The herpes simplex virus virion protein VP16 is a potent transcriptional activator that specifically activates viral immediate early gene expression (1, 2). As a transcriptional regulatory protein, it contains two functional domains. The amino-terminal portion of the protein, in association with host cellular proteins, binds to specific sequences upstream of the immediate early gene core promoters (3, 4). The transcriptional enhancement activity resides in the carboxy-terminal 78 amino acids (5, 6). This domain can strongly activate transcription in various systems when attached to the DNA-binding domain of transcriptional activators and suggest that ordered structure in the VP16 activation domain is induced upon interaction with target proteins.

The herpes simplex virus virion protein VP16 resides in the carboxyl-terminal 78 amino acids (residues 413–490). Fluorescence analyses of this domain indicated that critical amino acids are solvent-exposed in highly mobile segments. To examine interactions between VP16 and components of the basal transcriptional machinery, we incorporated (at position 442 or 473 of VP16) tryptophan analogs that can be selectively excited in complexes with other Trp-containing proteins. TATA-box binding protein (TBP) (but not transcription factor B (TFIIB)) caused concentration-dependent changes in the steady-state anisotropy of VP16, from which equilibrium binding constants were calculated. Quenching of the fluorescence from either position (442 or 473) was significantly affected by TBP, whereas TFIIB affected quenching only at position 473. 7-aza-Trp residues at either position showed a emission spectral shift in the presence of TBP (but not TFIIB), indicating a change to a more hydrophobic environment. In anisotropy decay experiments, TBP reduced the segmental motion at either position; in contrast, TFIIB induced a slight change only at position 473. Our results support models of TBP as a target protein for interactions between VP16 and putative co-activator or adaptor proteins (11, 21). Direct interactions between several of these target proteins and many other activation domains have also been shown (14). Although the physical interactions have been demonstrated, their relevance and role in transcriptional activation are still largely unknown.

Despite abundant functional studies of activation domains, little is known of their structures. No activation domain structure has yet been solved by x-ray crystallographic analyses or NMR. The limited biophysical studies of several AADs suggest that isolated AADs are unstructured (22–26). We recently performed fluorescence analyses employing chimeric proteins comprising the GAL4 DNA-binding domain (residues 1–147) fused to the VP16 activation domain (64). Trp residues were substituted for Phe at either position 442 or 473 of VP16, respectively. These mutants (17) later, VP16 was shown to directly interact with another basal transcription factor, TFIIB (18), although there is some discrepancy about the specificity of this interaction (11, 13, 19). Recently, a specific interaction between VP16 and a subunit of transcription factor H1 has been reported (20), as have interactions between VP16 and putative co-activator or adaptor proteins (11, 21). Direct interactions between several of these target proteins and many other activation domains have also been shown (14). Although the physical interactions have been demonstrated, their relevance and role in transcriptional activation are still largely unknown.

The activation mechanisms of eukaryotic transcriptional activators have been the focus of many studies (13, 14). In addition to alleviating chromatin-mediated inhibition (15), activators have been proposed to interact with components of the transcriptional apparatus to stimulate or stabilize the formation of the transcription initiation complex at the promoter. Biochemical approaches have identified several potential targets of activation domains, particularly for the AAD of VP16. TATA-box binding protein (TBP) was the first basal factor shown to directly bind to the VP16 AAD (16). The specificity of this interaction was demonstrated by a correlation between binding of VP16 mutants to TBP and the transcription activities of these mutants (17). Later, VP16 was shown to directly interact with another basal transcription factor, TFIIB (18), indicating a change to a more hydrophobic environment. In anisotropy decay experiments, TBP reduced the segmental motion at either position; in contrast, TFIIB induced a slight change only at position 473. Our results support models of TBP as a target protein for interactions between VP16 and putative co-activator or adaptor proteins (11, 21). Direct interactions between several of these target proteins and many other activation domains have also been shown (14). Although the physical interactions have been demonstrated, their relevance and role in transcriptional activation are still largely unknown.

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The abbreviations used are: AAD, acidic activation domain; TBP, TATA-box binding protein; TFIIB, transcription factor (RNA polymerase II) B; SHW, 5-hydroxytryptophan; 7AW, 7-azatryptophan.
conditions under which the AAD interacts with its target proteins. The AADs, therefore, have been hypothesized to adopt a specific conformation in the presence of their target proteins. However, no structural characterizations of these AADs have yet been carried out in the presence of their target proteins.

One major difficulty in studying protein-protein interactions by various biophysical means is that the signals from different proteins overlap and make the interpretation ambiguous. Recently, several groups reported that Trp analogs (5-hydroxytryptophan or 7-azatryptophan) can be successfully incorporated into proteins by using Trp auxotrophic Escherichia coli strains and supplementing the growth media with the relevant Trp analog (27–29). The excitation spectra of these Trp analogs are shifted to longer wavelengths compared with Trp itself. The excitations spectra of these Trp pancrealogs (27–29). The excitation spectra of these Trp pancrealogs (27–29). The excitation spectra of these Trp pancrealogs (27–29).

