Critical Amino Acids in the Transcriptional Activation Domain of the Herpesvirus Protein VP16 Are Solvent-exposed in Highly Mobile Protein Segments

AN INTRINSIC FLUORESCENCE STUDY*

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Eukaryotic transcriptional regulatory proteins typically comprise both a DNA binding domain and a regulatory domain. Although the structures of many DNA binding domains have been elucidated, no detailed structures are yet available for transcriptional activation domains. The activation domain of the herpesvirus protein VP16 has been an important model in mutational and biochemical studies. Here, we characterize the VP16 activation domain using time-resolved and steady-state fluorescence. Unique intrinsic fluorescent probes were obtained by replacing phenylalanine residues with tryptophan at position 442 or 473 of the activation domain of VP16 (residues 413-490, or subdomains thereof), linked to the DNA binding domain of the yeast protein GAL4. Emission spectra and quenching properties of Trp at either position were characteristic of fully exposed Trp. Time-resolved anisotropy decay measurements suggested that both Trp residues were associated with substantial segmental motion. The Trp residues at either position showed nearly identical fluorescence properties in either the full-length activation domain or relevant subdomains, suggesting that the two subdomains are similarly unstructured and have little effect on each other. As this domain may directly interact with several target proteins, it is likely that a significant structural transition accompanies these interactions.

Transcription initiation by RNA polymerase II in eukaryotic cells requires the assembly of a basal transcription complex containing the polymerase and several general transcription factors (1). The actual level of transcription, however, is regulated by gene-specific proteins termed transcriptional activators or repressors. These proteins usually contain two functional domains. One domain directs the gene-specific binding, or repression function (2–5).

VP16 is a virion protein of herpes simplex virus that specifically activates viral immediate early gene expression (6, 7). The amino-terminal region of this protein interacts with host DNA binding proteins to associate with the immediate early gene promoter sequences (8, 9). The activation function resides within the carboxyl-terminal 78 amino acids (10–12). As one of the most potent activators known, the VP16 activation domain has been studied widely in many systems and by various experimental designs. In light of these studies, several models have been proposed for the mechanisms of activation. Activators might function by relieving the repression effect of chromatin structure (13). Alternatively, they may interact with components of the basal transcription complex, directly or indirectly, to either speed up or stabilize the formation of the preinitiation complex (14–18). Some activators may affect initiation, promoter clearance, or transcriptional elongation (19–21).

Despite their central importance in gene regulation, the structures of the transcriptional activation domains remain a mystery. No activation domain structure has yet been solved by x-ray crystallographic analyses or NMR. Most clues to the structures of activation domains come from mutational analyses. Many activation domains are rich in acidic amino acids; in the case of VP16, 21 acidic residues are found in the 78-amino acid domain. Initially, an “acidic blob” random coil model was suggested for these acidic activation domains (AADs) (22). According to this model, AADs would function primarily through electrostatic interactions. Subsequent mutational analyses provided evidence against this model (23–25), in that no strict correlation between negative charge and activity was observed. An alternative, the so-called amphipathic α-helix model (26), was also refuted by mutational analyses of the VP16 AAD (23, 27). No relation was observed between predicted amphipathy and activity, and proline substitutions introduced into the putative helix had no effect on activity. Instead, particular aromatic and bulky hydrophobic residues were found important for function. These and other studies also suggested that VP16 AAD had two subdomains, namely, the N-terminal subdomain (residues 413–456) and the C-terminal subdomain (residues 453–490) (17, 27, 28). Phe442 was deemed the most critical residue in the N-subdomain, and its aromaticity was the most important feature. Although the pattern of amino acids surrounding Phe473 resembled that surrounding Phe442, Phe473 was not as sensitive to mutations. Thus, these two subdomains apparently depend on different patterns of residues and might function through different mechanisms.

Few biophysical studies of transcriptional domains have been reported. Both one- and two-dimensional NMR of the isolated VP16 AAD demonstrated that this domain lacked sta-
Fluorescence Spectroscopy of VP16 Activation Domain

ble secondary and tertiary structure (29). Similarly, circular dichroism (CD) experiments indicated that this isolated domain was devoid of any stable α-helical or β-strand structure (30), although more α-helical structure was induced under hypodofbic conditions or at low pH. Parallel studies by CD spectroscopy revealed that the AADs of yeast activators GAL4 and GCN4 were conformationally mobile at neutral pH and underwent a transition to β-sheet in acidic solution (31). Taken together, the limited biophysical studies have not detected the secondary structure of AADs under physiological conditions.

