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Structural Studies of the Acidic Transactivation Domain of the Vmw65 Protein of Herpes Simplex Virus Using ^{1}H NMR

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ABSTRACT: We have overproduced and purified the carboxy-terminal transactivation domain of Vmw65 (VP16) of herpes simplex virus, and studied potential folding of the domain by ^{1}H NMR. Two species of the acidic domain were obtained from the bacterial expression system, and we demonstrate that one of these represents read-through of the natural amber termination codon of the Vmw65 reading frame producing a larger polypeptide. Additional residues in the read-through product were identified by total amino acid analysis and by NMR. Study of the correctly terminated product by 1D NMR gave resonances which were clustered into groups around their random-coil chemical shift positions, and 2D NMR demonstrated that, even in mixed solvents containing up to 80% MeOH, there was very little evidence of secondary structure. Together these results indicate that the isolated acid domain has little if any α-helical content of any stable nature. We discuss these results with reference to the demonstrated activity of the acidic domain in a wide variety of polypeptide contexts.

Vmw65 (VP16) is a structural protein of herpes simplex virus (HSV) which is packaged into the tegument region of the virion at approximately 400-600 molecules per virion (Heine et al., 1974). In addition to its structural role, Vmw65 is a regulatory protein (Post et al., 1981; Campbell et al., 1984) which selectively induces the transcription of HSV immediate-early (IE) genes after virus infection by a complex interaction with at least two cellular factors including Oct-1 (McKnight et al., 1987; Kristie & Roizman, 1987; Gerster & Roeder, 1988; O'Hare & Goding, 1988; Stern et al., 1989; Preston et al., 1988) and CFF (VCAF-1, C1/C2) (Xiao & Capone, 1990; Kristie et al., 1989; Katan et al., 1990). A transcription complex containing these three proteins is selectively assembled on IE-specific, cis-acting regulatory elements (the TAATGARAT motif) present upstream of each IE gene (Mackem & Roizman, 1982a,b; Preston et al., 1984; Whitton et al., 1983; Treizenberg et al., 1988a; O'Hare & Hayward, 1987). Requirements within Vmw65 for transactivation are distinct and separable from those for complex formation (Treizenberg et al., 1988b; Greaves & O'Hare, 1989; Werstruck & Capone, 1989). Thus, removal of the acidic carboxy-terminal region of Vmw65 completely abrogates transactivation of IE genes but has no effect on complex formation. Moreover, this acidic region of Vmw65 will function in transcriptional activation when fused to a wide variety of different proteins, including, for example, the human estrogen receptor (Elliston et al., 1990), the chicken oncogene v-myb (Ibanez & Lipsick, 1990), and the yeast regulatory protein GAL4 (Sadowski et al., 1988; Cousens et al., 1989).

The carboxy-terminal region used in such "domain swap" experiments is highly acidic, containing 21 glutamic or aspartic residues and, from secondary structure predictions (Garnier et al., 1978), is proposed to form 2 helical regions separated by a poorly structured proline/glycine-rich loop. The prediction that this activation region of Vmw65 may adopt an α-helical conformation is consistent with the proposal of Ptashne and colleagues that acidic activation regions may have the general property of forming amphipathic helices having one hydrophilic face bearing the acidic residues, and one hydrophobic face (Ma & Ptashne, 1987; Giniger & Ptashne, 1987). However, although the activation regions of a number of other regulatory proteins are enriched in acidic residues (Hope et al., 1988; Hollenberg & Evans, 1988), there has been no direct examination of the physical structure of such regions and no analysis of the proposal for a requirement for α-helix formation.

Since the transcriptional function of the acidic region of Vmw65 is independent of the sequence of the DNA binding domain to which it is attached, we anticipated that any specification for folding would be entirely contained within the region itself. Here we report the absence of any major stable secondary structure of the domain and discuss this in light of current results from site-directed mutagenesis of the region and previous predictions on the requirement for α-helical formation for the function of acidic transactivation domains.

Materials and Methods

Plasmid Construction, Expression, and Purification. The coding region for the acidic carboxy-terminal 79 amino acids of Vmw65 protein of HSV-1 strain MP was fused in-frame

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to the coding sequence for glutathione *S*-transferase contained in the plasmid pGEX2T (Pharmacia). Insertion of the appropriate *Hin*cII to *Eco*RI fragment of pRG50 (Greaves & O'Hare, 1989) into the *Sma*I to *Eco*RI sites of pGEX2T resulted in a vector expressing a glutathione transferase–acid domain fusion protein from which the acid domain could be isolated by thrombin cleavage. Cleavage at the site engineered in the vector was predicted to result in an N-terminal extension of 3 amino acids, Gly·Ser·Pro, in front of the 79 amino acid region of Vmw65 (which commenced with threonine-412).

