under the experimental conditions of this study (Daugherty et al., 1999, 2000) and a single monomer binds to a TATA Box sequence (Khrapunov et al., 2002; Parkhurst et al., 1996, 1999; Petri et al., 1995, 1998). For the equilibrium $P_f + O_f \leftrightarrow PO$, P_f and O_f are the concentrations of free TBP and DNA, respectively, and PO is the concentration of the TBP-DNA complex. The concentration of PO is $K_a \times P_f \times O_f = K_a(P_{tot} - PO) \times (O_{tot} - PO)$, where P_{tot} and O_{tot} are the total concentrations of protein and DNA, respectively, and K_a is the equilibrium association constant. Since the concentration of the fluorescently-labeled oligonucleotide probe is sufficiently high relative to the binding affinity of the complex, the simplifying assumption $[ligand]_{total} \approx [ligand]_{free}$ cannot be used in the analysis of the titrations of the present study.

The fractional saturation of the DNA binding site, $\bar{Y}$, is PO/O_{tot}, which after substitution and transformation yields

$$\bar{Y}^2 \times K_a \times O_{tot} - \bar{Y} \times (1 + K_a \times (O_{tot} + P_{tot})) + K_a \times P_{tot} = 0. \quad (7)$$

Since R_{ET} is proportional to the concentrations of reactants and the product, the change in R_{ET} is a function of TBP binding to DNA, as

$$\bar{Y} = dR_{ET}/dR_{ET}^{max}, \quad (8)$$

in which $dR_{ET} = R_{ET} - R_{ET}^0$ and $dR_{ET}^{max} = R_{ET}^{max} - R_{ET}^0 \cdot R_{ET}^0$ and R_{ET}^{max} are the R_{ET} values measured for the free DNA and TBP-saturated DNA, respectively. Substituting Eq. 7 into Eq. 8 and relating $K_a = 1/K_d$ yields

$$(dR_{ET})^2 \times O_{tot} - (dR_{ET}) \times (dR_{ET}^{max}) \times (K_d + O_{tot} + P_{tot}) + (dR_{ET}^{max})^2 \times P_{tot} = 0. \quad (9)$$

Nonlinear least-squares fitting is used to determine dR_{ET}^{max} and K_d. Equation 7 reduces to

$$(\bar{Y})^2 \times O_{tot} - \bar{Y} \times (K_d + O_{tot} + P_{tot}) + P_{tot} = 0, \quad (10)$$

after scaling of the data to the fitted value of dR_{ET}^{max}. A similar equation can be derived for the Hill equation, $nP_f + O_f \leftrightarrow PO_n$,

$$(\bar{Y})^2 \times O_{tot} - \bar{Y} \times ((K_d)^{nH} + O_{tot} + (P_{tot})^{nH}) + (P_{tot})^{nH} = 0, \quad (11)$$

where n_H is the Hill coefficient (Hill, 1910). Equation 11 provides a phenomenological test of single-site binding since Eq. 11 reduces to Eq. 10 when $n_H = 1$. All of the titrations reported in this study are best fit by $n_H = 1.0 \pm 0.1$, consistent with the single-site binding model (analysis not shown).

RESULTS

The structures of TBP complexes with TATAAAAG and TITIIIIG

R_{ET} for TAMRA fluorescence was separately determined using Eq. 5 for the adenosine- and inosine-containing probes from a series of titrations of T14 against F14 (Fig. 3, for example). Upon titration of the two double-labeled duplexes by TBP, the emission spectrum upon excitation at 490 nm changes due to FRET (Fig. 4, *insert*; Parkhurst et al., 1996). In contrast, neither the fluorescein fluorescence at 520 nm nor the TAMRA fluorescence at 580 nm changes upon titration of the single-labeled duplexes with TBP (Fig. 1).

Titrations of the double-labeled A-AdMLP and I-AdMLP probes with TBP yield R_{ET} as a function of TBP concentration for each sequence (Fig. 4; Table 1). Differences between the two free probes are observed (Table 1). In addition, the maximum value of R_{ET} measured upon the addition of saturating concentrations of TBP (Fig. 4, *dashed lines*) are different for the adenosine- and inosine-containing probes. As discussed below, we conclude these differences