Fluorescence spectroscopy can provide a rich variety of information about protein conformation, including the local environment of specific residues, populations of protein conformers, and dynamics (32). Here, we describe a fluorescence analysis employing chimeric proteins comprising the DNA-binding domain of yeast protein GAL4 fused to the AAD of VP16. Trp residues were substituted for Phe (at either position 442 or 473) to provide unique intrinsic probes within each subdomain. The results of fluorescence quenching, time-resolved intensity decay and time-resolved anisotropy decay studies show that the VP16 AAD is largely unstructured. Moreover, the structure of either subdomain seems unaffected by the presence or absence of the counterpart subdomain, reinforcing the concept that the two subdomains have independent structures and activities.

EXPERIMENTAL PROCEDURES

Mutagenesis and Cloning—The original GAL4-VP16 fusion protein (12) contained a linker region of seven amino acids, including one Trp codon within the GAL4 DNA binding domain was changed to Ser in the following sequence, the acquisition reads Ser-Pro-Glu-Pro-Pro-Arg (12). A Trp codon within the GAL4 DNA binding domain was changed to a Val codon using oligonucleotide-directed mutagenesis (34). The altered DNA fragment was then subcloned into each of the three E. coli expression vectors, pSF12182, pAC-del456, and pLA31 Sma. Previ- outly mutated VP16 activation domains (F442W or F473W) were fur-

Expression and Purification of Proteins—Various GAL4W36V-VP16 proteins were expressed in E. coli XA90 cells under control of the hybrid tac promoter. A previously reported purification protocol (33) was signifi-

cantly improved to modify the purity. Cells containing the expres-

sion plasmid were grown at 37 °C in LB medium containing ampicillin at 50 μg/ml. At a cell density of A600 = 0.7, isopropyl-1-thio-

galactopyranoside was added to 1 mM to induce the synthesis of fusion proteins. Zinc acetate was added to 100 μM at this point to provide divalent cations for the GAL4 zinc binding domain. Three hours after induction, cells were harvested and resuspended in (40 μl/milliter culture) ice cold buffer A (20 mM HEPES, pH 7.5, 20 mM 2-mercaptoethanol, 10 μM zinc acetate, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 20 μM benzamidine, and 0.2 μM phenylmethylsulfon fluoride) plus 200 mM NaCl. The cells were then lysed by sonication, and cell debris was pelleted by centrifugation. Polyethyleneimine was added to the cleared lysate to 3 mg/ml. The precipitated proteins were resuspended in buffer A plus 750 mM NaCl and precipitated by the addition of solid ammonium sulfate to 0.194 g/ml for GAL4-VP16 and GAL4-VP16C and to 0.226 g/ml for GAL4-VP16N. The precipitate was centrifuged, resuspended in buffer A plus 650 mM NaCl and dialyzed against standard column buffer (SCB; 20 mM HEPES, pH 7.5, 10 mM zinc acetate, 1 mM dithiothreitol) plus 100 mM NaCl. The crude proteins were then loaded onto a pre-equilibrated Whatman P-11 column at 4 °C. The column was first washed with SCB plus 100 mM NaCl and then eluted with a linear gradient of 100 mM to 1000 mM NaCl in SCB. The relatively pure fractions (as judged by SDS-polyacrylamide gel electrophoresis) were combined and dialyzed against SCB plus 200 mM NaCl for GAL4-VP16 or 150 mM NaCl for GAL4-VP16N and GAL4-VP16C and then loaded onto a pre-equilibrated DE-52 column at room temperature. This column was washed with SCB plus 200 mM NaCl (for GAL4-VP16) or 150 mM NaCl (for GAL4-VP16N and GAL4-VP16C) first and then eluted with a linear gradient (to 400 mM NaCl in SCB). Fractions containing purified proteins (as judged by SDS-polyacrylamide gel electrophoresis) were pooled and stored as aliquots at –70 °C. GAL4-(3–147) was purified by a modified procedure of a method previously described (35).