Excitation spectra of these Trp pancrealogs (27–29). The excitation spectra of these Trp pancrealogs (27–29). The excitation spectra of these Trp pancrealogs (27–29).

Purification of Recombinant TBP—

Purification of 5-OH-Trp- or 7-aza-Trp-incorporated GAL4–VP16—Expression plasmids for GAL4-VP16 fusion proteins with unique Trp codons in the VP16 activation domain have been described (64). E. coli strain CY15077 was transformed with pMS421 and with an expression plasmid for one of the various GAL4-VP16 fusion proteins. Cell growth and Trp analog incorporation procedures were followed as described (27) with some modifications. The cells were maintained under ampicillin (100 μg/ml) and streptomycin (20 μg/ml) selection. An overnight culture was diluted 1:100 into M9 medium supplemented with 0.1 mM CaCl₂, 1 mM MgSO₄, 0.5% glucose, 0.1% thiamine, 1% casamino acids, 2.7 mM KCl) containing 8% glycerol (v/v). Protein concentrations were determined using the method of Lowry et al. (30) with minor modifications. E. coli BL21 (DE3) cells carrying the plasmids pLYS S and pKA9 were grown at 37 °C in LB medium containing 30 μg/ml chloramphenicol and 25 μg/ml ampicillin. TBP expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside when cell density reached an A₅₅₀ of 0.6. Cells were then collected by centrifugation and resuspended in the original volume of M9 medium, except that 0.25 mM MgSO₄, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol) plus 100 mM KCl. The crude protein fraction was loaded onto a Whatman P-11 column. The column was washed with buffer B plus 100 mM KCl and then washed with buffer B plus 300 mM KCl. The column was eluted with a linear gradient of 300–800 mM KCl in buffer B. Fractions containing TBP and TFIIB of highest purity (eluting between 620 and 1000 mM KCl) were pooled and dialyzed against buffer B plus 100 mM KCl and loaded onto a pre-equilibrated DE-52 column. The flow-through contained TFIIB at greater than 95% homogeneity. The protein was stored at −70°C.

Purification of Recombinant TFII-B—TFII-B was purified using modified published procedures (31). E. coli BL21 cells containing phII-B were grown in LB media containing 100 μg/ml ampicillin at 37 °C. The cells were harvested after 2 h of additional growth. The cell pellets were resuspended (50 ml/liter of culture) in a buffer comprising 20 mM HEPES, pH 7.9, 25 mM EDTA, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol) plus 100 mM KCl. The crude protein fraction was loaded onto a Whatman P-11 column. The column was washed with buffer B plus 100 mM KCl and then washed with buffer B plus 300 mM KCl. The column was eluted with a linear gradient of 300–800 mM KCl in buffer B. Fractions containing TFII-B of highest purity (eluting between 620 and 1000 mM KCl) were pooled and dialyzed against buffer B plus 100 mM KCl and loaded onto a pre-equilibrated DE-52 column. The flow-through contained TFII-B at greater than 95% homogeneity. The protein was stored at −70°C.

Gal4–VP16 Activity Assay—Activities of the various GAL4–VP16 fusion proteins were tested in vitro transcription reactions using yeast nuclear extracts as described (32).

Purification of TBP and TFII–Activity Assay—In vitro transcription assays using HeLa nuclear extracts were performed as described (33). The template plasmid pML containing the adenosine major late promoter was linearized with Smal. The activity of purified recombinant TBP was tested using HeLa nuclear extracts preincubated at 47 °C for 15 min to inactivate endogenous TBP (34). To test the activity of recombinant TFIIB, the HeLa nuclear extract was depleted of endogenous TFII-B as follows: 0.12 ml of agarose-conjugated antibodies directed against TFII-B (Santa Cruz Biotechnology) was equilibrated with a buffer comprising 20 mM HEPES, pH 7.9, 20% glycerol, 1 mM EDTA, 0.2 mM EGTA, 0.2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 500 mM KCl. 5 ml NaCl and 2% Triton X-100 was added to 120 ml of HeLa nuclear extract to bring the final NaCl concentration to 500 mM and the final Triton X-100 concentration to 0.02%. This extract was incubated with the equilibrated anti-TFIIB agarose bead at room temperature for 40 min and at 4 °C for an additional 1.5 h. The agarose beads were centrifuged at 2500 rpm for 5 min, and the supernatant was used as the TFII-B depleted-extract.

Spectroscopy—All proteins were dialyzed against phosphate-buffered saline (pH 7.4, 81.1 mM NaHPO₄, 1.4 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl) containing 8% glycerol (v/v). Protein concentrations were estimated from 280-nm extinction coefficients based on amino acid composition (35). The 280-nm extinction coefficients of 5-OH-Trp and 7-aza-Trp were used as described (29). Absorbance measurements were obtained using a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer. The steady-state fluorescence spectra were obtained with an SLM 8000 spectrophotometer. The excitation wavelength was 309 nm. The emission spectra titration experiments of 7-aza-Trp incorporated GAL4–VP16 were performed by recording the initial emission spectrum of the 4 μM 7-aza-Trp incorporated GAL4–VP16 and then adding small aliquots of concentrated TBP or TFII-B solution and recording emission spectra until no further change could be detected. The same amount of TBP or TFII-B were added to the buffer control, and these blank emission spectra were also recorded. Final emission spectra were corrected for blank control and for dilution.

