Reevaluation of Transcriptional Regulation by TATA-Binding Protein Oligomerization: Predominance of Monomers[†]

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ABSTRACT: The TATA-binding protein (TBP) plays an important role in transcriptional initiation by all three nuclear RNA polymerases. TBP contains a conserved C-terminal domain (cTBP) that binds DNA. Crystallographic studies of cTBP (i.e., TBP without the N-terminal domain) from various species and molecular biology studies of cTBP and mixed cTBP/TBP species have led to the view that DNA binding by TBP is regulated by TBP dimerization. Using sedimentation equilibrium, we show that yeast cTBP forms dimers in solution at 5 °C with a dissociation constant of 7 ± 1 μM. This observation of cTBP dimers in solution is in accord with the dimeric state observed in crystal structures of cTBP. In contrast, physiologically relevant, full-length yeast TBP is monomeric at 5 °C and forms dimers at 30 °C with a dissociation constant of 51 ± 16 μM. This dissociation constant precludes formation of stable full-length TBP dimers at physiological concentrations. In addition, we tested for yeast TBP oligomerization in the presence of TBP-associated factors in the context of TFIID. No evidence for TBP oligomers was found using immunoprecipitation techniques from yeast whole-cell extracts. We conclude that yeast TBP is predominantly monomeric under physiological conditions, arguing against a role for TBP dimerization in the regulation of transcriptional initiation.

Initiation of gene transcription on a TATA-containing RNA polymerase II promoter is a complex process, involving a large number of interactions between multiple transcription factors (*1*). The RNA polymerase II general transcription factor TFIID consists of both TBP[1] and TAFs. An initial regulatory step in transcription initiation by polymerase II is the binding of the TBP to DNA, which serves to nucleate preinitiation complex assembly (*1*, *2*), and is proposed to be a major rate-limiting step in RNA polymerase II transcription initiation (*3*−*5*). In addition, TBP is a central component in transcription by RNA polymerases I and III (*1*, *2*). Accurate prediction of transcription patterns and an understanding of the mechanism of transcription initiation will require a description of the factors that regulate TBP.

The DNA-binding region of TBP is located in the conserved 180-residue C-terminal domain (*2*). The crystal structures of yeast, human, and plant TBP C-terminal domains have been solved (*2*, *6*, *7*). In all cases, the TBP C-terminal domain crystallizes as a dimer (Figure 1). However, crystal structures of the TBP C-terminal domain bound to DNA show that the C-terminal domain binds DNA as a monomer (*2*, *6*, *7*). The dimerization interface of the C-terminal domain occludes the DNA-binding site, and so dimerization and DNA-binding by the C-terminal domain are mutually exclusive on structural grounds (Figure 1). Taken together with cross-linking and pull-down assays of solutions containing the TBP C-terminal domain and full-length TBP (*8*−*12*), these results have led to the view that DNA-binding by TBP is regulated by dimerization, and that TBP dissociation is a key rate-limiting step in transcriptional initiation (*8*−*12*).

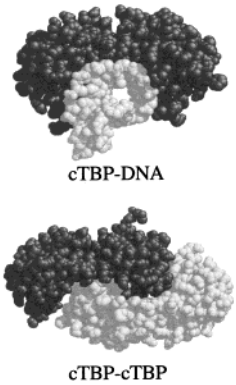

FIGURE 1: Crystal structures of yeast cTBP and the yeast cTBP−DNA complex (*6*, *7*). The dimerization interface of yeast cTBP occludes the DNA-binding surface. The figure was produced with RASMOL (*29*).

We report here the oligomerization properties of cTBP, corresponding to the yeast TBP C-terminal domain, and full-length yeast TBP using sedimentation equilibrium and immunological methods. Sedimentation equilibrium is a thermodynamically rigorous method to characterize protein self-association in solution (*13*). The technique is sensitive to small amounts of incompetent monomer that are unable to oligomerize, which is detected by a systematic increase

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[1] Abbreviations: K_d, dissociation constant; cTBP, C-terminal domain of yeast TBP (residues 49−240); TBP, TATA-binding protein; TAFs, TBP-associated factors.