In Vitro Transcription Assay—In vitro transcription reactions were performed as described (36). The DNA template, pC2Z3GAL, contains the yeast CYC1 gene promoter, which includes two TATA box sequences and multiple initiation sites. It also contains three tandem binding sites for GAL4. In vitro synthesized RNAs were analyzed by primer extension using avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL). Primer extension products were separated on 9% polyacrylamide, 7 M urea gel.

Fluorescence Measurements—All proteins were dialyzed against phosphate-buffered saline (pH 7.4, 8 mM NaHPO4, 1.4 mM KH2PO4, 137 mM NaCl, and 2.7 mM KCl). Concentrations of samples were determined using the following extinction coefficients derived from amino acid composition (37): e297 nm = 9890 cm−1 M−1 for GAL43W6V- VP16F442W, GAL4G36V-VP16F473W, and GAL4G36V-VP16C F473W; e290 nm = 7370 cm−1 M−1 for GAL4G36V-VP16N F442W. Absorbance measurements were obtained using a Perkin-Elmer Lambda 48 UV/VIS spectrophotometer. Concentrations of all proteins used in this study were in the range of 10–20 μM. The optical densities of all samples were less than 0.1 at the excitation wavelengths to avoid inner filter effects.

The steady-state fluorescence spectra were obtained on an SLM 8000 spectrophorimeter operated in a ratio mode. "Magic angle" configuration was used to avoid rotational artifacts (38). The bandwidths for excitation and emission slit were 4 nm. The excitation wavelength was 297 nm.

Quenching experiments were performed at an excitation wavelength of 297 nm. All probes of stock quenching solutions (4 mM KI, 4 mM CsCl, and 8 mM acrylamide) were added to 1.4ml protein samples. The values of fluorescence emission intensity at 350 nm were corrected for dilution prior to data analysis. Quenching data were analyzed by the Stern-Volmer equation for dynamic quenching,

\[
F_0/F = 1 + K_{sv}[Q] \tag{Eq. 1}
\]

where \(F_0\) and \(F\) are the fluorescence intensity in the absence and presence of quencher, \([Q]\) is the quencher concentration, \(K_{sv}\) is the Stern-Volmer dynamic quenching constant, and \(V\) is the static quenching constant (or "active volume"). \(K_{sv}\) and \(V\) values were determined using least-squares regression (IGOR, WaveMetrics, Lake Oswego, OR). The bimolecular collisional quenching constant \(k_q\) was calculated from the following equation:

\[
k_q = K_{sv}/(c) \tag{Eq. 3}
\]

where \(c\) is the mean (intensity-weighted) fluorescence lifetime obtained from time-resolved measurements.

Time-resolved fluorescence was measured on a single photon counting fluorometer (39). A synchronously pumped, mode-locked, cavity-dumped dye laser (Spectra-Physics 3520) was used as the light source, providing pulses of width ~10 ps at 297 nm with a repetition rate of 4 MHz and an average power of 200 μW. The vertically polarized UV pulses were obtained by frequency doubling of horizontally polarized dye laser pulses. The exciting light time profile was obtained with a light-scattering suspension (Ludox, DuPont). The intensity decay profiles were collected through an emission sheet polarizer oriented 55° from the vertical symmetry axis (38). Emission was selected by computer-controlled J YH10 monochromator with the bandwidth set at 8 nm, and a glass slide was added to further reject stray excitation as needed. Decay curves were recorded at 5-nm intervals across the emission bands (310–460 nm) by using standard time-correlated single photon counting modules and an Ortec ADCAM multichannel analyzer under computer control. The decay-associated spectra were obtained from global analysis (40, 41). The fluorescence intensity decay, \(I(\tau)\), was fit to a sum of exponentials,

\[
I(\tau) = \sum \alpha_i(\tau) \exp(-\tau/\tau_i) \tag{Eq. 4}
\]