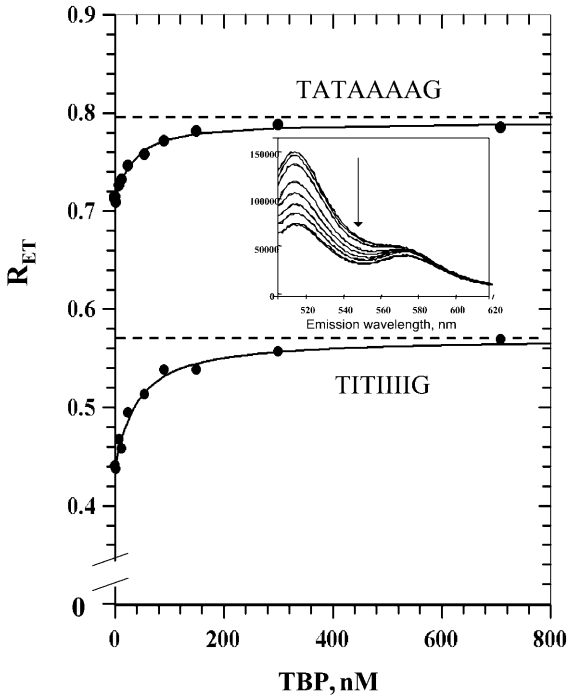


FIGURE 4 Isotherms obtained for the binding of TBP to the duplex double-labeled with fluorescein and TAMRA bearing the sequences TATAAAAG (*upper panel*) or TITIIIIG (*lower panel*). R_{ET} is a relative portion of the acceptor fluorescence due to FRET. The dashed lines denote the maximum values of R_{ET} $(R_{ET})_{max}$ for the two sequences. The solid lines represent the best fits to Eq. 9. (*Inset*) The emission spectra obtained after excitation at 490 nm of a TBP titration of the TATAAAAG-bearing duplex double-labeled with fluorescein and TAMRA. The arrow indicates the consequences of the increasing concentrations of TBP ranging from 0 to 1.8 μM.

TABLE 1 Spectroscopic parameters determined for the 14-bp duplexes bearing the TATAAAAG and TITIIIIG "TATA Box" sequences

Sequence	$\varepsilon_T,555/\varepsilon_F,490$*	$(R_{ET})_{min}$†	$(R_{ET})_{max}$‡	E_f§	E_b§	$K_d(A)/K_d(I)$¶
TATAAAAG	1.35	0.71	0.79	0.44	0.66	0.77
TITIIIIG	2.13	0.44	0.57	0.23	0.42	

*$\varepsilon_T,555/\varepsilon_F,490$ is the ratio of the extinction coefficients of TAMRA (T) and fluorescein (F) at the indicated wavelengths for the double-labeled duplexes.

†$(R_{ET})_{min}$ (calculated from Eq. 5) is the parameter R_{ET} for the free double-labeled duplexes.

‡$(R_{ET})_{max}$ is the parameter R_{ET} for the TBP-bound double-labeled duplexes (see Fig. 4).

§E_f and E_b (calculated from Eq. 6) are the energy transfer efficiencies from fluorescein to TAMRA for the free and TBP-bound double-labeled duplexes, respectively.

¶$K_d(A)/K_d(I)$ is the relative affinity of TBP for two sequences in the experimental buffer free of osmolyte, where $K_d(A)$ and $K_d(I)$ are the dissociation constants of TBP for the TATAAAAG and TITIIIIG sequences, respectively.

are due to differences in the critical distance, R_o, not the end-to-end distances of the two probes.

Many factors influence R_o for a given pair of probes (Eq. 3). We note that upon duplex formation the fluorescein fluorescence increases for A-AdMLP and decreases for I-AdMLP (Fig. 1) and that there are differences in the spectral overlap integral (Table 1, column $\varepsilon_T,555/\varepsilon_F,490$). However, R_o is constant for a given oligonucleotide as a function of TBP binding (Wu et al., 2001a). Therefore the relative protein-induced change of end-to-end distance can be calculated for both oligonucleotides from Eq. 2 using E_f and E_b (Table 1). The relationships between end-to-end distances of DNA free (R_f) and bound to TBP (R_b), $R_f = 1.17 \cdot R_b$ and $R_f = 1.16 \cdot R_b$ for A-AdMLP and I-AdMLP, respectively, are identical within experimental error and in excellent agreement with the results obtained for an A-AdMLP oligonucleotide in which the top strand was double-labeled and annealed with the unlabeled compliment (Parkhurst et al., 1996; Wu et al., 2001a).

A change in either R_o or in R results in a change in the value of E (Eq. 2). Consider that an A-tract, such as that present in the A-AdMLP sequence, induces curvature to the helical axis of DNA (Crothers and Drak, 1992; Davis et al., 1999). An overall helix axis bend of 19° (calculated as the radius of curvature of the DNA) was measured in an NMR solution study of a dodecamer containing a 6-bp A-tract (MacDonald et al., 2001). For a 14-bp oligonucleotide, this angle of curvature yields the length ratio between bent and linear DNA, L_b/L_l of 0.996, assuming a simple isotropic bending rod model of DNA (Wildeson and Murphy, 2000). A 0.4% change in end-to-end length is not detectable by our assay. Since the curvature of I-AdMLP is greater than that of random-sequence DNA but less that of an A-tract of comparable length (Koo and Crothers, 1987; Shatzky-Schwartz et al., 1997), the difference between the end-to-end distances of the A-AdMLP and I-AdMLP probes will be undetectable in our measurements. Thus, differences in the critical distance, R_o, must be responsible for the difference in the measured value of E between the I-AdMLP and A-AdMLP oligonucleotide duplexes (Fig. 4, Table 1).