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in K_d with increasing protein concentration (*14*). In contrast to chemical cross-linking, sedimentation equilibrium will not generate artificially high levels of an oligomer due to a reactive cross-linking reagent. In addition, problems due to selectivity in pull-down assays and anomalous mobility during gel filtration are avoided. Using sedimentation equilibrium we find that, while cTBP forms dimers, dimerization of full-length TBP is thermodynamically unfavorable at physiological concentrations. In addition, immunological methods provide no evidence for dimerization of TBP in the context of TFIID. Our results challenge the notion that TBP dimerization plays an important role in regulating transcription initiation.

EXPERIMENTAL PROCEDURES

Proteins. Full-length yeast TBP was expressed in *Escherichia coli* strain BL21 (DE3) with a pET11a vector (called pYTBP) and purified from the soluble fraction with Q, SP, and Heparin HiTrap columns (Pharmacia) equilibrated in buffer A (10 mM sodium phosphate, 100 mM KCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM DTT, pH adjusted to 7.5). TBP flowed through the Q column and was eluted from the SP and Heparin columns using linear NaCl gradients in buffer A. Final purification of TBP was by gel filtration with Sephacryl S-100 equilibrated in 10 mM sodium phosphate, 250 mM Na_2SO_4, 5 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol, pH adjusted to 7.5. cTBP (residues 49−240) was prepared from purified TBP by trypsin (Sigma T-1426) cleavage of the N-terminal domain (*15*) and further purified with an SP HiTrap column (Pharmacia) using a linear NaCl gradient in buffer A. Purified TBP and cTBP were stored as aliquots at −70 °C and were not freeze−thawed more than once, since it has been reported that repeated freeze−thaw cycles affect significantly the oligomerization state of yeast TBP (*16*). N-terminal sequencing indicated that the N-terminal Met of TBP was processed, as observed for authentic TBP from yeast (*17*). The identities of TBP and cTBP were confirmed with electrospray mass spectrometry, with the expected and observed masses agreeing to within 2 Da.

DNA-Binding Assays. Electrophoretic mobility−shift assays were performed using a ^{32}P-labeled 45-base-pair fragment containing the adenovirus early 1B TATA box as described previously (*18*). TBP (60 pM to 22 nM) and DNA (5.4 nM) were incubated at 25 °C for 30 min in 15 μL of 20 mM Tris (pH 7.5), 40 mM HEPES (pH 7.9), 100 mM KCl, 1 mM DTT, 0.5 mM PMSF, 5 mM $MgCl_2$, 10% glycerol. TBP−DNA complexes were separated from unbound DNA by 6% nondenaturing acrylamide gel electrophoresis and quantified by phosphorimaging.

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed with a Beckman XL-I and An60-Ti rotor. Data were collected using 12 mm path length six-sector centerpieces at wavelengths of 276 and 280 nm for cTBP and TBP, respectively. Equilibrium was judged to be reached when scans collected 3 h apart were indistinguishable, and was generally attained within 12 h. Solvent densities of 1.060 g mL^{-1} at 5 °C and 1.056 g mL^{-1} at 30 °C and partial molar volumes of 0.754 mL g^{-1} for cTBP and 0.746 mL g^{-1} for TBP were calculated as described elsewhere (*19*).

Data were analyzed with Origin (*20*) and fit to ideal single-species, monomer−dimer, monomer−trimer, monomer−tetramer, and monomer−octamer models. The mathematical expression used by Origin is described in detail elsewhere (*20*, *21*). Discrimination between the different models was based on the distribution of residuals and the variance, which approaches 1 as the fit improves (*20*). Parameters that were allowed to vary during fits to the ideal single-species model were molecular mass and baseline offset, unless stated otherwise. Parameters that were allowed to vary during fits to the self-associating models were the log of the dissociation constant and the baseline offset, with the molecular mass held constant at the known monomeric value (21 279.9 Da for cTBP and 26 871.7 Da for TBP). In all fits the second virial coefficient was held constant at zero, and terms for oligomers not considered in the fit were made insignificant by constraining the association constant to 10^{-20} (*21*).

Samples were dialyzed at 4 °C against the reference buffer (10 mM sodium phosphate, 250 mM Na_2SO_4, 5 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol, pH adjusted to 7.5, unless stated otherwise) for at least 12 h. For cTBP, data were collected at 5 °C using multiple cTBP loading concentrations spanning 6−60 μM, rotor speeds of 20, 30, and 40 krpm, and cTBP from three independent preparations. For TBP, data sets were collected at 5 and 30 °C using multiple TBP loading concentrations spanning 6−60 μM, and rotor speeds of 30, 35, and 40 krpm, and TBP from four independent preparations.