where \(\tau_i\) is the emission wavelength independent decay time of the ith
FIG. 1. **Schematic representations of the various transactivators used in this study.** All proteins contain the GAL4 DNA binding domain (residues 1–147) with a tryptophan to valine substitution at position 36, designated as GAL4W36V. All proteins also contain a 2- or 3-amino acid linker between GAL4 domain and VP16 domain. GAL4W36V-VP16 (413–490), GAL4W36V-VP16 F442W, or GAL4W36V-VP16 F473W contain the in-frame fused wild-type full-length VP16 activation domain (residues 413–490) or phenylalanine to tryptophan substitution at position 442 or 473, respectively. GAL4W36V-VP16 F 442W contains the in-frame fused VP16 activation N subdomain (residues 411–456) with the tryptophan substitution at position 442. GAL4W36V-VP16C F 473W contains the in-frame fused VP16 activation C subdomain (453–490) with the tryptophan substitution at position 473.

decay component, and $a_i$ is its preexponential term at emission wavelength $\lambda$. The fractional fluorescence, $f(\lambda)$, of the $i$th component at wavelength $\lambda$ is defined by the following equation (42).

$$f(\lambda) = a_i(\lambda)\tau_i \sum \alpha_i(\lambda)\tau_i$$  \hspace{1cm} (Eq. 5)

The mean lifetime $<\tau>$ is defined by the following equation.

$$<\tau> = \sum \alpha_i \tau_i^2 \sum \alpha_i\tau_i$$  \hspace{1cm} (Eq. 6)

The confidence limits for decay-associated spectra (DAS) have been explored by Boens et al. (43); for our experimental conditions, we anticipate that these parameters will be recovered within 5%.

Anisotropy decay curves were obtained by alternatively recording emission oriented parallel and perpendicular to the plane of excitation at an emission wavelength of 350 nm. Time per channel was 90 ps, and 512 channels were recorded. Data were analyzed by the "sum and difference" method (44). The anisotropy decay curve, $r(t)$, was obtained from the difference curve and total intensity curve by the following.

$$r(t) = \left( \frac{I_{\|} - I_{\perp}}{I_{\|} + 2I_{\perp}} \right)$$ \hspace{1cm} (Eq. 7)

where $I_{\|}$ and $I_{\perp}$ are emission intensities measured parallel and perpendicular to the excitation plane, respectively. Data were corrected for the G factor, even though a depolarizer in our system makes the G factor very close to unity.

$$r(t) = \sum \beta_i \exp(-t/\phi_i)$$  \hspace{1cm} (Eq. 8)

where $\phi_i$ is the rotational correlation time of the $i$th component and $\beta_i$ is its preexponential term. A fixed 50-ps component was introduced to compensate for both scattering and color shift artifacts. If one assumes segmental motion can be reconciled with the "wobbling in cone" model (45, 46), the cone semiangle, $\Theta$, is given by the following.

$$\beta_i = r_0 \left( \frac{1}{2} \sqrt{\cos(\Theta)(1 + \cos(\Theta))} \right)^2$$  \hspace{1cm} (Eq. 9)

where $\beta_i$ is the preexponential term for the global rotation of the macromolecule and $r_0$ is the limiting ("time zero") anisotropy.

**RESULTS**

Production of GAL4-VP16 Fusion Proteins with Unique Trp Substitutions—The chimeric transactivator GAL4-VP16, which contains the DNA-binding domain (residues 1–147) of GAL4 and the activation domain of VP16 (residues 413–490 or derivatives thereof), was utilized in our study. The GAL4 domain initially had a tryptophan at position 36. To exclusively study the fluorescence properties of the VP16 activation domain, Trp$_{36}$ was replaced with Val using oligonucleotide-directed mutagenesis. This substitution was chosen because Val is present at homologous positions in proteins related to GAL4 (47). Gel mobility shift assays demonstrated that GAL4W36V-VP16 produced in E. coli bound to DNA containing the GAL4 recognition sequence as well as did GAL4-VP16 (data not shown), implying that this Trp to Val substitution did not significantly change the structure of the GAL4 DNA binding domain.