Steady-state fluorescence anisotropy was measured using an L-format detection configuration. The excitation bandpass was 340 nm, and the emission detection was 800 nm moved by 8 nm on each side. The same amount of Trp or 7-aza-Trp was added to the Schiff base to 300 nm, and emission was at 360 nm. Every data point was measured at least eight times. Data were fit to the equations describing formation of the 1:1 binary complex between GAL4–VP16 and TBP (36),

\[ r = \frac{r_0 - r_{11}}{1 - f_{11} f_{12} + (1 - f_{11}) f_{12} + f_{11} f_{12}} \]  

(Eq. 1)
form (complex with TBP), \(r_q\) and \(r_r\) are the anisotropy of the free and bound fluorophores, \(f_q\) and \(f_r\) refer to the fraction of the total fluorophore that is present in the bound and free forms, and \(I_q\) and \(I_r\) are the fluorescence intensities of the fluorophore in bound or free forms, and,

\[
\frac{I_q}{I_r} = \frac{\left(1 + K_{d}[Q]\right)}{\left(1 + K_{d}(1-f_q)[Q]\right)}
\]

where \([Q]\) is the quencher concentration, \(K_{d}\) is the dissociation constant for the association between GAL4 and the VP16 domain. \(G\) is the quencher concentration, and \(T_0\) is the concentration of the added 5-hydroxytryptophan at position 442 or 473, respectively. N-5HW442 contains a truncated VP16 activation domain (amino acids 413–490) with the incorporation of 5-hydroxytryptophan at position 442. GV-7AW442 and GV-7AW473 contain the full-length VP16 activation domain (amino acids 411–456) with 5-hydroxytryptophan at position 442 or 473, respectively. N-5HW442 contains a truncated VP16 activation domain (amino acids 413–490) with the incorporation of 5-hydroxytryptophan at position 442. GV-7AW442 and GV-7AW473 contain the full-length VP16 activation domain (amino acids 413–490) with the incorporation of 5-hydroxytryptophan at position 442 or 473, respectively.

**RESULTS**

Incorporation of 5-OH-Trp and 7-aza-Trp into GAL4W36V-VP16 Proteins—By using a Trp auxotrophic E. coli strain, 5-OH-Trp or 7-aza-Trp were biologically incorporated into various GAL4-VP16 proteins (Fig. 1). These proteins were purified to more than 95% homogeneity and were functionally active when tested by in vitro transcription assays (data not shown).

Therefore, the structural features revealed by these proteins should reflect those of the wild-type VP16 AAD. Fig. 2A shows the peak normalized absorbance spectra of GAL4-VP16 proteins with Trp or its analogs incorporated at position 442 of the VP16 AAD. The absorbance spectrum of the protein containing 5-OH-Trp demonstrated a characteristic shoulder between 290 and 320 nm, while that of the protein containing 7-aza-Trp showed extended low energy absorbance. The fluorescence excitation spectra of the same GAL4-VP16 fusion proteins (Fig. 2B) demonstrate that the Trp analogs at position 442 can be selectively excited at 310 nm. Fig. 2C shows the normalized emission spectra of these fusion proteins. GAL4-VP16 containing 5-OH-Trp had an emission maximum at 340 nm, the same maximum observed for the free amino acid analog. GAL4-VP16 containing 7-aza-Trp showed an emission maximum centered at 396 nm, close to that of 7-aza-Trp in aqueous solution (398 nm). Absorbance spectra, excitation spectra, and emission spectra of GAL4-VP16 proteins with Trp analogs incorporated at position 473 or at position 442 in a truncated activation domain all showed similar properties, indicating that both Trp analogs were successfully incorporated into all the proteins.
Moreover, the concordance of the spectra of the labeled proteins with the spectra of free amino acid analogs supports our observation that these residues of the VP16 activation domain are largely solvent-exposed (64).

The presence of 5-OH-Trp or 7-aza-Trp in the GAL4-VP16 proteins enables the fluorescence of the fusion proteins to be selectively excited at 310 nm in the presence of other Trp-containing proteins. Recombinant basal transcription factors TBP and TFIIB were purified from E. coli, and their transcriptional activities were confirmed using specifically depleted nuclear extracts (data not shown). As expected, these proteins were not efficiently excited using 310-nm light; the fluorescence observed for a 2-fold molar excess of TBP or TFIIB when excited at 310 nm amounted to less than 10% of the signal observed for GAL4-VP16 proteins bearing Trp analogs in the presence of a 2-fold molar excess of TBP or TFIIB.