Expression of the fusion protein was induced by addition of 0.5 mM IPTG to large-scale fermentations (2–10 L) of *Escherichia coli* (HB101) containing the recombinant plasmid (pPO70). Preliminary experiments demonstrated that virtually all of the fusion protein was soluble under mild solubilization conditions (sonication in PBS containing 1% NP40) and the fusion protein was purified on glutathione–Sepharose affinity columns and eluted in a buffer containing 50 mM Tris-HCl, pH 8.0, and 5 mM reduced glutathione. Routinely, 5 mL of buffer was used to solubilize the pellet from 1 L of bacterial culture. Bound material was eluted in 3-mL fractions, 10 μL of which was loaded on SDS–polyacrylamide gels for analysis.

The purified fusion protein was cleaved with thrombin in a buffer containing 100 mM NaCl and 2.5 mM $CaCl_2$, using 1 μg of thrombin to 1 mg of fusion protein. After complete cleavage was ensured, the material was applied directly to an FPLC MonoQ column, equilibrated in 50 mM Tris, pH 7.5, and 20 mM NaCl, and eluted with a linear gradient of 20 mM–1 M NaCl. Desalting and buffer exchange were performed using Amicon Centricon units with nominal cutoff values of 3000 Da.

NMR Spectroscopy. NMR samples contained typically 2 mg of protein in 450 μL of D_2O at pH 6.8 ± 0.2. pH adjustments in aqueous solution were made via microliter additions of 1 M solutions of ^2HCl and NaO^2H. The acidic domain is insoluble at these concentrations below pH 5.3 but redissolves on raising the pH. There is no significant change in the 1D NMR spectrum between pH 5.3 and pH 8.0. Samples in methanol–water mixtures were prepared by first freeze-drying the protein from water at pH 6.8, followed by dissolution in the desired solvent mixture without further adjustment.

^1H NMR spectra were recorded at 400 MHz using a Bruker AM400 spectrometer. Typical 2D spectra were collected with 96–160 scans per t_1 increment and with data sizes of 4096 × 400 points ($t_2 \times t_1$). Free induction decays were multiplied by a 45°-shifted sine-bell function before Fourier transformation and zero-filled to 4096 × 1024 points ($F2 \times F1$) during processing. Base lines were optimized by careful adjustment of the receiver phase (Marion & Bax, 1988) and ADC delay (Hoult et al., 1983), and no further correction was applied. TOCSY spectra employed a 36-ms, 13.6-kHz WALTZ-16 spin-lock, flanked by 1.5-ms trim pulses. Double-quantum-filtered COSY spectra were obtained using the phase cycling scheme of Derome and Williamson (1990).

Other Methods. Polyacrylamide gel electrophoresis was performed using denaturing conditions and buffers as previously described (Laemmli, 1975). N-Terminal sequencing was performed using conventional Edman degradation of the appropriate purified species by Jaytee Biosciences Ltd., Kent, U.K.

Results

Protein Production and Purification. The acidic domain (residues 412–490) of Vmw65 was expressed in bacteria as a fusion protein with glutathione *S*-transferase and the product purified as described under Materials and Methods by affinity chromatography on glutathione–Sepharose columns (Figure 1A). One major product with a molecular weight of approximately 38K and one minor product of approximately 43K were observed in the purified fractions (Figure 1A, solid arrows lanes 1–6). The 38K protein was of the size predicted, and minor lower molecular weight products were considered to be heterogeneous degraded products. Thrombin cleavage of the purified fusion protein resulted in three main products: one of 29K (the size of glutathione *S*-transferase) and two smaller products with molecular weights of approximately 18K and 14K [Figure 1B, lanes (–) and (+) Thr]. These products could be separated by anion-exchange chromatography (Figure 1B) and ultimately purified to virtual homogeneity. N-Terminal sequencing of the two cleavage products gave identical results, and the sequence was exactly as predicted for the three residue extension reading into the acidic domain region, i.e., Gly·