The wild-type VP16 AAD has no indigenous tryptophan residues. To obtain unique intrinsic fluorescence probes at key positions within the VP16 AAD, Phe to Trp mutations were introduced at either position 442 or 473. These mutations had modest or no effects on transcriptional activation when tested in transient transfection assays (27). For this study, these mutations were transferred to the expression vector for the GAL4-VP16 fusion protein as both full-length AAD and as relevant subdomains (413–456 or 453–490). These fusion proteins (represented in Fig. 1) were purified to more than 95% homogeneity as judged by SDS-polyacrylamide gel electrophoresis. These proteins were transcriptionally active when tested by in vitro transcription assays (Fig. 2). For this study, the excitation wavelength at 297 nm was chosen to avoid excitation of tyrosine fluorescence. The emission maximum of the various GAL4W36V-VP16 proteins are presented in Fig. 3. The emission maximum of the various GAL4W36V-VP16 proteins are presented in Fig. 3. The emission maximum of Trp$_{442}$ in both the full-length activation domain or in the N subdomain was centered at 350 ± 2 nm. The Trp$_{473}$ in the full-length activation domain context displayed an emission...
maximum centered at 349 ± 2 nm, while a C subdomain yielded an emission maximum centered at 348 ± 2 nm. All of these emission wavelength maxima resemble those of fully exposed Trp residues, suggesting that both Trp442 and Trp473 are accessible to solvent.

To further assess the solvent access to the surroundings of Trp442 and Trp473, quenching studies were undertaken using anionic (iodide), cationic (cesium), and neutral polar (acrylamide) quenching agents. The results of these studies are given in Fig. 4, and the results of the analysis in terms of Equations 1–3 are given in Table I. The Stern-Volmer plot of acrylamide for GAL4W36V-VP16F442W was linear (Fig. 4A), giving a Stern-Volmer quenching constant ($K_{sv}$) of 15.7 M$^{-1}$. The Stern-Volmer plot of acrylamide for GAL4W36V-VP16F473W showed upward curvature (Fig. 4A); a single-species dynamic-static model fit the data significantly better than did a pure dynamic model. This analysis gave a $K_{sv}$ of 6.6 M$^{-1}$ and a static quenching constant ($V$) of 2.3 M$^{-1}$. $V$ reflects the strength of the ground state complex between the quencher and Trp. The quenching rate constants for GAL4W36V-VP16F442W and GAL4W36V-VP16F473W were 4.2 M$^{-1}$ ns$^{-1}$ and 2.0 M$^{-1}$ ns$^{-1}$, respectively, within the range (2–4 M$^{-1}$ ns$^{-1}$) typically seen for exposed Trp.

**TABLE I**

| Proteins            | Quenchers | $K_{sv}$ M$^{-1}$ | $k_q$ M$^{-1}$ ns$^{-1}$ | $V$ M$^{-1}$ |
|---------------------|-----------|------------------|-------------------------|--------------|
| GAL4W36V-VP16F442W  | Acrylamide| 15.7             | 4.2                     |              |
|                     | KI        | 3.1              | 0.8                     |              |
|                     | CsCl      | 2.3              | 0.6                     | 2.3          |
| GAL4W36V-VP16F473W  | Acrylamide| 6.6              | 2.0                     | 2.3          |
|                     | KI        | 2.2              | 0.7                     |              |
|                     | CsCl      | 1.2              | 0.4                     |              |
| GAL4W36V-VP16N      | KI        | 2.9              | 0.7                     |              |
| F442W               |           |                  |                         |              |
| GAL4W36V-VP16C      | KI        | 2.1              | 0.5                     |              |
| F473W               |           |                  |                         |              |
residues in proteins with little secondary structure (48). These results suggest that both Trp residues are highly exposed.

Using iodide as a quenching agent (Fig. 4B), the Stern-Volmer constants $K_{sv}$ for GAL4W36V-VP16F442W and GAL4W36V-VP16F473W were 3.1 M$^{-1}$ and 2.2 M$^{-1}$, respectively, significantly lower than $K_{sv}$ values for acrylamide. Acrylamide and iodide typically yield similar $K_{sv}$ results when shielding by the protein matrix is determined by steric effects only (32). The lower $K_{sv}$ values seen for iodide quenching in the present experiments presumably reflect the fact that both probes (at 442 and 473 nm) are surrounded by numerous acidic residues. The Stern-Volmer constants $K_{sv}$ of KI for the two VP16 subdomains (GAL4W36V-VP16NF442W and GAL4W36V-VP16C F473W) were 2.9 M$^{-1}$ and 2.1 M$^{-1}$, respectively. These constants are very close to those observed for the full-length activators. Thus, truncation of the activator has no effect on the extent of exposure of Trp$^{442}$ and Trp$^{473}$.