Interaction between TBP and VP16 AAD Changes the Polarity of the Environments Surrounding 7AW-442 and 7AW-473—The emission spectrum of 7-aza-Trp is very sensitive to the polarity of the environment (29). In aqueous solution, the emission maximum is near 400 nm, but in hydrophobic environments a maximum at 370 nm is observed. To test whether TBP or TFIIB could change the polarity around residues 442 and 473 in the VP16 AAD, increasing amounts of TBP or TFIIB were added to GV-7AW442 or GV-7AW473, and emission spectra were recorded. In the absence of either basal transcription factor, both GV-7AW442 and GV-7AW473 showed the characteristic 396-nm emission maximum of exposed 7-aza-Trp. With the addition of increasing amounts of TBP to either labeled protein, the relative intensity around the 370-nm region increased gradually and eventually reached saturation (Fig. 3, A and B). To quantitate the spectra shift, the ratio of the emission intensities at the two wavelengths (I_{376}/I_{396}) was calculated at each concentration of TBP or TFIIB (Fig. 3, C and D). These ratios increased from 0.81 to 0.96 with the addition of TBP. Thus, residues at both positions are found in more hydrophobic environments in the presence of TBP. In contrast, the addition of TFIIB did not increase the relative intensity around the 370-nm region of these proteins. The I_{376}/I_{396} ratio was unchanged by the addition of increasing amounts of TFIIB (Fig. 3, C and D), suggesting that even if TFIIB interacts with the activator, the polarity of the environments surrounding both 442 and 473 remains the same.

It should also be noted that the quantum efficiency of GV-7AW442 and GV-7AW473 increased modestly in the presence of TBP. The relative intensity around 370 nm region increased; however, the emission maximum is still at 396 nm. These fluorescence properties most closely match those of the model compound 7-azaindole in alcohol rather than those of 7-azaindole in aprotic solvents such as acetonitrile (41). Hydroxyl groups in alcohols induce tautomerization of 7-azaindole, resulting in two populations of fluorescing molecules (29, 41). The results of this study imply that the surroundings of both 7AW-442 and 7AW-473 become more hydrophobic; however, either the solvent is not totally excluded from these residues or there are nearby polar residues that hydrogen bond to the 7AW to
cause tautomerization.

Interaction between Basal Factors and VP16 AAD Alters the Solvent Accessibility of Residues at Amino Acid Positions 442 and 473—Acrylamide quenching experiments were performed to test whether the presence of TBP or TFIIB affected the solvent accessibility of the fluorophores at residues 442 and 473. Activator proteins labeled with 5-OH-Trp were mixed with saturating amounts of TBP or an equivalent amount of TFIIB in the presence of increasing concentrations of acrylamide. The Stern-Volmer plots of these quenching experiments are shown in Fig. 4, and the best fit parameters are summarized in Table I. In the absence of basal transcription factors, the Stern-Volmer plots of both GV-5HW442 (Fig. 4A) and GV-5HW473 (Fig. 4B) showed upward curvature. These data were best fit to a model invoking both dynamic and static quenching, yielding Stern-Volmer constants ($K_{sv}$) of 6.8 $M^{-1}$ and 6.4 $M^{-1}$ and static quenching constants ($V$) of 1.3 $M^{-1}$ and 1.5 $M^{-1}$ for the proteins labeled at 442 and 473, respectively. The dynamic quenching constants for GV-5HW442 and GV-5HW473 were both 2.6 $M^{-1}ns^{-1}$. The Stern-Volmer plot of the free amino acid analog 5-OH-Trp also showed upward curvature, with a $K_{sv}$ of 22.8 $M^{-1}$ and $V$ of 1.5 $M^{-1}$ (data not shown), yielding a dynamic quenching constant of 6.3 $M^{-1}ns^{-1}$. Dynamic quenching constants for both GV-5HW442 and GV-5HW473 are thus of the same order of magnitude as that for free 5-OH-Trp. Consistent with the quenching study of GAL4-VP16 containing natural Trp (64), these results suggest that residues 442 and 473 are solvent-exposed.

In the presence of saturating amounts of TBP, the response of both GV-5HW442 and GV-5HW473 to acrylamide changed significantly. The Stern-Volmer plots of both proteins were linear, and the data were best fit to the purely dynamic quenching model, with a $K_{sv}$ of 8.2 $M^{-1}$ and 6.3 $M^{-1}$, respectively. In this case, the dynamic quenching rate constant was 2.6 $M^{-1}ns^{-1}$ for GV-5HW442 and 2.2 $M^{-1}ns^{-1}$ for GV-5HW473. In the presence of a similar amount of TFIIB, the Stern-Volmer plots of both proteins showed upward curvature, as observed for those of the labeled fusion proteins alone. The analysis gave a $K_{sv}$ of 5.7 $M^{-1}$ and 3.9 $M^{-1}$ and a static quenching constant $V$ of 1.0 $M^{-1}$ and 1.6 $M^{-1}$, respectively. The dynamic quenching rate constant was 2.2 $M^{-1}ns^{-1}$ for GV-5HW442 and 1.5 $M^{-1}ns^{-1}$ for GV-5HW473. Although both TBP and TFIIB altered the quenching effect of acrylamide for both probes, the nature of the effect is very different in the two cases. The presence of TBP eliminated the static quenching process, whereas it did not change the dynamic quenching process significantly. In contrast, in the presence of TFIIB both static and dynamic quenching remain, albeit somewhat altered. Moreover, the addition of TBP to the truncated activator N-5HW442 altered the quenching rate by acrylamide as it did to the full-length AAD, whereas the addition of TFIIB had no effect on accessibility (data not shown). Thus, if there is any interaction between TFIIB and the activators, the effect of that interaction on the structure of the VP16 AAD is apparently different from that seen with TBP.