Monomer−dimer association constants ($1/K_d$) were converted from values obtained from the fits in absorbance units (K_{abs}) to molar values (K_{conc}) using $K_{conc} = K_{abs}\epsilon l/2$, where ϵ is the extinction coefficient and l is path length (*21*). Reported errors in K_d values were derived from the standard deviation of the mean K_d value obtained from the fits and the error in the extinction coefficient. Extinction coefficients for cTBP and TBP were experimentally determined in 10 mM sodium phosphate, 250 mM Na_2SO_4, 5 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol, pH adjusted to 7.5, from a linear fit of the change in absorbance with protein concentration to the Beer−Lambert equation. Measurements were made three times to obtain extinction coefficients for cTBP at 276 nm of 10 474 ± 3% M^{-1} cm^{-1} and for TBP at 280 nm of 13058 ± 3% M^{-1} cm^{-1}. Concentrations of the stock solutions were determined by absorbance in 6 M GuHCl, 10 mM sodium phosphate, pH adjusted to 6.5, using extinction coefficients for cTBP at 276 nm of 8700 M^{-1} cm^{-1} and for TBP at 280 nm of 13 370 M^{-1} cm^{-1} (*22*).

Analytical Gel Filtration. Apparent molecular masses were determined at room temperature (approximately 20 °C) by gel filtration with a TosoHaas G2000SW_{XL} column equilibrated in 10 mM sodium phosphate, 250 mM Na_2SO_4, 5 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol, pH adjusted to 7.5. A flow rate of 0.5 mL min^{-1} was used. The column was calibrated with lysozyme, chymotrypsinogen A, ovalbumin, albumin, and blue dextran.

Immunoprecipitations. Yeast stains expressing TBP and coexpressing TBP and mycTBP were generated from yeast strain BYΔ2 as described previously (*23*). The mycTBP protein contains an N-terminal triple myc tag (GEQKLISEEDLNx3). Preparation of whole-cell extracts, immunoprecipitations, and immunoblotting were performed as described previously (*24*) except that 422 μg of extract was

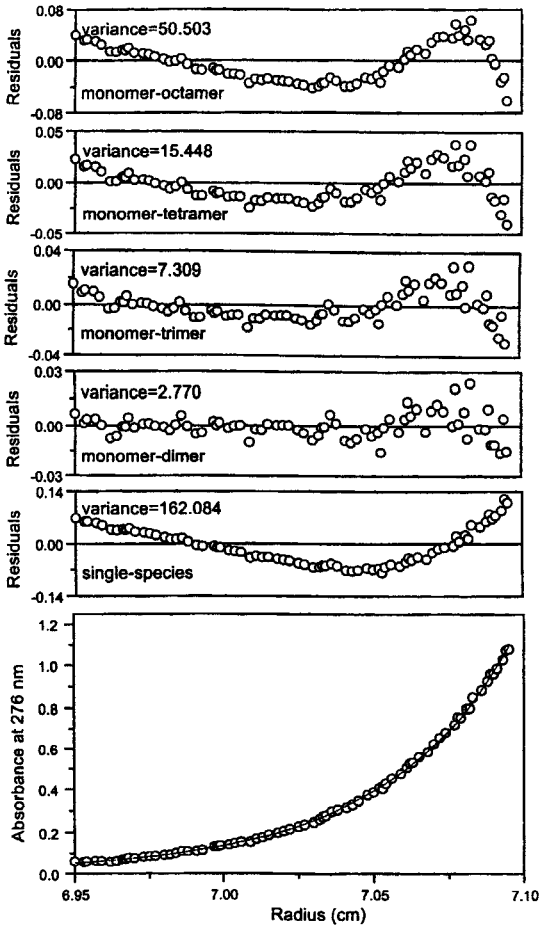

FIGURE 2: Sedimentation equilibrium indicates that cTBP exists in a monomer−dimer equilibrium at 5 °C. Results of the fits of sedimentation equilibrium data for cTBP at 5 °C from a single channel to single-species monomer, monomer−dimer, monomer−trimer, monomer−tetramer, and monomer−octamer models are shown. The residuals are random for the monomer−dimer fit, and exhibit systematic deviations for the other fits. The variance is closest to 1 for the monomer−dimer fit. The residuals for the single-species monomer are indicative of a self-associating system (*21*).

used with monoclonal antibodies generated to the myc tag (Mab 9E10). Complexes were harvested and analyzed by either 10% (TBP and TAF68) or 7.5% (TAF130) SDS−PAGE, electroblotted to nitrocellulose, and probed with polyclonal antibodies specific to TBP, TAF68, or TAF130.