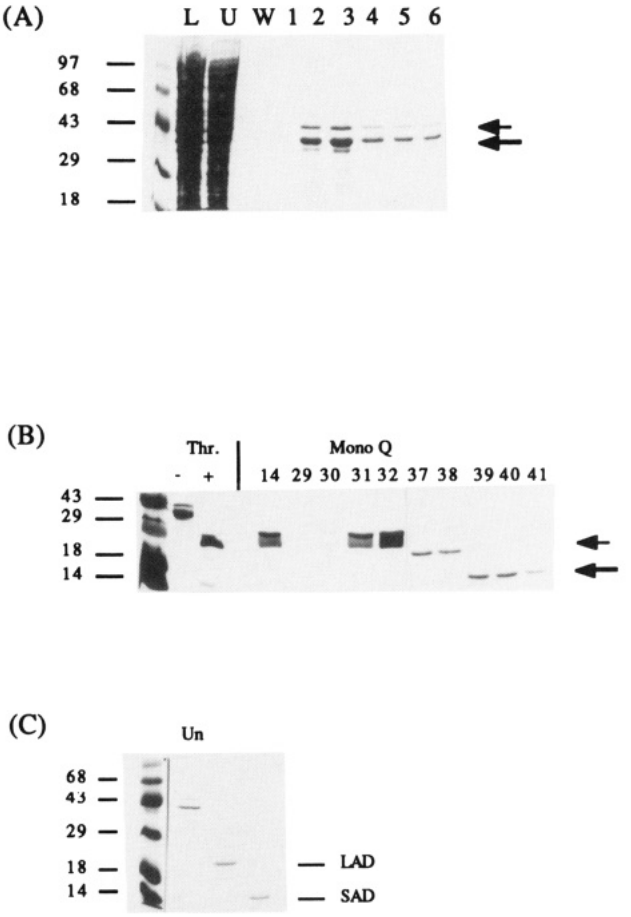

FIGURE 1: Purification of the acid domain of Vmw65. (A) A soluble extract was prepared from bacterial cells as described under Materials and Methods and applied to a glutathione–Sepharose affinity column, the unbound material was collected, and the column was washed. Bound material was eluted in a buffer containing 50 mM Tris, pH 8.0, and 5 mM glutathione. Lanes indicate load (L), unbound (U), wash (W), and fractions 1–6 (10 μL out of 3-mL fractions). (B) Typical results of cleavage are shown in the left two tracks [(–) and (+) thrombin]. After cleavage, the sample was loaded onto a MonoQ column as described under Materials and Methods. One-milliliter fractions were collected, and 2 μL was analyzed by SDS–polyacrylamide gel electrophoresis. GST eluted mainly in the unbound fraction (fractions 10–14) although a proportion of GST eluted at higher salt concentrations (fractions 31/32). The two cleavage products (arrowed) eluted in overlapping peaks from 0.3 to 0.4 M NaCl. (C) Although the elution profiles of LAD and SAD overlapped, the peaks were distinct, and LAD could be efficiently separated from SAD. The figure shows a summary of the uncleaved fusion protein (Un), and two peak fractions containing purified LAD and SAD as indicated.

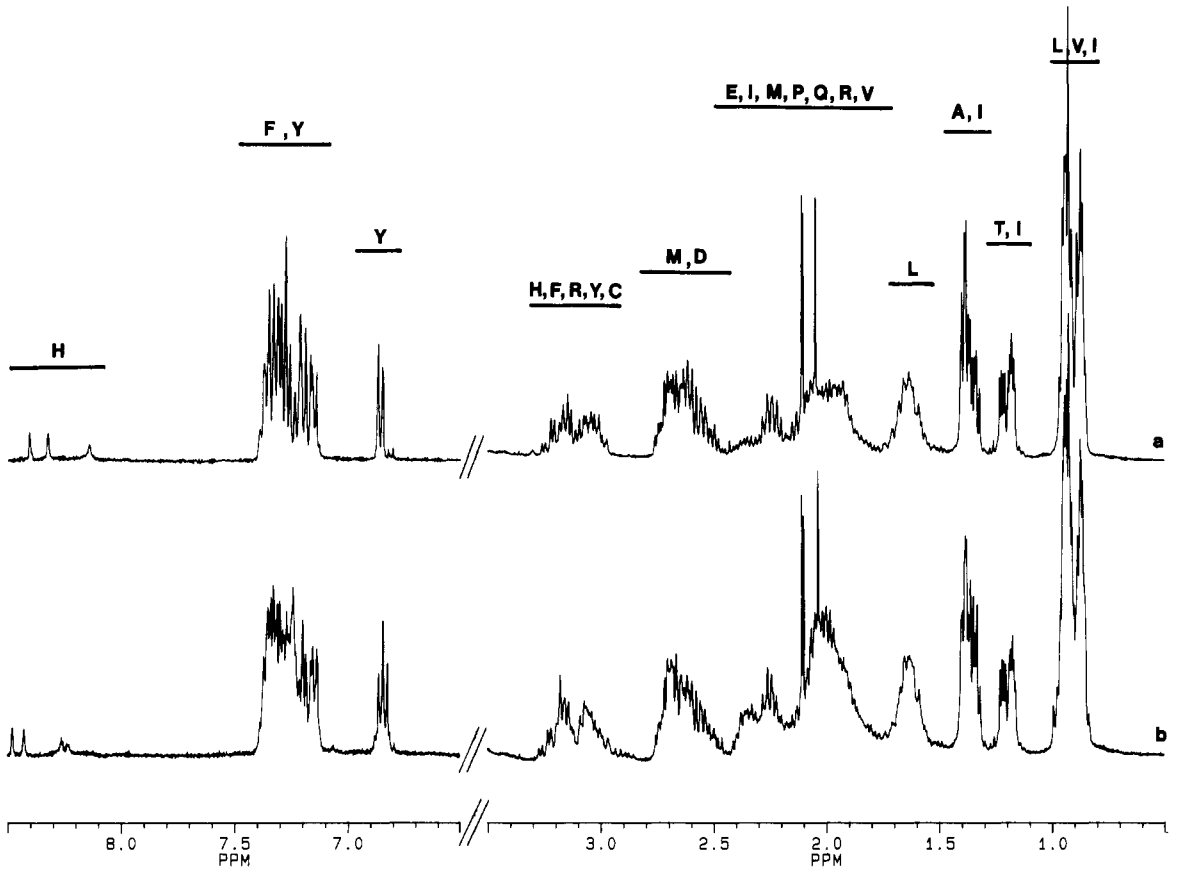

FIGURE 2: ^{1}H NMR spectra of (a) the small acidic domain (SAD) and (b) the large acidic domain (LAD) protein products from expression of the Vmw65 acidic domain in *E. coli*. Random-coil chemical shift ranges are illustrated for some amino acid types (indicated by single-letter codes).