Comparison of the electrostatic, enthalpic, and entropic components of TBP binding to A-AdMLP and I-AdMLP

To ascertain whether electrostatics differentially contribute to TBP binding the adenosine- and inosine-substituted TATA Box sequences, a series of TBP-binding isotherms were determined as a function of KCl concentration (Fig. 5). This linkage analysis provides an estimate of the number of the counterions released upon TBP binding (Record, Jr. et al., 1998). The slopes of the data shown Fig. 5 for A-AdMLP and I-AdMLP are identical within experimental error, 1.64 ± 0.27 and 1.65 ± 0.35, respectively. These values are less than that previously determined for the A-AdMLP within a long DNA restriction fragment (3.5; Petri et al., 1998). This difference is unsurprising due to documented electrostatic end-effects upon binding to oligonucleotides (Zhang et al., 1999) and the potential for protein-DNA contacts with the DNA surrounding a specific binding sequence (Holbrook et al., 2001).

Partitioning of the enthalpic and entropic contributions to the ΔG° of binding was accomplished using the van't Hoff equation $d\ln K/d\ln(1/T) = \Delta H^\circ/R$ for TBP-binding isotherms obtained as a function of temperature at 100 mM KCl (Fig. 6). The observed standard enthalpy changes, ΔH°, determined for the A-AdMLP and I-AdMLP sequences of 19.3 ± 2.9 and 18.5 ± 1.8 kcal M^{-1}, respectively, are identical within experimental error. The linearity of these van't Hoff analysis over this limited range of temperatures is consistent with previous studies conducted using DNA

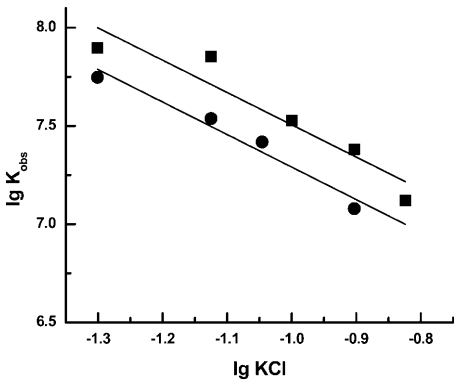


FIGURE 5 The salt dependence of the equilibrium binding of TBP with the double-labeled oligonucleotides bearing TATAAAAG (*squares*) or TITIIIIG (*circles*). K_{obs} is an association constant; concentration of KCl in M.

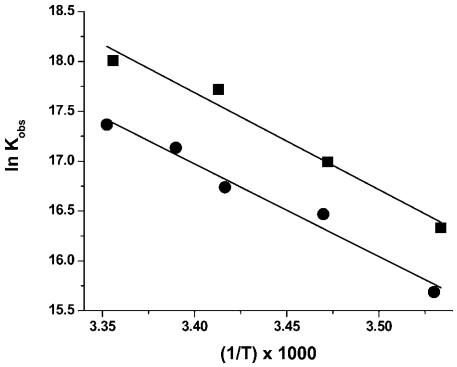


FIGURE 6 A van't Hoff analysis of the binding of TBP with the double-labeled oligonucleotides bearing TATAAAAG (*squares*) or TITIIIIG (*circles*). K_{obs} is an association constant; T is absolute temperature.

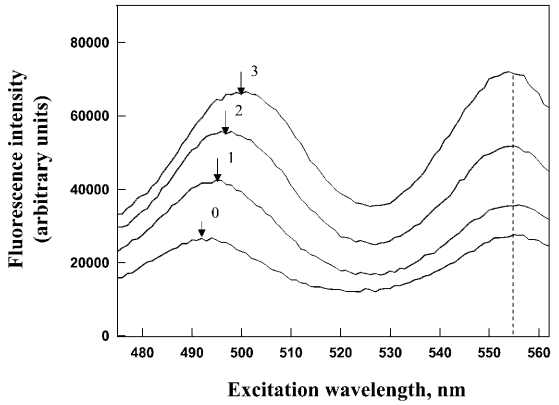


FIGURE 7 The fluorescence excitation spectra obtained by monitoring emission 580 nm for the duplex double-labeled with fluorescein and TAMRA bearing the TATAAAAG sequence as a function of the molar concentration of triethylene glycol (TEG). The arrows indicate the shift in the λ_{max} of donor (fluorescein) excitation at the indicated molar concentration of TEG. The dashed line indicates the constant λ_{max} of acceptor (TAMRA) excitation.

restriction fragments (Petri et al., 1995, 1998) or oligonucleotides (Parkhurst et al., 1999).