RESULTS

Sedimentation equilibrium data for cTBP were collected at 5 °C using cTBP from three independent preparations. Twenty-five data sets were fit individually to an ideal, single-species model in which the molecular mass was allowed to vary. In each case, the apparent molecular mass was higher than expected for a monomer but less than expected for a dimer, indicative of a self-associating species (*21*). Therefore, each data set was fit with the molecular mass held constant at the known monomeric value to single-species, monomer−dimer, monomer−trimer, monomer−tetramer, and monomer−octamer models. In every case the data were fit best by a monomer−dimer model, as indicated by the random distribution of residuals and variances closest to 1 (Figure 2). Equivalent results were obtained for cTBP that was either never frozen or freeze−thawed once.

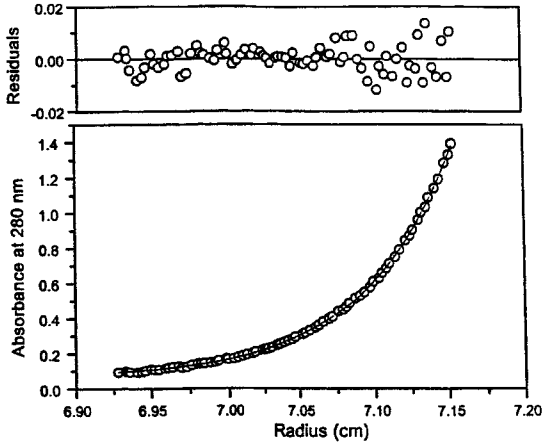

FIGURE 3: Sedimentation equilibrium indicates that TBP is monomeric at 5 °C. The data fit a single-species model, as indicated by the random distribution of residuals, with a molecular weight expected for a monomer.

The mean K_d values for the cTBP monomer−dimer equilibrium obtained from fits of the single data sets is 7 ± 1 μM. Global fits of data collected for each of the three cTBP preparations yielded a mean K_d of 7.9 ± 1 μM. The value of the monomer−dimer K_d for cTBP did not vary systematically in the 25 independent data sets collected over a 10-fold range of total cTBP concentration and at multiple rotor speeds, indicating that cTBP was essentially fully active for dimerization (*14*). These results indicate that cTBP in solution exists in a monomer−dimer equilibrium at 5 °C at low micromolar concentrations, in accord with the dimeric state of cTBP observed in the crystal structure (Figure 1).

In contrast to the behavior observed for cTBP, sedimentation equilibrium measurements indicate that full-length yeast TBP is monomeric at 5 °C. TBP from four independent preparations was used, and 16 data sets were fit to an ideal single-species model in which the molecular mass was allowed to vary. In each case, the residuals were random (Figure 3), indicating that TBP behaves as a single ideal species at 5 °C. The average molecular mass of TBP from 16 independent measurements is 27.52 ± 0.79 kDa, which is within experimental error of the expected monomer mass (26.87 kDa). The apparent molecular mass of TBP did not increase systematically with total TBP concentration in the range 6−60 μM, indicating that TBP does not oligomerize significantly at 5 °C (*25*). Thus, at 5 °C, TBP does not form dimers or higher-order oligomers at micromolar concentrations.

Sedimentation equilibrium studies of full-length TBP from four independent preparations were also performed at 30 °C (a standard yeast-culturing temperature). Twenty-one data sets were first fit individually to an ideal single-species model in which the molecular mass was allowed to vary. In each case, the apparent molecular mass was higher than expected for a monomer but less than expected for a dimer, indicative of a self-associating species (*21*). The data were therefore fit to ideal single−species, monomer−dimer, monomer−trimer, monomer−tetramer, and monomer−octamer models in which the molecular mass was held constant at the known value for TBP.