Ser·Pro·Thr·Ala·Pro·Pro·Thr·Asp·Val.... Consequently these two products were named large acidic domain (LAD) and small acidic domain (SAD), and we originally anticipated that the SAD was a specific proteolysis product of LAD. Overall we obtained 5–10 mg of each species, and each was purified to at least 95% homogeneity as determined by Coomassie Brilliant Blue staining of SDS–polyacrylamide gels.

NMR Spectroscopy. One-dimensional ^{1}H NMR spectra of the long acidic domain (LAD) and short acidic domain (SAD) proteins show obvious similarities (Figure 2). In spectra of both LAD and SAD, resonances are clustered into groups around the "random-coil" chemical shift positions of amino acid residues. Each group of signals has a similar intensity in both LAD and SAD spectra, with the exception of resonances around 2.0 ppm, which are more intense in the spectrum of LAD. Resonances from Glu, Pro, Ile, Gln, Arg, Val, and Met amino acids occur in this region. However, both samples have three intense singlet resonances in this region of the spectrum which arise from the $C_\epsilon H_3$ groups of three Met residues. This is entirely consistent with the expected amino acid composition, and these can be discounted as a source of heterogeneity between the two samples.

In the aromatic region of the spectrum, resonances of histidine C2H protons and the C3H/C5H doublets of tyrosine residues are separated from the remainder. Signals from three His residues are observed in SAD while four residues are apparent in LAD. The variation in line width among these signals, due to chemical exchange effects can be attributed to a minor variation in pK_a among these residues. In all cases, the observed pK_a is close to the value (6.6) observed in small peptides. At least two tyrosines are present in both samples, although in SAD the two C3H/C5H doublets overlap almost exactly. However, two-dimensional, double-quantum-filtered COSY (DQF-COSY) spectra can be used to resolve, at least partially, this overlap.

Figure 3a shows the cross-peaks arising from correlations between C3H/C5H and C2H/C6H proton resonances of tyrosine residues in SAD. Although the C3H/C5H proton resonances of the tyrosines are degenerate, the C2H/C6H resonances are resolved, and two antiphase patterns are evident in the cross-peak above the spectrum diagonal. These patterns are not resolved in the cross-peak below the diagonal due to the poorer digitization of the spectrum in the F1 dimension. The presence of two tyrosine residues in SAD is consistent with the predicted composition. However, the spectrum of LAD in this region is more complex (Figure 3b). Apparent phase distortions are due to the combined effects of resonance overlap and mild resolution enhancement. It is possible to discern two antiphase multiplets in the cross-peak above the diagonal; however, one multiplet is approximately twice as intense as the other. Close inspection of the more intense antiphase multiplet reveals a small splitting in the F1 dimension, which is more clearly resolved in the F2 dimension of the cross-peak below the spectrum diagonal. This splitting, together with the increase in intensity, indicates that two tyrosine residues with almost degenerate resonances overlap at these chemical shifts. Thus, LAD contains at least three tyrosine residues in total.

Of the two tyrosine residues in the acidic domain sequence, one is two residues from the C-terminus. Since both LAD and SAD contain at least two tyrosines, any differences between them are unlikely to arise from C-terminal truncation of the acidic domain sequence. This conclusion is supported by the observation of a $C_\beta H/C_\gamma H3$ correlation from a single isoleucine residue in each sample (Figure 4). The acidic domain