Analysis of cesium quenching (Fig. 4C) for the full-length proteins with Trp$^{442}$ or Trp$^{473}$ using the dynamic quenching model gave $K_{sv}$ values of 2.3 M$^{-1}$ and 1.2 M$^{-1}$, respectively. The quenching efficiency of cesium for an indole ring is much lower than that of iodide (48). The similar values of $K_{sv}$ of both quenchers for both proteins again indicate that the microenvironments surrounding both tryptophans are negatively charged. Additional studies of the ionic strength dependence and pH dependence of the quenching reactions would be needed to further characterize the local electric potentials in the vicinity of these tryptophans (49).

Time-resolved Fluorescence Intensity Decay—The fluorescence decays for all the proteins studied were recorded as a function of wavelength across the emission spectrum. By using global analysis, nanosecond time-resolved DAS were obtained. Fig. 5 shows the resolved DAS for various samples. A summary of these recovered DAS parameters is shown in Table II. In all cases, three decay times and a fourth short-lived fixed component (compensating for any scattered excitation or color shift) gave the best fit. The three decay times (0.9, 3.0, and 5.9 ns) are very similar for all proteins. Furthermore, in all four proteins, the relative intensity of the intermediate lifetime contributed about half of the total emission intensity, whereas the long lifetime contributed one-third and the short lifetime contributed one-sixth. The DAS for short lifetimes in all proteins were blue-shifted, with emission maxima near 340 nm. The middle and long lifetimes in both full-length VP16 activation domains had emission maxima at 350 nm, the same as the steady-state emission maxima. In the truncated VP16 N subdomain, the emission maximum of middle lifetime of Trp$^{442}$ was slightly red-shifted (to 355 nm), while the long lifetime maximum was at 350 nm. For the C subdomain, the emission maximum of long lifetime of Trp$^{473}$ was slightly blue-shifted (to 345 nm), while the middle lifetime maximum was at 350 nm. Despite these fairly small differences, the environments surrounding Trp$^{442}$ and Trp$^{473}$ seem to be very similar.

Time-resolved Fluorescence Anisotropy Decay—Time-resolved fluorescence anisotropy decay was analyzed for all these proteins. Fig. 6 shows the anisotropy decay curves for various samples. The resulting decay parameters are summarized in Table III. The steady-state anisotropy ($r^ss$) values were also calculated using the lifetime instrument in the steady-state photon-counting mode. These small values, in the range of 0.059–0.079, suggest that probes at either position were associated with fast segmental motion as seen in the flexible polypeptide adrenocorticotropic, where $r^ss = 0.06$ (50). By using “sum and difference” analysis, we found that these decays were best represented by two decay components. In all cases, a “fast” rotation component with a subnanosecond rotational cor-

![Fig. 5. Resolution of the total fluorescence spectrum into the DAS. Total, r1, r2, and r3 indicate the total spectrum, the spectrum associated with the short lifetime component, the spectrum associated with the middle lifetime component, and the spectrum associated with the long lifetime component, respectively. In panel A, the spectra of GAL4W36V-VP16F442W are denoted by the solid lines, and the spectra of GAL4W36V-VP16F473W are denoted by the dotted lines. In panel B, the spectra of GAL4W36V-VP16F442W are denoted by the solid lines, and the spectra of GAL4W36V-VP16C F473W are denoted by the dotted lines.](http://www.jbc.org/)

![Image](http://www.jbc.org/)

relation time contributed 60% of the anisotropy decay, while a “slow” component (associated with a rotational correlation time greater than 10 ns) accounted for about 40% of the depolarizing process. The longer correlation time has a magnitude roughly consistent with the rotation of the entire protein. The short correlation time reflects localized motion of a smaller protein segment including the Trp. If one assumes 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 semiamplitude magnitude. The cone semiamplitudes for these four proteins are large (in the range of 41–45°) and are comparable with those of known flexible polypeptides such as adrenocorticotropic (41°) and glucagon (41°) (32). For these flexible polypeptides, this range of motion has been modeled in terms of a persistence length of 7–10 residues (32). As a rule of thumb, the mass of the rotating moiety can be estimated as twice the numerical value of the rotational correlation time; thus, the rotational correlation times observed for Trp at either position in the VP16 AAD (0.46–0.8 ns) can be associated with a very flexible motion of a peptide segment in the range of 0.9–1.6 kDa, or approximately 7–12 residues.
suggesting that both subdomains are similarly unstructured.