The similarity of the salt and temperature dependences of TBP binding of the A-AdMLP and I-AdMLP TATA Box sequences suggests similar electrostatic and enthalpic contributions to these association reactions. However, despite the similarity and thermodynamics of these two TBP-DNA complexes, the affinity of TBP for A-AdMLP is higher than that of I-AdMLP (Table 1). This result is, on the surface, surprising due to the greater flexibility of I-AdMLP, a factor considered as critical for recognition and binding (Davis et al., 1999; Qian et al., 2001). To further probe the molecular mechanism of TBP recognition, the role of water activity in TBP binding to the adenosine- and inosine-containing TATA box sequences was compared.

The influence of osmolytes on the optical properties of the labeled oligonucleotides

Although the osmolytes used in the osmotic-stress experiments are considered neutral in an osmotic-stress analysis (Parsegian et al., 1995), preferential interactions (Courtenay et al., 2000; Parsegian et al., 2000; Record Jr., et al., 1998; Tang and Bloomfield, 2002) with osmolytes might perturb the optical properties of the fluorophores probes. The intensity of the excitation spectra of the double-labeled A-AdMLP increases with increasing concentration of TEG, with the wavelength maximum of the short-wavelength peak also increasing (Fig. 7). This result is not surprising, since the characteristics of the medium can affect quantum yield, extinction and/or anisotropy of the chromophores, either directly or indirectly. The short-wavelength excitation peak of Fig. 7 reflects both direct TAMRA excitation and FRET, whereas the long-wavelength peak results from direct TAMRA excitation since emission was monitored at 580 nm.

To distinguish between direct and indirect effects of osmolytes on fluorescence, the fluorescence intensity and anisotropy of the double-labeled duplex were determined (Fig. 8). TAMRA fluorescence intensity increases monotonically with TEG concentration whereas that of fluorescein increases between 0 and 1 molar TEG, then remains constant. TAMRA anisotropy is constant with increasing TEG concentration whereas that of fluorescein increases. Inspection of Fig. 8, together with Fig. 9 showing results obtained

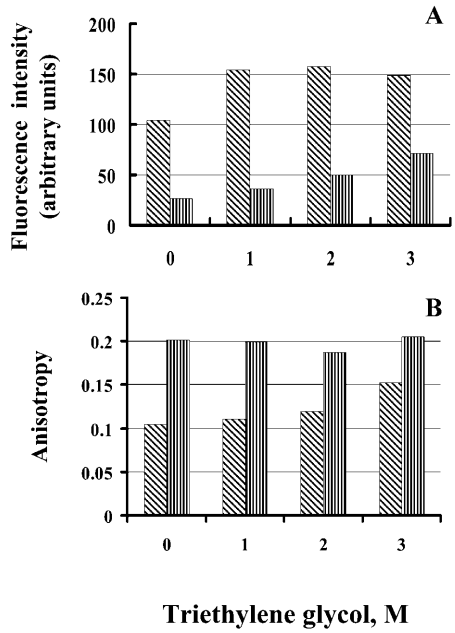


FIGURE 8 Fluorescence intensity (*A*) and anisotropy (*B*) of the duplex double-labeled with fluorescein and TAMRA bearing the TATAAAAG sequence as a function of molar concentration of TEG. The columns with inclined lines denote fluorescein fluorescence measured at 520 nm after excitation at 490 nm. The columns with vertical lines denote TAMRA fluorescence measured at 580 nm after excitation at 555 nm. The labels denote the molar concentration of TEG.