**TABLE I**

| Proteins | Dynamic model* ($K_{sv}$) | Dynamic and static model* ($K_{sv}$) | Fluorescence intensity parameters |
|----------|---------------------------|-------------------------------------|---------------------------------|
|          | $K_{sv}$ $M^{-1}$ | $V$ $M^{-1}$ | $\langle r \rangle$ ns | $k_d$ $M^{-1}ns^{-1}$ |
| GV-5HW442 | 6.8 | 1.3 | 2.6 | 0.26 | 2.6 |
| GV-5HW442 + TBP | 6.4 | 1.5 | 2.4 | 0.26 | 2.6 |
| GV-5HW473 | 3.9 | 1.6 | 2.6 | 0.26 | 2.6 |
| GV-5HW473 + TBP | 8.2 | 1.0 | 2.6 | 0.26 | 2.6 |
| GV-5HW473 + TFIIB | 6.3 | 1.5 | 2.6 | 0.26 | 2.6 |

*a Data best fit to this model.

**FIG. 4.** Stern-Volmer plots for the quenching of the fluorescence of GV-5HW442 (panel A) and GV-5HW473 (panel B) by acrylamide. 2 $\mu M$ of the activators and 4 $\mu M$ of TBP or TFIIB were used in these experiments. Closed circles, activator alone; triangles, in the presence of TBP; squares, in the presence of TFIIB. Data sets were compared with the various quenching models described under “Experimental Procedures.” The solid line represents the quenching model to which the data are best fit.
The activator proteins containing 7-aza-Trp were also tested in acrylamide quenching assays (Fig. 5, A and B). Stern-Volmer plots of acrylamide quenching of GV-7AW442 and GV-7AW473 were linear, yielding a $K_{sv}$ of 2.3 M$^{-1}$ and 3.3 M$^{-1}$, respectively (Table II). Acrylamide was a less efficient quencher for 7-aza-Trp than for Trp or 5-OH-Trp. The presence of TBP altered the solvent accessibility of both residue 442 and 473. The downward curvatures of the Stern-Volmer plots were best fit to a two-species model, with approximately 40% of the probe molecules being inaccessible to acrylamide (assumed $K_{sv}$ of 0) and an accessible fraction of approximately 60% having a $K_{sv}$ of 4.0 M$^{-1}$ or 7.0 M$^{-1}$ for GV-7AW442 or GV-7AW473, respectively. In contrast, the presence of TFIIB with GV-7AW442 or GV-7AW473 did not change the quenching mechanism, nor did it change significantly $K_{sv}$. However, TFIIB did alter the solvent accessibility of GV-7AW473. Its Stern-Volmer plot was downward curved; in a two-species model, approximately 30% of the probe was inaccessible, and the accessible fraction had a $K_{sv}$ of 4.9 M$^{-1}$. In this case, TFIIB caused a change similar to that seen with TBP.

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Interaction between TBP and VP16 Restricts the Segmental Motion in the AAD—The findings from steady-state anisotropy analysis were further confirmed by time-resolved anisotropy decay measurements. The anisotropy decay curves are shown in Fig. 7, and the fitted parameters are summarized in Table III. The anisotropy decays of the activator proteins (tested in the absence of target proteins) were best fit to two components: a subnanosecond fast decay component representing segmental motion around the 5-OH-Trp fluorophore and the slower decay component in the range of 2–6 ns. In all of these proteins, the segmental motion contributed at least 60% of the anisotropy change. The extent of these segmental motions were comparable with those of the known most flexible proteins (42). These results are consistent with our previous results using GAL4-VP16 bearing natural Trp and taken together suggest that both VP16 subdomains are very mobile (64). The rotational correlation times ($\phi$) for the slower decay components reflect a molecular size smaller than that predicted for a globular GAL4-VP16 protein and presumably represent the size of the activation domain itself tethered to the GAL4 DNA binding domain.
domain by a highly flexible linker.

The anisotropy decay of these proteins was then measured in the presence of a 2-fold molar excess of TBP or TFIIB. Under these conditions, binding between GAL4-VP16 and TBP reached saturation as indicated by steady state anisotropy titration experiments. In the presence of TBP, the anisotropy decay of GV-5HW442 is greatly slowed (Fig. 7A). As for the activator fusion protein alone, data were also best fitted to two decay components. However, the contribution of the segmental motion ($\beta_1$) was dramatically reduced from 75 to 40% of the total, and the slow component dominated this decay process (Table III). If one assumes segmental motion can be reconciled with the "wobble in cone" model (i.e. the localized motion of Trp is a wobbling of its transition moment within a cone), the extent of this motion can be described by the cone semiangle magnitude (43, 44). The cone semiangle for the GV-5HW442 alone (52°) is larger than or comparable with those of many known flexible polypeptides such as apocytochrome C with a reported semiangle of 47° (42). However, the presence of TBP reduced the calculated cone semiangle to 35°. This result indicates that the segmental motion around residue 442 in the VP16 AAD is restricted in the presence of TBP. Moreover, the rotational correlation time of the slow component ($\phi_2$) increased, reflecting the change in molecular mass from the activation domain alone to a complex containing GAL4-VP16 and TBP together. In contrast, TFIIB did not cause any change in the anisotropy decay of GV-5HW442 (Fig. 7A). The lack of any effect on $\phi_2$ suggests that TFIIB did not interact with the VP16 AAD or that if any interaction does exist the mode of association has no effect on the overall rotation of the AAD.