At either of the two subdomains had very similar properties, suggesting that it is poorly structured. The intrinsic probes placed at either of the two subdomains had very similar properties, suggesting that both subdomains are similarly unstructured.

The reduced $\chi^2$ for the global fit ($\chi^2 = 1$ for an ideal fit).

| Proteins                  | $\tau_1$ | $f_1$ | $\lambda_{\text{max}}$ | $\tau_2$ | $f_2$ | $\lambda_{\text{max}}$ | $\tau_3$ | $f_3$ | $\lambda_{\text{max}}$ | $\gamma$ | $\chi^2$ |
|---------------------------|----------|-------|-------------------------|----------|-------|-------------------------|----------|-------|-------------------------|---------|---------|
| GAL4W36V-VP16             | ns       | 0.92  | 12.9                    | 340      | 3.05  | 50.5                    | 350      | 5.97  | 36.6                    | 350     | 3.77    | 1.25 |
| GAL4W36V-VP16 F442W       | 0.94     | 17.5  | 340                     | 2.89     | 55.2  | 350                     | 5.80     | 27.3  | 350                     | 3.35    | 1.27    |
| GAL4W36V-VP16 F473W       | 0.86     | 12.3  | 340                     | 3.01     | 50.9  | 355                     | 5.99     | 36.8  | 350                     | 3.89    | 1.30    |
| GAL4W36V-VP16N F442W      | 0.81     | 14.7  | 340                     | 2.73     | 49.1  | 350                     | 5.96     | 36.2  | 345                     | 3.95    | 1.28    |
| GAL4W36V-VP16C F473W      | 0.92     | 17.5  | 340                     | 3.05     | 50.5  | 350                     | 5.97     | 36.6  | 350                     | 3.77    | 1.25    |

Each showed the characteristic “exposed” fluorescence spectrum with $\lambda_{\text{max}}$ around 350 nm, consistent with highly exposed Trp. Rate constants for quenching by acrylamide for both probes were comparable with those of proteins with exposed Trp residues and little secondary structure. KI quenching for the two probes indicated that their microenvironments were negatively charged, consistent with the primary structures of these subdomains. Time-resolved intensity decay yielded similar lifetime species with similar contributions for these two Trp residues. Anisotropy decay measurements suggested that both Trp residues were associated with highly flexible, disordered segments. Noteworthy is that each probe experienced the same environment whether in the full-length context or in truncated subdomains. We infer that deletion of either subdomain had no gross structural effect on the other subdomain. The fact that individual DAS components for each protein were not identical but were distinguishable in these proteins shows that some structures persist, at least on nanosecond timescales (39). On the other hand, the Trp multiexponentiality and anisotropy results point toward multiple conformers that intermix; no evidence for rapid (ns) exchange is seen, however. At this juncture, the most attractive view is one of a flexible but “lumpy” structure whose features switch and vary in microseconds. In summary, these fluorescence properties closely resemble those of the well-characterized class of polypeptides such as adrenocorticotropic hormone, bombesin, and glucagon that have little persistent three-dimensional structure and behave nearly as flexible coils (50–52). Recent mutational analyses of this domain further suggested the importance of residues Phe$^{475}$ and Phe$^{479}$ Trp substitution mutants at these positions can be subjected to the same kinds of studies. We expect similar results will be obtained to illustrate the disordered structure of this AAD.