Seventeen data sets exhibited a concentration change of >0.2 absorbance unit across the cell, which is necessary for a reliable discrimination between different models (*25*). All

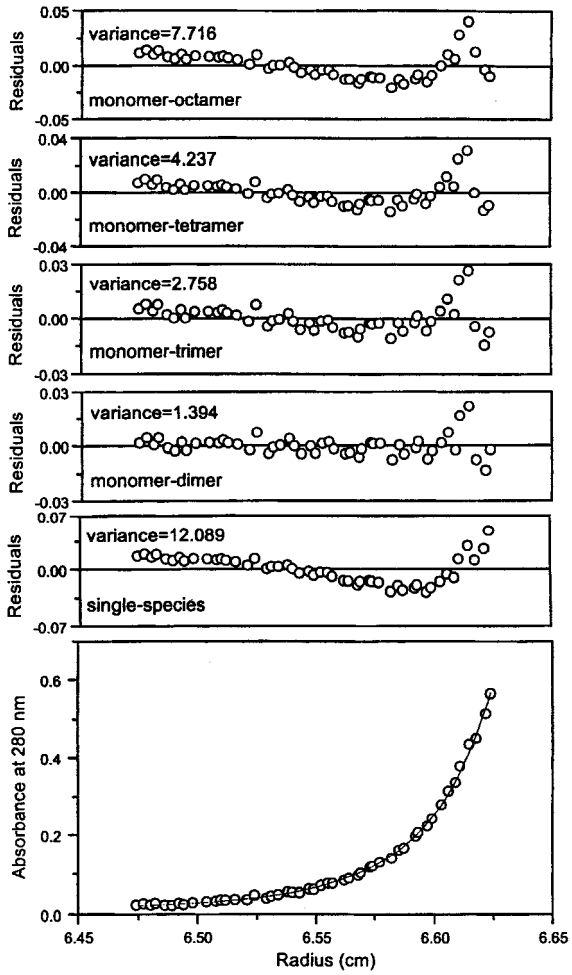

FIGURE 4: Sedimentation equilibrium indicates that TBP exists in a monomer−dimer equilibrium at 30 °C. Results of the fits of sedimentation equilibrium data for TBP at 30 °C to single-species monomer, monomer−dimer, monomer−trimer, monomer−tetramer, and monomer−octamer models are shown. The residuals are random for the monomer−dimer fit, and exhibit systematic deviations for the other fits. The variance is closest to 1 for the monomer−dimer fit. The residuals for the single species monomer are indicative of an associating system (*21*).

of these data sets were accounted for best by a monomer−dimer model, as indicated by a random distribution of residuals and variances closest to 1 (Figure 4). The four data sets collected at the lowest total concentration (6−15 μM) spanned only a limited concentration range across the cell (approximately 0.1 absorbance unit) and exhibited limited curvature. These data sets could also be accounted for by a monomer−dimer model. Equivalent results were obtained for TBP that was either never frozen or freeze−thawed once.

The mean of the monomer−dimer K_d determinations from individual fits for TBP at 30 °C is 51 ± 16 μM. The K_d did not vary systematically over a 10-fold range of total TBP concentration and at multiple rotor speeds (Figure 5), indicating that the TBP was essentially fully active for dimerization (*14*). Global fits of data collected for each of the four TBP preparations yielded a mean K_d of 63 ± 16 μM.

A preponderance of TBP monomers was also observed under conditions of different ionic strength (20 mM HEPES, 120 mM KCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, pH adjusted to 7.5, and 10 mM sodium phosphate, 150 mM Na_2SO_4, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, pH adjusted to 7.5). In addition, the functional activity of TBP was not altered during sedimentation equilibrium, since the TBP was equally active for DNA-binding before and after sedimentation equilibrium (Figure 6).