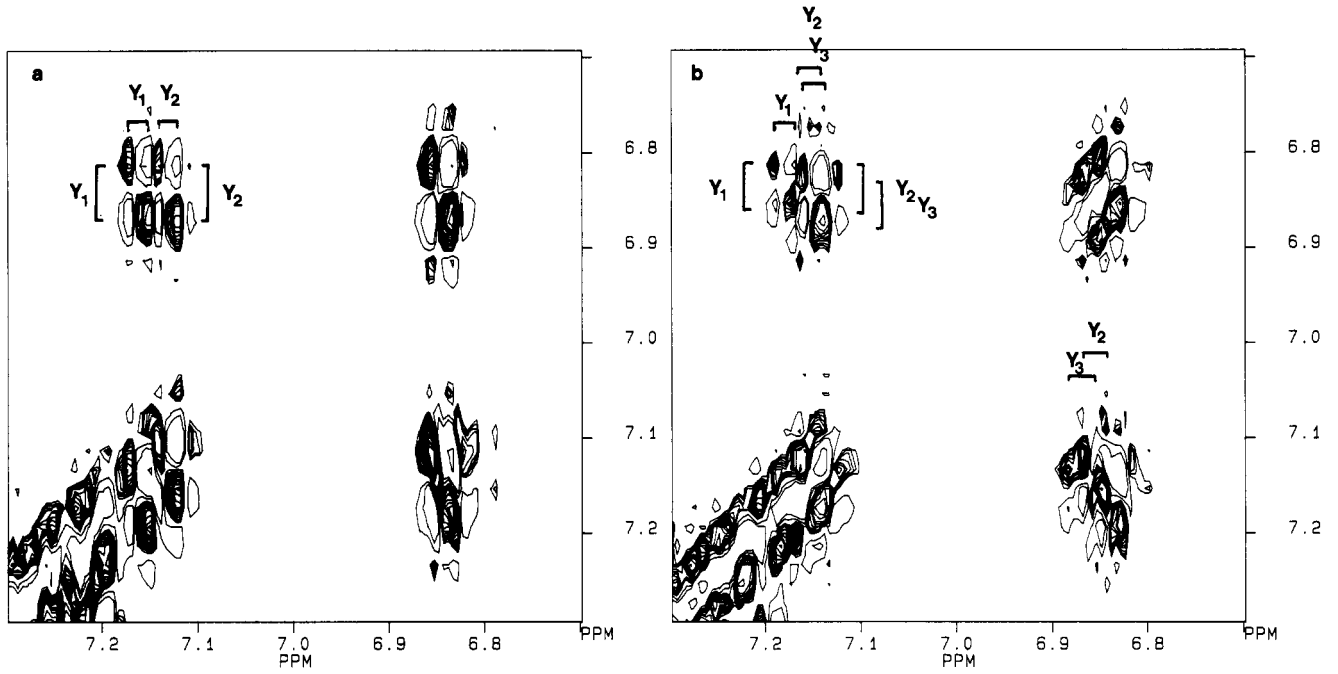

FIGURE 3: DQF-COSY spectra of (a) SAD and (b) LAD. Cross-peaks arising from C3H/C5H–C2H/C6H correlations of two tyrosines (Y_1 and Y_2) are evident in (a), while three, highly-overlapped cross-peaks are evident in (b) (Y_1–Y_3). Positive contours are drawn at intervals of $2^{1/2}$ while negative contours are drawn at intervals of 4.

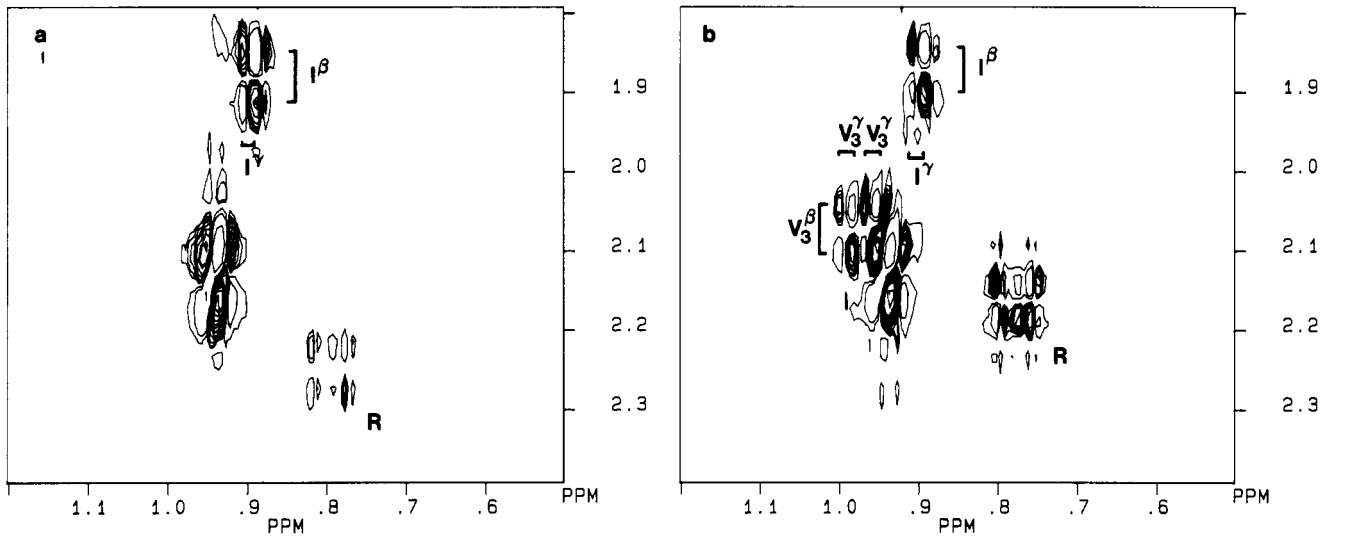

FIGURE 4: DQF-COSY spectra of (a) SAD and (b) LAD. Cross-peaks arising from isoleucine and valine β–γ correlations are shown. A single isoleucine residue is evident in both spectra while LAD contains an additional valine residue (V_3), whose β–γ correlations are labeled. The cross-peak denoted R in each spectrum arises from a reference compound.

sequence contains a single isoleucine residue, which is five residues from the C-terminus. As indicated above, N-terminal peptide sequencing over the first five residues indicates that both LAD and SAD have identical, and correct, N-terminal sequences, thus eliminating N-terminal cleavage as a source of the LAD/SAD heterogeneity.