These results are consistent with results from the previous CD and NMR studies of the isolated VP16 AAD (29, 30), in which no significant secondary structure was detected. In those studies, an isolated AAD peptide fragment was used. Structural analyses of the GAL4, GCN4, and glucocorticoid receptor AADs also employed protein fragments (31, 53). In the present work, we used the chimeric GAL4-VP16 proteins and determined the transcription activities of these proteins. The concordant results suggest that the presence of the GAL4 DNA binding domain does not induce or confer any specific structure in the VP16 AAD.

Our results indicate that the VP16 AAD is largely disordered in solution, and the two aromatic amino acids at positions 442...
...and 473 are solvent-exposed. In the primary structure of the VP16 AAD, abundant acidic residues are found near these aromatic residues. These acidic residues may increase the solvility of this domain.

The disordered structure of the VP16 AAD may be fundamental to the nature of the activation process. Eukaryotic transcriptional activation is tremendously complicated, involving a large number of protein-protein interactions. Biochemical and genetic studies have suggested multiple target proteins of activators (14-18), and many activators enhance transcription synergistically (54, 55). To promote such complicated macromolecular associations in vivo, an unstructured polymeric domain may have many advantages over a specific structured domain (56). For example, flexible, weakly interacting, relatively unstructured polymeric domains can promote the rapid renaturation of complementary DNA strands (57, 58). In such weak interactions, charged groups and hydrophobic residues in an unstructured polymeric domain have been thought to provide a suitable interaction force in the promotion of macromolecular associations (56). For many AADs, transcriptional activities generally correlate with the number of acidic residues and are also dependent on the bulky hydrophobic residues (5, 23). According to this model, these residues in the AAD are important to enhance the large number of macromolecular associations in many steps of the transcription process, mainly through nonspecific interactions. Relatively unstructured domains in activators may permit interaction with any of several different target proteins and thus may function at several steps in transcription activation (15).

Recent studies show that distinct regions of the large subunit of RNA polymerase II share features in common with either acidic activators or a proline-rich activator (59, 60). On the basis of the present and other structural studies, we believe these shared domains are relatively unstructured. These domains in the polymerase may interact with the same target proteins as those of activators. A tether-and-competition model for activation has been proposed (59, 60), in which the dynamic exchange of numerous protein-protein interactions allows the assembly and the disassembly of the transcription complex. Thus, these unstructured domains may facilitate the dynamic exchange interactions in the activation process.

The lack of structure of the VP16 AAD inferred from biochemical studies seems contradictory to the mutational analyses of the VP16 AAD, which showed that its activity is critically dependent on certain types of hydrophobic residues in certain positions. A hypothesis to explain this paradox is that whatever structural element is needed for this specificity is formed during interaction with in vivo targets and that certain hydrophobic residues in the AAD are critical for this transition. The α-helix structure in the VP16 AAD observed under more hydrophobic and low pH conditions and the β-sheet structure induced in the AADs of GAL4 and GCN4 in acidic solution support this hypothesis (29-31). This hypothesis can be further tested by studying the biophysical properties of an AAD in the presence of its putative target proteins. To this end, we have examined the fluorescence properties of the VP16 AAD, labeled with Trp analogs as intrinsic probes, in the presence of various general transcription factors (61). These experiments provide biophysical evidence for “target-induced structure,” and important qualitative and quantitative insights have been gained from this approach. Thus, time-resolved fluorescence may cast new light on mechanisms of transcriptional activation.

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**TABLE III**

| Proteins         | r<sup>m</sup> | β<sub>1</sub> | φ<sub>1</sub> | β<sub>2</sub> | φ<sub>2</sub> | r<sub>j(app)</sub> | θ  | χ<sup>2a</sup> |
|------------------|-------------|-------------|-------------|-------------|-------------|-----------------|-----|--------------|
| GAL4W36V-VP16    | 0.061       | 0.102       | 0.46        | 0.065       | 0.167       | 43.6            | 1.23|              |
| F442W            | 0.072       | 0.098       | 0.76        | 0.058       | 0.156       | 44.6            | 1.63|              |
| GAL4W36V-VP16    | 0.059       | 0.073       | 0.80        | 0.056       | 0.129       | 41.2            | 1.49|              |
| F473W            | 0.079       | 0.085       | 1.32        | 0.052       | 0.137       | 44.1            | 1.51|              |

<sup>a</sup>The reduced χ<sup>2</sup> for the fit.
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