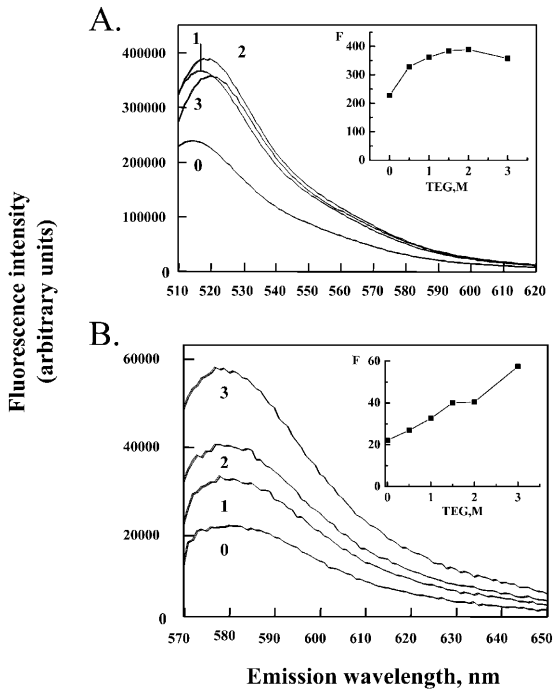


FIGURE 9 (*A*) The fluorescence emission spectra after excitation at 490 nm of the fluorescein-only labeled A-AdMLP duplex as a function of [TEG]. (*Inset*) The fluorescence emission intensity measured at 520 nm as a function of [TEG]. (*B*) The fluorescence emission spectra after excitation at 555 nm of the TAMRA-only labeled A-AdMLP duplex as a function of TEG concentration. (*Inset*) The fluorescence emission intensity measured at 580 nm as a function of [TEG]. The labels for each spectra denote the molar concentration of TEG.

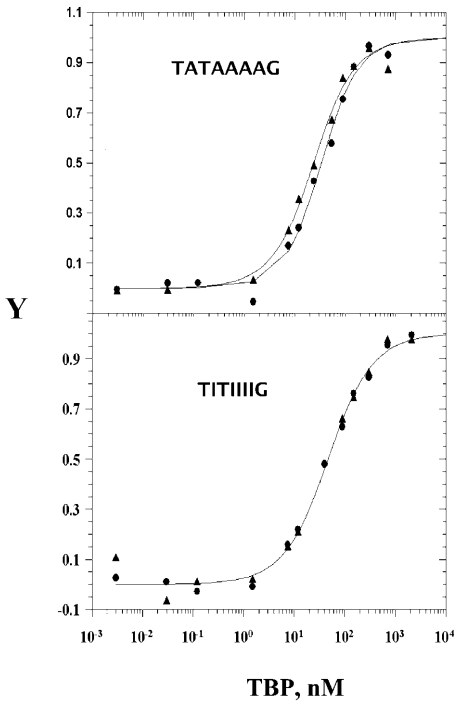


FIGURE 10 Isotherms of TBP binding to the double-labeled oligonucleotides bearing TATAAAAG (*upper panel*, $K_d = 30.8 \pm 3.0$ nM and 21.8 ± 2.7 nM for solutions without osmolytes or with 1 M TEG, respectively) or TITIIIIG (*lower panel*, $K_d = 39.8 \pm 3.9$ nM). Circles denote the standard buffer and triangles denote the standard buffer to which 1 M TEG was added. The solid lines represent the best fit to Eq. 11.

for the single-labeled duplexes, indicates that fluorescein, but not TAMRA, undergoes a change in its interaction with the DNA at high TEG concentration. Similar results were obtained while using I-AdMLP and for methyl glucoside (data not shown). Since the relative orientation and mobility of the dyes used as any donor-acceptor pair are important to the accuracy of the derived donor-acceptor distance by FRET (Eq. 3), concentrations of osmolytes <1 M were used for the analysis of water release from adenosine and inosine TATA box sequences upon the binding of TBP.

Osmotic sensitivity of the TBP-DNA interaction

The inosine for adenosine substitutions change the hydration of the minor groove surface bound by TBP but do not change the chemical surfaces interacting with the protein (Pastor et al., 1999). A comparative approach (Sidorova and Rau, 2001) was used to measure the hydration changes at the TBP-DNA interface for A-AdMLP and I-AdMLP so as to circumvent possible preferential interactions of solutes with TBP. The differential sensitivity of TBP-DNA affinity to osmolytes of different identity allows the discrimination between direct cosolute-macromolecule interactions and osmotic-stress effects (Fried et al., 2002; Parsegian et al., 2000). Therefore we have chosen two neutral solutes with distinct chemical nature: comparatively nonpolar triethylene

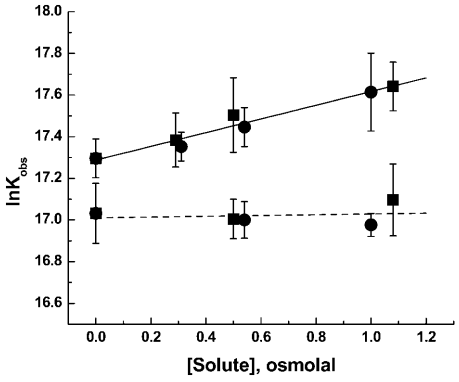


FIGURE 11 The osmotic sensitivity of TBP binding to the double-labeled oligonucleotide bearing TATAAAAG (*solid line*) or TITIIIIG (*dashed line*). TEG (*squares*) or methyl glucoside (*circles*) was added at the indicated concentrations to the standard buffer. K_{obs} is an association constant in the presence of the respective concentration of solutes, respectively. The error bars represent the standard deviation for each value of $\ln(K_{obs})$. The lines represent a least-square fit to Eq. 12. The slopes of the graphs translate into change of 18.3 ± 1.5 and -2.9 ± 1.5 water molecules bound to the two sequences with 95% confidence limits calculated including propagation of the error of the individual measurements.