The anisotropy decay curves of the chimeric protein containing the VP16 AAD N-subdomain in the presence of TBP and TFIIB are shown in Fig. 7B. TBP formed a complex with N-5HW442, evidenced by the increase in the rotational correlation time ($\phi_2$) of the slow decay component (Table III). TBP also reduced the amplitude ($\beta_1$) of the segmental motion around residue 442, although the magnitude of the effect is less than that seen for the full-length protein (GV-5HW442). In contrast, TFIIB had much less of an effect on the anisotropy decay of N-5HW442, consistent with the results seen for TFIIB with the full-length protein labeled at position 442.

The anisotropy decay curves of GAL4-VP16 labeled at position 473 of the full-length activation domain (GV-5HW473) in the presence of TBP or TFIIB are shown in Fig. 7C. As observed...
with the probe at amino acid 442, TBP greatly restricted the segmental motion around residue 473 ($\beta_2$, Table III). In addition, the apparent size of the segment associated with this fluorophore at position 473 became larger, as shown by the increment of the rotational correlation time of the fast decay component ($\phi_2$). The rotational correlation time of the slow component ($\phi_3$) also increased, albeit not to the extent seen with the probe at position 442. In this case, the slow component may represent the “freezing” of a subdomain surrounding position 473 rather than the size of the entire GAL4-VP16 complex. This result suggests that binding of TBP may have different effects on the flexibility of the two subdomains of the VP16 AAD.

In this experiment, the presence of TFIIB also affected the anisotropy decay, reflected in an increase in the rotational correlation time of the slow component ($\phi_3$). The magnitude of this parameter was smaller than that expected for the TFIIB-GV-SHW473 complex, and thus probably represents only a subdomain of that complex. TFIIB also moderately restricted the motion surrounding residue 473 ($\beta_1$). The extent of restriction was much smaller than that caused by TBP; TBP reduced the cone semiangle of local motion from 47° to 31°, whereas TFIIB only reduced it to 43°.

**DISCUSSION**

Previous structural characterization of the AADs of VP16, GAL4, GCN4, NF-κB p65, and glucocorticoid receptor by CD and NMR studies revealed that these domains were unstructured in aqueous solution under neutral pH (22–26). However, the AADs of VP16, NF-κB p65, and glucocorticoid receptor all form an α-helix conformation in less polar solvent (22, 23, 25, 26). The AADs of GAL4 and GCN4 form β-sheet in lower pH solution or in a hydrophobic solvent (24). Authors of these reports all speculate that in the process of transcriptional activation, the AADs adopt higher-order structure upon contacting their target molecules by an “induced fit” mechanism. The present report provides biophysical evidence to support that speculation, in that the local structure surrounding key residues of the VP16 AAD was significantly constrained upon interaction with TBP, and to a lesser extent, with TFIIB. The induced conformation in transcription factors have been previously shown only in the DNA binding basic region of leucine zipper proteins and the arginine-rich RNA binding domain of human immunodeficiency virus Tat protein (45–47). The finding of the induced ordered structure in the VP16 activation domain will likely lead to a more refined analysis of the specific secondary and tertiary structures induced by its target proteins.

In this study, Trp analogs with unique fluorescent properties were incorporated at key positions of the VP16 transcriptional activation domain. These spectrally enhanced proteins were used to study the interactions between this activation domain and the basal transcription factors TBP and TFIIB. In the absence of these factors, studies of the VP16 AAD containing 5-OH-Trp or 7-aza-Trp at positions 442 or 473 showed that both residues are solvent-exposed and are associated with highly mobile protein segments, consistent with previous fluorescence analyses of the VP16 AAD containing natural Trp at these positions. The presence of TBP induced a significant change in the VP16 AAD, with a more ordered or constrained structure becoming apparent using fluorescent probes at either position. In contrast, effects of TFIIB interaction were observed only for probes at position 473 of the VP16 AAD, and those effects were weaker than those induced by TBP. Probes placed at positions 442 and 473 showed similar changes in the presence of basal transcription factor TBP. Probes at position 442 either in the full-length AAD or in the truncated subdomain also showed similar changes upon interaction with TBP.