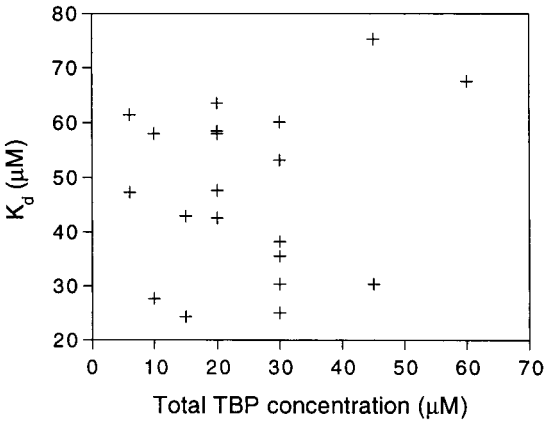

FIGURE 5: The K_d for the TBP monomer−dimer equilibrium obtained from the single-channel fits does not vary systematically with total TBP concentration.

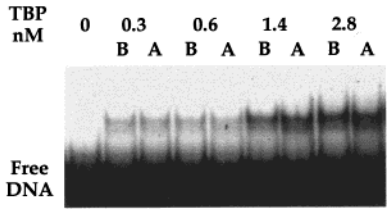

FIGURE 6: TBP exhibited no detectable difference in binding the adenovirus early 1B TATA element (5.4 nM) before (B) or after (A) ultracentrifugation at 30 °C. The intensities of the TBP−DNA bands at a particular TBP concentration are the same to within 3%.

A previous sedimentation equilibrium study reported that yeast TBP forms tetramers and octamers (*26*). The study was performed at total TBP concentrations of 9 and 15 μM and at two rotor speeds (16 and 24 krpm) (*26*). The final pH of the buffer used in the study (*26*) was not reported, and so we repeated the experiments using the same buffer components (20 mM HEPES, 120 mM KCl, 1 mM DTT, 1 mM EDTA) with the pH adjusted to 7.5. The TBP aggregated visibly in this buffer in two independent attempts. The experiments were not pursued further, since others have reported that TBP is rapidly inactivated in the buffer where tetramers and octamers were observed (*12*), and the physiological relevance of residual, soluble TBP under aggregating conditions is questionable. Inclusion of 10% glycerol in the buffer alleviated the aggregation and gave results that fit a monomer−dimer equilibrium, as described above.

The apparent molecular mass of TBP was also estimated with gel filtration. A single peak was observed, with an apparent molecular mass of 31.6 kDa (Figure 7). This mass is within 18% of the known monomeric mass of TBP. The gel filtration results are consistent with the conclusions from the sedimentation equilibrium analysis that TBP is predominantly monomeric at micromolar concentrations.

TBP is associated in vivo with TAFs to form the general transcription factor TFIID (*2*). To test whether the presence of TAFs could favor TBP dimerization, immunological methods were used to evaluate whether TBP dimerizes in the context of TFIID. Since TBP monomers and oligomers

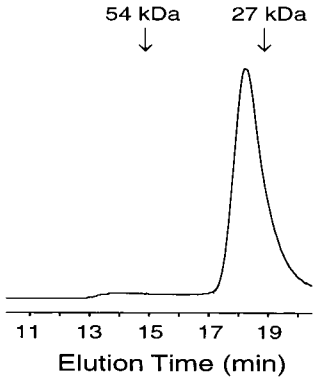

FIGURE 7: TBP elutes during gel filtration as a single peak with an apparent molecular mass of 31.6 kDa. Expected elution times for monomeric and dimeric TBP are indicated.

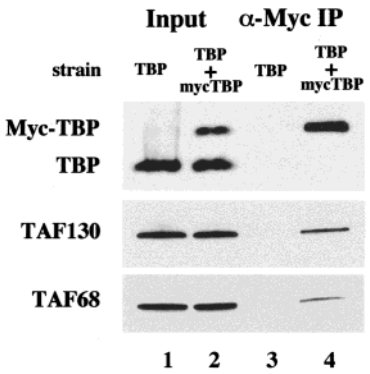

FIGURE 8: Immunoprecipitation assays indicate that TBP is not dimeric in yeast. Whole-cell extracts were prepared from two different strains, one that expresses only TBP (labeled TBP; lanes 1 and 3) and one that expresses both TBP and mycTBP (labeled TBP+mycTBP; lanes 2 and 4). The extract starting material is indicated (labeled Input; lanes 1 and 2). Extracts were immunoprecipitated using myc antibodies (denoted α-Myc IP, lanes 3 and 4), and probed with polyclonal antibodies specific to TBP, TAF68, or TAF130. As expected, immunoprecipitation with anti-myc antibodies of extracts derived from the strain expressing only TBP did not result in isolation of TBP or TFIID (lane 3). Immunoprecipitation of extracts derived from strains coexpressing TBP and mycTBP resulted in isolation of only mycTBP and TAFs and not TBP (lane 4).

cannot be distinguished by immunoprecipitation or immunodetection, TBP was tagged with a myc epitope (mycTBP) to allow isolation and detection of putative mycTBP/TBP heterodimers.