Comparison of Figure 4a with Figure 4b indicates the presence of an additional valine residue in LAD whose $C_\beta H/C_\gamma H3$ antiphase correlation multiplets are clearly visible to one side of a more intense peak produced by the overlap of four such multiplets arising from two valine residues. These amino acid compositions can be confirmed by inspection of a TOCSY spectrum in which valine and isoleucine $C_\alpha H/C_\gamma H3$ correlations are present (Figure 5). Due to the greater chemical shift dispersion of the $C_\alpha H$ resonances, these correlation peaks (which are in-phase multiplets in TOCSY spectra) are better resolved from each other than the corresponding $C_\beta H/C_\gamma H3$ correlations. Thus, three valine (and one isoleucine) residues are evident in LAD while two valine residues (and one isoleucine) are present in SAD. From the predicted sequence, the acid domain should contain two valine residues.

This region of the TOCSY spectrum also contains signals arising from Ala $C_\alpha H/C_\beta H3$, Thr $C_\alpha H/C_\gamma H3$, and Thr $C_\beta H/C_\gamma H3$ correlations. There are nine Ala and five Thr residues in the acidic domain sequence, and most of their correlation peaks are clustered into two groups (boxed) whose chemical shift coordinates are centered on 4.25, 1.22 ppm (Thr $C_\alpha H/C_\gamma H3$ and Thr $C_\beta H/C_\gamma H3$) and 4.32, 1.40 ppm (Ala $C_\alpha H/C_\beta H3$). However, the $C_\alpha H$ resonances of one Thr and two Ala residues are shifted significantly downfield from these values while the corresponding Thr $C_\beta H$ resonance is shifted upfield, in spectra from both LAD and SAD. While such secondary shifts are commonplace in structured proteins, ar-

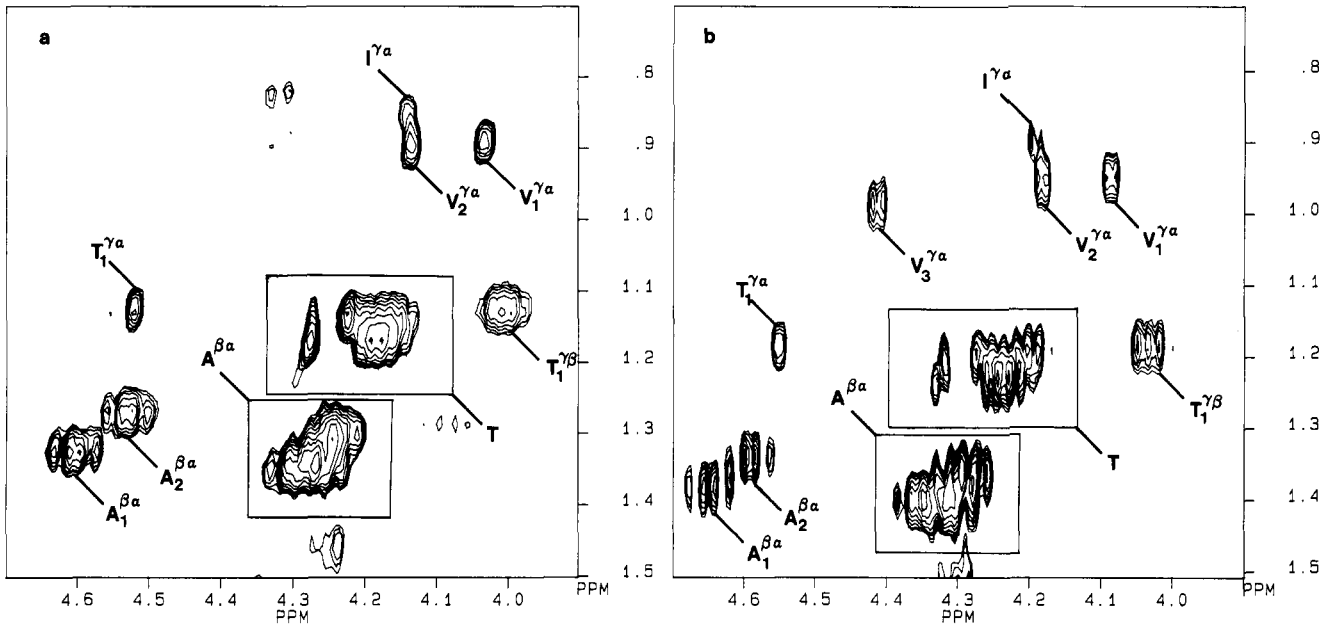

FIGURE 5: TOCSY spectra of (a) SAD and (b) LAD. A portion of the spectrum is shown which contains cross-peaks due to isoleucine α–γ, valine α–γ, threonine α–γ, threonine β–γ, and alanine α–β correlations. Two valine α–γ correlations are identified in SAD while an additional cross-peak is present in LAD. Downfield shifts are evident on the $C_\alpha H$ protons of one threonine (T_1) and two alanine (A_1, A_2) residues in both spectra.