Table III shows the fluorescence anisotropy decay parameters of the GAL4-VP16 proteins labeled with 5HW in the absence and presence of basal transcription factors TBP and TFIIB. Anisotropy decay data were analyzed by the “sum and difference” method (40). The rotational correlation times ($\theta$) for motions faster than 300 ps. $\theta_2$ was used to calculate the cone semiales $\chi^2$. The $\chi^2$ for the fit. $^a$ The reduced $\chi^2$ for the fit.

| Proteins       | $\phi_1$ | $\phi_2$ | $\phi_3$ | $\theta$ | $\chi^2$ |
|----------------|----------|----------|----------|----------|----------|
| GV-SHW442      | 0.169    | 0.37     | 0.054    | 5.37     | 0.223    | 52.3     | 1.36 |
| GV-SHW442 + TBP| 0.086    | 0.39     | 0.108    | 39       | 0.194    | 34.9     | 1.29 |
| GV-SHW442 + TFIIB| 0.175 | 0.30     | 0.075    | 4.86     | 0.25     | 48.7     | 1.68 |
| N-SHW442      | 0.206    | 0.20     | 0.108    | 4.34     | 0.314    | 46.1     | 1.48 |
| N-SHW442 + TBP| 0.111    | 0.54     | 0.083    | 37       | 0.194    | 41.5     | 1.28 |
| N-SHW442 + TFIIB| 0.137 | 0.39     | 0.079    | 6.01     | 0.216    | 44.9     | 1.98 |
| GV-SHW473     | 0.224    | 0.14     | 0.110    | 1.96     | 0.334    | 47.0     | 1.02 |
| GV-SHW473 + TBP| 0.073    | 1.24     | 0.128    | 16.1     | 0.201    | 30.8     | 1.83 |
| GV-SHW473 + TFIIB| 0.115 | 0.65     | 0.079    | 10.5     | 0.194    | 42.6     | 1.60 |

* The reduced $\chi^2$ for the fit.

Target-induced Structure in VP16 Activation Domain

Fluorescence anisotropy decay parameters of the GAL4-VP16 proteins labeled with 5HW in the absence and presence of basal transcription factors TBP and TFIIB. Anisotropy decay data were analyzed by the "sum and difference" method (40). The rotational correlation times ($\theta$) for motions faster than 300 ps. $\theta_2$ was used to calculate the cone semiales $\chi^2$. The $\chi^2$ for the fit. $^a$ The reduced $\chi^2$ for the fit.
pletely buried residues.

The steady-state anisotropy of GV-5HW442, N-5HW442, and GV-5HW473 increased substantially in the presence of TBP. Dissociation constants were calculated from these analyses. Dissociation constants between TBP and GV-5HW442 or N-5HW442 were both in the range of $3 \times 10^{-7}$ M, while that between TBP and GV-5HW473 was in the range of $3 \times 10^{-8}$ M. The differences in these dissociation constants may correspond to differences in transcriptional activities as a result of the Phe \rightarrow Trp mutations at positions 442 and 473. The substitution mutant F442W retains 70% activity as a full-length AAD, whereas the F473W mutation has a negligible effect on activity (10). An affinity capture method had previously yielded an apparent dissociation constant of $2 \times 10^{-7}$ M between the VP16 AAD and $^{35}$S-labeled yeast TBP (17). The 10-fold difference in the results may be due to inherent differences between spectroscopic and capture-type assays, or to differences in the fusion protein constructs used in these experiments.

Time-resolved anisotropy decay measurements demonstrate that the mobility of protein segments surrounding positions 442 and 473 is markedly reduced in the presence of TBP (Fig. 7 and Table I). When the VP16 AAD was labeled with 5-OH-Trp at either position, the fraction of the anisotropy associated with fast decay ($\beta_1$) was reduced by roughly 50% by binding to TBP, while the fraction associated with slow decay ($\beta_2$) was increased. Assuming that segmental motion can be correlated with the fluorophore wobbling within a cone (43, 44), the calculated cone semi-angle ($Q$) is reduced from approximately 50° to approximately 30°, representing a considerable constraint on the segmental motion. Moreover, the increase in the rotational correlation time for the slow decay component ($\phi_2$) in the presence of TBP indicates that this component is moving with a much greater mass. The decrease of $\phi_1$ in the presence of TBP was observed on the anisotropy decay of GV-5HW442. In particular, the cone semi-angle ($\phi_2$) reduction caused by TFIIB is much smaller than that caused by TBP. The magnitude of the effect on the rotational correlation times for both the fast and slow decay components was approximately half that observed with TBP, implying that the sizes of the domains responsible for these components were not dramatically altered. This lack of any significant change in steady-state anisotropy in the presence of TFIIB (Fig. 6C) might further suggest that the VP16-TFIIB interaction is weak.

Taken together, these results indicate that the interaction of the VP16 AAD with TBP is very different from its interaction with TFIIB. TBP altered the fluorescence of probes at both 442 and 473, whereas TFIIB affected only probes at 473. Moreover, the magnitude of the effects induced by TBP was also consistently greater than those induced by TFIIB. While these results do not rule out the ability of TFIIB to interact with the VP16 AAD entirely, it is striking that few if any effects are observed on the properties of amino acids at or near positions critical to the transcriptional function of the VP16 AAD.

Comparisons with Other Model Systems—The disordered structure of acidic activation domains of transcriptional activators and their structural transitions in the presence of target binding proteins have precedents in other biological systems. For example, the carboxyl-terminal regions of several isofoms of tubulin are very acidic, and most experimental results suggest that these regions are extended and unstructured. However, α-helical structure was observed in the presence of hydropobic solvents or low pH, consistent with secondary structural predictions (48). No evidence yet indicates whether such a secondary structure is induced when these regions bind to other proteins.