glycol (TEG) and polar methyl glucoside (MG) to probe changes in hydration during TBP-DNA complex formation. TBP affinity to the natural promoter sequence TATAAAAG is enhanced by increasing concentrations of both TEG and MG. In contrast, the affinity of TBP for the inosine-substituted sequence TITIIIIG is not increased by the osmolytes (Fig. 10). The binding free energy of TBP for the A-AdMLP sequence increases linearly with the osmolal concentration; in contrast, the TBP affinity for I-AdMLP decreases slightly (Fig. 11).

Since two osmolytes with different chemical structures yield the same results, within experimental error, we conclude that the results presented in Figs. 10 and 11 are predominantly due to osmotic effects with negligible contribution of preferential interactions (see Discussion). Thus, an estimate of the change in the number of solute-excluding water molecules accompanying the binding of TBP to two promoters can be estimated from Fig. 11 by

$$\frac{d\ln(K_{obs})}{d[osmolal]} = -\frac{N_w}{55.6}, \tag{12}$$

where K_{obs} values are the equilibrium association constants in the presence of different concentrations of solutes, [*osmolal*] is the osmolal concentration of the cosolute, 55.6 is the number of moles in 1 kg of water, and N_w is the change in the net number of water molecules (Parsegian et al., 1995). Fig. 11 yields N_w equal to 18.3 ± 1.5 and −2.9 ± 1.5, respectively, for TBP binding to the A-AdMLP and I-AdMLP sequences. Thus, the favorable entropically driven release of water molecules upon binding the natural promoter is absent for binding of the inosine-substituted TATA box sequence.

DISCUSSION

Utilization of the parameter R_{ET}

Two FRET approaches are presently used to study the structure of nucleic acids. A variety of approaches have been developed and published for steady-state FRET (see Cantor and Schimmel, 1980; Clegg, 1992; Lakowicz, 1999, for general discussions). However, uncertainties remain with this approach with regard to issues such as the variability induced by particular experimental conditions and the differences in probe concentration among samples. Steady-state methodology relates the measured average donor-acceptor distance to a change in the structure, curvature, and bending of DNA consistent with the helical geometry of the DNA molecule (Clegg, 1992; Gelfand et al., 1999; Stuhmeier et al., 2000; Toth et al., 1998; Wildeson and Murphy, 2000). Time-resolved methodology exploits the distribution of donor-acceptor distances and has been used to study changes in the DNA structure depending on sequence, extent of hybridization, and protein binding (Klostermeier and Millar, 2001; Parkhurst et al., 1996; 2001–2002; Wu et al., 2001a). The mean donor-acceptor distance and the dispersion of the distribution are extracted from the distribution of donor-acceptor distances accessible to time-resolved studies. The time-resolved method is particularly informative due to its physical connection to DNA dynamics in solution.

An issue general to the quantitative interpretation of both approaches is their dependence upon the critical distance, R_o, which in turn depends on the orientation factor, κ, of a donor-acceptor pair and J, the spectral overlap integral (Eq. 3). Variation in the published values of R_o for the fluorescein-TAMRA donor-acceptor pair reflect the different contributions of these physical parameters (Parkhurst et al., 1996; Stuhmeier et al., 2000, 1997; Toth et al., 1998; Wildeson and Murphy, 2000; Wu et al., 2001a; Yang and Millar, 1996). Although the orientation of fluorescein is generally observed to be random, its behavior depends on the chemistry of linker connecting it to the DNA (Hill and Royer, 1997).

Substitution of inosine for adenine in the interior of an oligonucleotide resulted in opposite changes in the fluorescence of fluorescein attached to the DNA upon hybridization despite the identity of the terminal bases (Fig. 1; see Materials and Methods). The behavior of DNA-linked TAMRA is more problematic, including the observations of high anisotropy (Fig. 6; see also Clegg, 1992; Stuhmeier et al., 2000, 1997) and quenching by adjacent guanines (Edman et al., 1996; Eggeling et al., 1998; Stuhmeier et al., 2000, 1997; Wennmalm et al., 1997). The fluorescence quantum yield of both fluorescein and TAMRA depends on the 5′-basepair to which they are linked (Hill and Royer, 1997; Yang and Millar, 1996) and whether the dye is attached to 3′ or 5′ end of the DNA (unpublished observations). Single molecule studies also reveal influences of DNA sequence on TAMRA fluorescence (Edman et al., 1996; Eggeling et al., 1998; Wennmalm et al., 1997). Thus, only upon careful consideration of R_o (e.g., Klostermeier and Millar, 2001; Parkhurst et al., 2001–2002; Stuhmeier et al., 2000) can reliable structural parameters be extracted from FRET measurements (Stuhmeier et al., 2000, 1997; Wu et al., 2001a; Yang and Millar, 1996).