TBP and mycTBP were coexpressed in yeast, and the presence of mycTBP/TBP heterodimers was probed by immunoprecipitation of mycTBP followed by immunoblotting with anti-TBP antibodies. If TBP dimerizes in the context of TFIID, then immunoprecipitation of mycTBP should result in coimmunoprecipitation of TBP. We found that TBP was not present in the mycTBP immunoprecipitations, indicating that TBP does not oligomerize with mycTBP (Figure 8). Dilution experiments of the extract containing TBP and mycTBP indicate that 2% dimer formation could have been detected (data not shown). Moreover, TFIID formation is not disrupted by immunopreciptation or the myc tag, since TAF130 and TAF68 are present in the immunoprecipitate (Figure 8). Furthermore, the myc tag does not interfere with TBP functions since yeast strains in which TBP or mycTBP were the sole source of TBP were identical for growth at 30 and 37 °C, on alternative

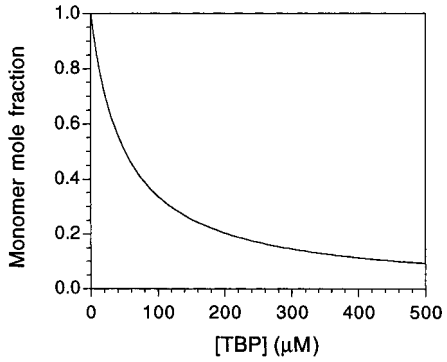

FIGURE 9: Change in mole fraction of monomeric TBP with total TBP concentration. The fraction monomer was calculated for a monomer−dimer equilibrium with a K_d of 51 μM.

carbon sources (raffinose and galactose), in high levels of copper (500 μM) and on aminotriazole (15 mM). Thus, TBP does not self-associate in the context of TFIID.

DISCUSSION

The C-terminal domains of TBP from several species, including yeast, are dimeric in crystal structures (*2*, *6*). Comparison of the free and DNA-bound yeast cTBP crystal structures shows that the TBP dimerization interface occludes the DNA-binding surface (Figure 1). This structural feature raises the possibility that TBP is regulated by dimerization, and that slow dissociation of dimers provides a regulatory step in DNA-binding by TBP and transcription initiation. Indeed, several experimental studies have been interpreted on the basis that TBP is regulated by dimerization (*8*−*12*).

In accord with the dimeric state of yeast cTBP observed in the crystal structure (*6*), we find that, in solution, cTBP forms dimers at 5 °C with a K_d of 7 ± 1 μM. In marked contrast to the dimerization properties of cTBP at 5 °C, the physiologically relevant, full-length TBP is monomeric at micromolar concentrations. Even at 30 °C, TBP dimerization is thermodynamically unfavorable at physiological concentrations. Since the mean K_d for TBP dimerization is 51 μM at 30 °C, and the nuclear concentration is estimated to be 1−6 μM (*9*, *27*), TBP will be predominantly monomeric at physiological concentrations (Figure 9). Moreover, TBP dimers were not observed in immunoprecipitates in the context of TFIID, suggesting that the TAFs or other proteins present in vivo do not promote TBP or TFIID oligomerization.

Our results challenge the model for a regulatory role for TBP dimerization in transcription initiation. Our results also demonstrate that TBP dimerization is unfavored by the presence of the N-terminal domain. Since the dimerization interface of cTBP coincides with the DNA-binding surface (Figure 1), and the N-terminal domain interferes with TBP dimerization, it is possible that the N-terminal domain occludes the dimerization interface of TBP in solution. Indeed, removal of the N-terminal domain of yeast TBP results in faster binding to DNA and a more stable TBP−DNA complex (*15*, *28*). It is an intriguing possibility that any transcription factor or TBP mutation capable of disrupting the interaction between the N- and C-terminal domains will result in potentially elevated levels of transcription.

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