ising from the spatial proximity of magnetically anisotropic groups such as aromatic rings, there is little evidence of such structure under the conditions of Figures 2–5. In extended polypeptides, downfield shifts on $C_\alpha H$ protons are commonly observed in residues which precede a proline in the amino acid sequence (G. Williams, unpublished data) and are probably due to restriction of free rotation around the $C_\alpha H$–CO bond, caused by steric interactions of the proline NCH_2 group. In the acidic domain, one threonine and two alanine residues precede proline, and thus the observed shifts in both LAD and SAD are consistent with the predicted sequence at these points.

TOCSY spectra have revealed an additional set of correlations which are present in LAD and absent in SAD. The resonances involved have chemical shifts which correspond to those expected for the β, γ, and δ protons of an arginine residue and occur in a region of the spectrum which is well separated from other signals (data not shown). No such residue is expected from the acidic domain sequence.

Overall then, the one-dimensional and two-dimensional DQF-COSY and TOCSY spectra of SAD are consistent with the amino acid composition of the expected product of the expression system. In addition, confirmation of the amino acid composition of SAD has been provided using negative-ion, electrospray mass spectrometry (Dr. Walter Vetter, Hoffmann–La Roche AG, Basel). The relative molecular mass predicted from the amino acid sequence of the construct is 8519.0 while that observed for SAD is 8518.5. However, ^1H NMR spectra of LAD indicate the presence of additional residues in LAD which include one Val, one Arg, one His, one Tyr, and, by integration of Figure 2, approximately seven Pro or Glu residues. Due to the extensive overlap in both 1D and 2D spectra, this is not likely to represent a complete list.

Since there is no evidence of heterogeneity in the sequence of the gene from which these proteins products are derived, it is unlikely that the additional residues of LAD arise from an insertion which maintains the correct reading frame. However, the extra residues observed in LAD are consistent with an extension to the sequence at the C-terminus which would result from inefficient termination at the single *stop* codon employed in the construct and translation of the DNA sequence until the next *stop* is reached. This adds the sequence XGARPDPHPPSGFSPPVTGSYPQ, where X represents the amino acid resulting from the translation of the *stop* codon and neatly accounts for the presence of the additional resonances observed in ^1H NMR spectra of LAD. Those additional residues which have not been identified by NMR (Ala, Thr, Phe, Glu, three Gly, and three Ser) have resonances which are obscured by overlap with those of similar residues or which occur in inaccessible regions of the spectrum (e.g., under the HOD resonance).

Amino acid analysis of the peptides supports these general conclusions: LAD contains significantly larger amounts of Pro, Ser, and Gly than SAD.

The absence of secondary and tertiary structure in aqueous solution at 35 °C, which is implied by Figure 2, is confirmed by inspection of two-dimensional NOE (NOESY) and rotating-frame NOE (ROESY) spectra of SAD and LAD. Intraresidue and $C_\alpha H_i$–NH_{i+1} sequential NOEs are observed, characteristic of an extended polypeptide chain; however, no sequential NH_i–NH_{i+1} NOEs, which are indicative of helical or nonhelical turns, are observed. Small changes in chemical shift are seen on the addition of salt (20 mM $CaCl_2$ or 100 mM NaCl), but there is no evidence of secondary structure formation under these conditions.

Addition of a nonaqueous solvent and reduction of temperature have been shown to promote structure formation in a variety of peptides. SAD is soluble in methanol–water mixtures containing up to 80% methanol. A portion of the NOESY spectrum of SAD at 7 °C in 80% methanol is shown in Figure 6. Strong intraresidue NOEs are evident between the C2H/C6H and C3H/C5H resonances of the two tyrosines: a less intense cross-peak arises from chemical exchange between the two $N_\delta H$ protons of the single glutamine residue, caused by slow rotation about the side chain C–N amide bond. However, while some interresidue NH–NH NOEs are observed, these are considerably weaker. The dominant conformer is thus still extended although some turn-containing conformers are probably present. However, these are populated in relatively small amounts (<20%), and it has not been possible to assign the turns to specific regions of the primary