However, the determination of relative changes in DNA structure requires only the accurate measurement of relative changes in the efficiency, E, of energy migration. The parameter R_{ET} (Eq. 5) is simply E multiplied by a proportional coefficient, F_{abs}, that is specific for a donor-acceptor pair (Eq. 6). The use of the R_{ET} for monitoring equilibrium and structural changes of macromolecules has several advantages. 1), It is a titration approach that directly determines the stoichiometric ratio of the labeled oligonucleotides that comprise the duplex. Since R_{ET} is insensitive to excess donor-labeled oligonucleotide, measurements can be made at acceptor-donor ratios of unity or less (0.85–0.95), avoiding an excess of the acceptor-labeled oligonucleotide in the reaction mixture. 2), Absorption measurements, which may be a source of error due to uncertainty in the extinction coefficient of the tethered dyes, are not required. 3), The

fluorescence intensities at different combinations of wavelengths required for determination of R_{ET} are acquired in the same experiment for one particular sample. 4), Since intensity ratios rather than absolute values are utilized, R_{ET} is insensitive to fluctuations in experimental conditions or probe concentrations. 5), Probe purification after annealing of the single strands and measurement of the concentration of double-labeled duplex is not required. 6), R_{ET} facilitates measurement of relative changes in the conformation of DNA when R_0 is unchanged.

The relative structures of TBP-TATAAAAG and TBP-TITIIIIG complexes

The sequence-specific binding of TBP to DNA is considered a defining example of *indirect readout* in which macromolecular structure and conformational accommodation are key elements of binding. The TBP-DNA interaction displays unusual functional as well as structural characteristics. Its high-equilibrium binding affinity masks unusually slow rates of DNA association and dissociation (Hoopes et al., 1992; Parkhurst et al., 1996; Perez-Howard et al., 1995; Petri et al., 1995) and a complex two-intermediate reaction pathway (Parkhurst et al., 1999; Powell et al., 2001).

DNA deformability is believed to play an important role in protein-DNA interactions (Sivolob and Khrapunov, 1995). The binding and stability of TBP with the extreme bend introduced into the bound DNA by TBP plays an important, if not dominant, role in its ability to facilitate transcription initiation. TBP binds with higher affinity to DNA that is prebent toward the major groove (Parvin et al., 1995), presumably favoring TBP's minor groove interaction. Cyclization kinetics studies of TBP-DNA complexes suggests that the AdMLP TATA box DNA is bent in a direction opposite to the bend induced by TBP and is anisotropically flexible (Davis et al., 1999), although the latter conclusion has been questioned (Pastor and Weinstein, 2000). It is claimed that overall DNA flexibility correlates with transcriptional activity (Qian et al., 2001), although the extent of this correlation seems minimal. In contrast, a strong correlation was reported between the solution bend angles of DNA within the TBP-DNA complex and relative transcription activity (Wu et al., 2001a). However, further study has revealed a complex dependence of TBP-induced bend angle upon the TATA box sequence and osmolyte concentration (Wu et al., 2001b). Thus, comparisons of transcriptional activity with TATA box sequences must consider this myriad array of factors.

The feature of the TIT-for-TAT (Pastor et al., 1999) substitution that stimulated the present experimental analysis is that substitution of inosine for adenine changes the chemical structure of the major but not the minor groove (Saenger, 1984). Long before atomic resolution structures of TBP were solved, substitution of inosine for adenosine was used to establish that TBP recognized and bound the minor groove of DNA; these studies concluded that the interaction of TBP with the sequences CICIIII and TATAAAA were indistinguishable (Starr and Hawley, 1991). Efficient preinitiation complex formation and transcription initiation is supported by inosine:cytosine basepair-substituted TATA box sequences (Lee et al., 1997).