A second analogy is with the binding of the basic pancreatic trypsin inhibitor to trypsin and to trypsinogen, with dissociation constants of $10^{-13}$ and $10^{-5}$ M, respectively (49). X-ray crystallographic analysis shows that trypsinogen in complex with basic pancreatic trypsin inhibitor complex acquires a trypsin-like conformation (i.e. with a rigidly structured binding domain). The reduced affinity of trypsinogen for basic pancreatic trypsin inhibitor is a consequence of the energy required to order the binding domain. Thermodynamic studies and struc-

Target-induced Structure in VP16 Activation Domain

4835
tural comparisons have demonstrated a large negative heat capacity change associated with local or more extensive folding when a protein binds its ligand (or another protein) (50). In such systems, the binding energy from the interaction creates part or all of the binding sites or even drives folding beyond the interface. Binding of a target protein to a flexible segment such as the VP16 AAD requires the reduction of its conformational entropy at the expense of association energy. Therefore, this kind of interaction, in which the flexible segment must be stabilized before it can provide optimal noncovalent interaction, is weaker than interaction with a rigid, stereochemically comple-
mentary surface. Nonetheless, an unstructured charged do-
main may have many advantages over a specific structured domain (51). At neutral pH in aqueous solution, charge repulsion between the many ionized residues in these domains may inhibit formation of specific structure. These domains are therefore flexible and extend away from the proteins. The flex-
ible and extended nature of these domains may increase the possibility of encountering the target proteins, and the charged amino acid side chains may provide a suitable force for promoting macromolecule association. Target proteins such as TBP and TFIIB present many surface-exposed basic amino acids that might serve to neutralize the acidic residues of the VP16 AAD and thereby help induce a specific conformation.

In addition to the charged or strongly polar amino acids commonly found in transcriptional activation domains, hydrophobic (and particularly aromatic) residues are often critical for the function of transcriptional activators (14). Aromatic residues have been shown in several cases to provide the bind-
ing docking force for protein-ligand interactions (52-54) or to be directly involved in protein-protein interactions (55-57). We speculate that the critical aromatic residues in the VP16 AAD participate directly in the binding of target proteins, providing some degree of binding stability and specificity.

The unusual potency of VP16 as a transcriptional activator has been attributed to its ability to bind to a number of target proteins in the transcriptional apparatus (58), which may allow VP16 to act during multiple steps of preinitiation complex assembly (59). The results of this report do not contradict this multiple-target model. Although our results demonstrate most clearly a specific interaction between TBP and VP16 AAD, a weaker and more limited interaction with TFIIIB was also ob-
erved. Interestingly, a TBP mutant deficient in interacting with TFIIIB was shown to be deficient in GAL4-VP16 activated transcription (60). This result suggests that in addition to interacting with TBP directly, the AAD interacts with the TBP-TFIIIB-promoter complex (61). Thus, the weak intrinsic interaction between VP16 AAD and TFIIIB may be strengthened in the presence of TBP.

Transcriptional activation is likely not to result from simple static interactions of activators with basal transcription factors but rather may involve the dynamic exchange of interactions among activation domains, basal factors, and coactivators. Re-
cent studies have shown distinct regions of the large subunit of RNA polymerase II share features in common with either acidic or proline-rich activators (62, 63). The activation do-
main and the RNA polymerase II domains may compete for interaction with the same basal transcription factors or coac-
vitators. If these interactions were to occur between rigid, stereochemically complementary protein surfaces, the binding might be so strong that exchange of such tight interactions would be difficult. In contrast, interaction of a target protein with flexible segments is weaker since association energy must be spent to compensate for the reduction of the conformational entropy. Thus, the transitions between ordered and disordered structures in activation domains (and their cognates in RNA polymerase II) may be a means to facilitate the dynamic interaction exchanges and hence to regulate the activation process.

Acknowledgments—We thank Dr. Charles Yanofsky for providing the E. coli tryptophan auxotrophic strain CY15077 (W3110trpE2A) and the lac"-bearing plasmid pH5421. We thank Dr. Fred Winston for providing the plasmid pKA9 carrying the s. cerevisae-25PT15 gene. We thank Dr. Danny Reinberg for providing the plasmid pIIIB expressing the human TFIIIB and for providing the procedure of making TFIIIB-depleted HeLa nuclear extract. We thank Dr. Shelley Berger for providing DNA template, primer, and yeast nuclear extract for testing the activity of GAL4-VP16. We thank Dr. Chun-Hsiung Chang and Dr. Audrey Burton for providing the HeLa cell nuclear extracts and plasmid pIIIB for testing the activity of TBP and TFIIIB. We thank Dr. Michael Brenowitz for providing procedures for TBP purification. We thank Dr. Alexander Ross for suggestions on the biological incorporation of Trp tags.
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J. Biol. Chem. 1996, 271:4827-4837.
doi: 10.1074/jbc.271.9.4827

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