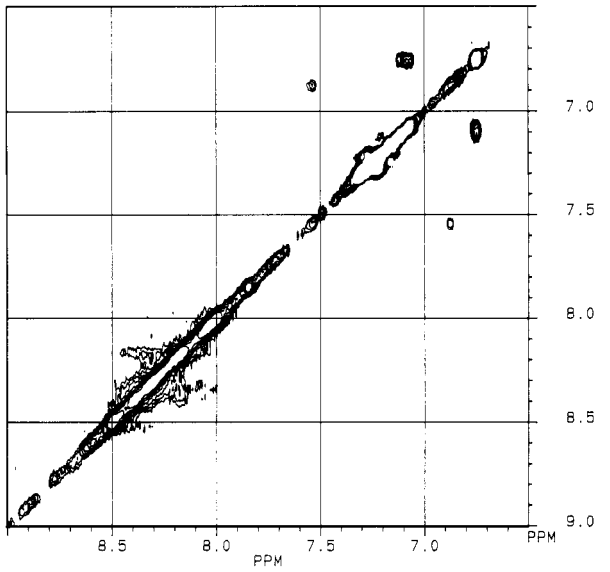

FIGURE 6: Aromatic region of the NOESY spectrum of SAD. The spectrum was recorded at 7 °C in 80% methanol-d_3 and with a mixing time of 200 ms. NOEs are clearly observed between the C3H/C5H and C2H/C6H resonances of the two tyrosine residues, and a moderately strong cross-exchange cross-peak (7.55, 6.87 ppm) is observed between the amide protons of the single glutamine residue. However, only weak cross-peaks are observed between backbone amide protons (signals close to the diagonal line between 8 and 9 ppm), indicating that there is little helical structure under these conditions.

sequence.

Discussion

The carboxy-terminal region of Vmw65 is one of the most potent activation regions yet described, and as such has been the subject of intense study as a paradigm for the mechanism of action of regulatory proteins and their interactions with the basal transcriptional apparatus. Although there has been much speculation on the structural requirements of acidic activation domains, to date none of these regions has been the subject of direct physical analysis.

The carboxy-terminal acid region of Vmw65 is absolutely required for transcriptional transactivation of target IE genes, but dispensable for the proper interaction of Vmw65 with at least two cellular proteins and the formation of a specific DNA binding complex (Greaves & O'Hare, 1989; Treizenberg et al., 1988b; Xiao & Capone, 1990; Ace et al., 1988) which is assembled as an intermediate on the pathway to transactivation. The functional autonomy of the acidic region has been demonstrated by many "domain fusion" experiments where it has been shown to be active in a wide variety of proteins of human, yeast, and bacterial origin [e.g., see Sadowski et al. (1988) and Cousens et al. (1989)].

In addition, varicella zoster virus encodes a protein which is highly related to Vmw65 with the exception that it completely lacks a region corresponding to the acidic domain of Vmw65 (Davison & Scott, 1986; Dalrymple et al., 1985). From these considerations, it is reasonable to propose that the functional properties of the acidic region of Vmw65 are based upon its presence as a physical domain and if its function in transcription requires any specific secondary structure, as has been proposed, the formation of that structure would be independent of the primary sequence of the polypeptide in which it was located.

Nonetheless, our present work indicates that the isolated acidic domain contains little, if any, stable secondary structure and, in particular, little α-helix formation.

Recent results of site-directed mutagenesis have produced consistent results. Cress and Treizenberg (1990), using a truncated version of Vmw65 which terminates at residue 456 and exhibits less than a 2-fold reduction in activity, placed helix-disrupting proline residues at positions 432 and 436. These positions were chosen because they were located around the region of highest probability for helix formation, yet the mutations had no detectable effect on Vmw65 transactivation. In addition, the HSV-2 homologue of Vmw65 contains a natural proline at position 436 (Greaves & O'Hare, 1991; Cress & Triezenberg, 1991). Although a proline substitution for phenylalanine at position 442 did completely disrupt function, it was the specific requirement for an aromatic residue at that position rather than the helix-disrupting property of proline which was important (Cress & Treizenberg, 1990).

The NMR data reported here for the Vmw65 acidic domain indicate that there is, at most, a small population of nonextended conformers under a wide range of solution and solvent conditions which range from pH 5.3 to pH 8.0, 0–0.1 M NaCl and 0–0.02 M $CaCl_2$ in D_2O and 0–80% methanol–D_2O mixtures. The weak NH_i to NH_{i+1} NOE's observed at 7 °C in 80% methanol solution could be accounted for either by the presence of α-helical region(s) or by the formation of a number of isolated turns in a minority of conformers. It is possible that this "nascent" structure is stabilized when the acid domain is incorporated with a DNA binding domain into a functional protein but the heterologous nature of the fusion proteins makes this unlikely. Any formation of secondary structure would then depend on an interaction with the target protein(s) involved in transactivation, and any requirements for specific residues in the acid domain sequence, particularly the hydrophobic residue identified by Cress and Treizenberg (1990), would reflect these intermolecular interactions rather than reflect a requirement for intramolecular folding. Similar conclusions on another acidic activation region have recently been made by Van Hoy et al. (1992). Although they were using a small 19 amino acid synthetic peptide, which corresponded to a subregion of the yeast GCN4 activation domain, and had only modest transcriptional activity (Webster et al., 1988), the authors demonstrated by spectroscopic analysis that it contained no discernible secondary structure. We propose that the absence of structure may be a general feature of acidic activation domains and are currently attempting to verify this proposal by a structural study of the acidic domain in various fusion proteins.

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CORRECTION

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