This study demonstrates that the end-to-end distance (R_{bound}/R_{free}) of the A-AdMLP and I-AdMLP DNA complexed to TBP is indistinguishable despite the higher flexibility and lower affinity of the I-tract compared with A-tract (Table 1). The sensitivity of R_{bound}/R_{free} to end-to-end distance of DNA changes is shown in studies of subtle and not-so-subtle DNA bends including those modeled as smoothly bent (MacDonald et al., 2001; Wildeson and Murphy, 2000), single-kinked (Hardwidge et al., 2002), and double-kinked (Wu et al., 2001a). The equivalence of the TBP-induced DNA bend inferred from the I-AdMLP and A-AdMLP data are consistent with the observed correlation between solution bend angles and transcription activity of the TBP-DNA complex (Wu et al., 2001a). Thus, deformability of DNA can be considered as an important (Parkhurst et al., 2001–2002) but not the sole factor leading to larger TBP-induced DNA bend angles. Both structural and thermodynamic features of TBP-DNA complexes modulate the affinity of the protein and DNA for each other (Table 1).

A role for water release in the stability of the TBP-DNA complex

The detailed thermodynamic analysis (Figs. 5 and 6) shows that neither the enthalpic nor electrostatic components of binding contribute noticeably to the differential affinity of TBP for a native TATA sequence compared with an inosine-substituted sequence. The osmotic-stress and preferential-interactions approaches to describing the effect of neutral solutes on biochemical equilibria are extensively discussed in the literature (Courtenay et al., 2000; Parsegian et al., 2000; Tang, 2002; Timasheff, 1998). The central point of the discussion is the unknown distribution of cosolute at macromolecule surfaces as well as in the bulk and local domains of the solution. Record and co-workers have proposed measurement of ''iso-osmolal preferential interaction coefficients'' for each solute (Courtenay et al., 2000; Record, Jr., et al., 1998). Although this approach is attractive, because of its clear physicochemical basis it is difficult to implement. From a practical point of view, the difference between osmotic stress and preferential interactions methodologies reduces to substitution of N_w in Eq.12 with $N_w * (K_p - 1)$ where K_p is the local-bulk partition coefficient for a nonelectrolyte solute (Courtenay et al., 2000; Parsegian et al., 1995, 2000; Tang, 2002; Timasheff, 1998). Both approaches are equivalent when K_p is 0. If K_p is not 0, data such as was shown in Fig. 11 report only the lower limit of N_w.

By conducting our experiments identically except for the oligonucleotide substitution of inosine for adenosine, we have

circumvented this ambiguity since the quantity of interest is the difference in water release between the two binding reactions. Rau and co-workers developed a similar differential method by using a competitive assay to compare DNA sequence-specific and nonspecific protein binding. The amount of water sequestered by two nonspecific protein-DNA complexes—the synthetic polymer poly (dI-dC)·poly (dI-dC) and the oligonucleotide differing from the specific recognition site by only two basepairs—is closely similar (Parsegian et al., 2000; Sidorova and Rau, 1996, 2001). As noted above, the chemical constituents in the minor grooves of the A-AdMLP and I-AdMLP sequences are identical, as are the changes in their end-to-end distances induced by TBP binding. Since the protein is the same in both sets of experiments, the water-accessible surfaces of the separate reactants and the TBP-DNA complex are presumably the same for the A-AdMLP and I-AdMLP TBP-binding reactions.

The minor groove of the DNA within the TBP-DNA complex is completely dehydrated in the co-crystal complexes (Kim et al., 1993a,b). Molecular dynamics simulations have shown that both enthalpic and entropic forces contribute to the stabilization of the TBP-DNA complex (Pastor et al., 2000). The enthalpic penalty associated with the dehydration of the DNA minor groove accompanying TBP binding is compensated for by the increased entropy resulting from water release. Thus, the entropic advantage is predicted to be higher for A-AdMLP relative to I-AdMLP based upon the excess of 19 water molecules bound within the minor groove of A-AdMLP that are displaced upon TBP binding (Pastor et al., 1999). This prediction is consistent with the greater affinity of TBP for A-AdMLP than for I-AdMLP (Fig. 10, Table 1).

Approximately 18 water molecules are released upon binding TBP to the A-AdMLP as compared with slight binding (in the range of experimental error for the associate constant determination) of water to the I-AdMLP sequence (Fig. 11). That difference is in a good agreement with the theoretically predicted excess of 19 water molecules bound in the minor groove of A-AdMLP (Pastor et al., 1999). The difference in amount of water released that accompanies TBP binding to two promoters can be largely attributed to the steric exclusion of water from the interacting surface of the TBP-DNA complex. An excess of water in the minor groove of the TATA box is thermodynamically expensive (Dunitz, 1994) and its release upon TBP binding makes the TATA box energetically preferential. Thus, it appears that despite the structural advantage of the TITIIIIG sequence with regard to its greater flexibility, the binding of TBP to the natural promoter binding is favored by entropic advantage due to the release of water upon complex formation.

SUPPLEMENTARY MATERIAL

Online supplements to this article can be found by visiting BJ Online at http://www.biophysj.org.

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