Conformational Lability of Herpes Virus Protein VP22

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Structural Characterization of VP22
Summary

The herpesvirus protein VP22 traffics between cells, being exported from expressing cells in a non-Golgi dependent manner and localizing in the nuclei of surrounding cells. This transport is retained in certain VP22 fusion proteins, making VP22 a candidate for use in macromolecular drug delivery. In an effort to understand the physical basis for this activity, we have initiated structural studies of VP22.C1, the C terminal half of VP22, which possesses the full transport activity of the native protein. CD and FTIR indicate a secondary structure consisting of approximately 30% α-helix, 17% β-sheet, and 51% disordered and turn structure. Unfolding studies conducted by CD, DSC, and fluorescence reveal a series of discrete structural transitions in the range of 20°C to 60°C. CD and fluorescence studies of interactions between VP22.C1 and divalent cations and model polyanions indicate that Mg++, Zn++, oligonucleotides and heparin interact with the protein, causing changes in secondary structure and thermal stability. Additionally, the interaction of VP22.C1 with model lipids was examined. Fluorescence titrations of the protein with trans-parinaric acid at various temperatures suggest the binding of one to two molecules of parinaric acid to VP22.C1 at temperatures >40°C, suggesting the possibility of conformation dependent membrane interaction under physiological conditions.
Introduction

VP22 is a 38kD, 301 amino acid residue protein encoded by the UL49 gene of herpes simplex virus 1 (HSV-1) and found in the tegument region of the virion located between the capsid and the viral envelope. The precise role of VP22 in the HSV-1 infection process is not presently understood, although it has been shown that the protein is modified by phosphorylation and induces bundling of cytoskeletal microtubules. The property that has stimulated the most interest in the protein, however, is the unique transport activity demonstrated in initial function studies. In transfection assays, the protein was observed in unusually high numbers of cells (up to 100% of the population). Further analysis indicated that this activity was due to spread of the protein from the subpopulation of cells in which it was actively synthesized, where it localized in the cytoplasm in a filamentous pattern, to surrounding cells where it accumulated in the nucleus and could be observed binding to chromatin. VP22 does not contain a conventional signal sequence and spread appeared to be via a Golgi-independent mechanism. Uptake in the surrounding cells did not involve classical endocytosis, and the nuclear import occurred in the absence of a conventional nuclear localization sequence (although portions of the protein's sequence are highly basic).

Thus, VP22 joins a small but growing group of proteins which utilize non-classical secretory mechanisms to traverse lipid bilayers, including acidic Fibroblast Growth Factor (FGF-1), interleukin-1β, the Antennapedia homeodomain and the HIV-1 Tat protein. These proteins are secreted or taken up by cells in the absence of conventional signal sequences, without a clear explanation for the underlying structural mechanisms involved. It has been postulated that this non-classical export utilizes an ATP driven protein transporter. Another explanation for these transport processes involves the adoption of a
molten globule-like state by the proteins. In a molten globule state there is a substantial loss of tertiary structure content with retention of the majority of the secondary structure present in the native protein. This state has been induced in select proteins under conditions of low pH, high salt content, and elevated temperature(13,14), and has been shown to be an intermediary in some protein folding pathways(15,16). It has also been postulated that the molten globule state may play an important role in the insertion and translocation of proteins across lipid bilayers(17). A conformational change into a molten globule-like state may allow the hydrophobic core of the protein to interact with and partially insert into the lipid bilayer. Molten globule-lipid interactions have been reported for a number of proteins(18-21); this type of process could eliminate the need for a signal sequence. Additionally, the interaction may be aided by initial electrostatic attraction between these proteins, which are often highly basic, and cell surface polyanions such as the proteoglycans. However, the presence and possible role of a molten globule state in the mechanism of VP22 transport has yet to be established.

The efficiency of transport, as well as the lack of reliance on conventional cell transport mechanisms, makes VP22 an intriguing candidate for macromolecular drug delivery. Studies have shown that various target proteins including green fluorescent protein(4,22), HSV thymidine kinase(23) and p53(24) can be linked to either the N- or C-terminus of VP22, with the chimeric proteins retaining both the function of the target protein and the transport activity of VP22, are taken up by cells. Such experiments have been successfully conducted in multiple cell lines (e.g. COS-1, BHK-21, HeLa, Vero(4)), in which spread of the protein occurs both within and between individual cell types. VP22 uptake from cell media has also been observed(4). Therefore, intercellular transport can be considered an intrinsic property of the protein itself, not the experimental system, encouraging the exploration of the protein as a drug delivery vehicle.
To this effect, VP22 has been successfully used in both in vitro and in vivo suicide gene therapy experiments(23).

In this work, we initiate investigations of the unusual transport properties of VP22, focusing on its underlying structural characteristics. Utilizing VP22.C1, a truncated version of VP22 consisting of the C terminal half that retains the transport activities of the native protein, we provide the first structural analysis of the protein. We establish the spectroscopic profile of VP22.C1 utilizing UV absorption, Fourier transform infrared (FTIR), fluorescence and circular dichroism (CD) spectroscopies. The structural properties of the protein in solution in both the native and temperature perturbed state are examined. Additionally, we describe a complex series of conformational transitions observed in VP22.C1 in the range of 20°C to 60°C that results in a post-transition state of the protein that retains a significant amount of secondary structure. The interaction of the protein with metal cations, model polyanions, and model lipids is also described, and possible mechanisms of VP22 transport are discussed.

**Experimental Procedures**

All errors are reported as standard error of the mean (SEM).

*Materials* - Phosphorothiolate oligonucleotides [5'-CCC CCA CCA CTT CCC CTC TC-3'] were synthesized by Genset. *trans*-Parinaric acids were purchased from Molecular Probes, Inc. High molecular weight heparin (MW 12,000 to 20,000) was purchased from Sigma. Dialysis materials were obtained from Pierce. SDS-PAGE buffers, stains, pre-cast gels, and other reagents were obtained from Novex. All other reagents were obtained from Sigma and Fisher.
**Protein Expression/Purification** - The bacterial expression construct pVP24 contains VP22 residues 159-301 in the background of the commercial vector pEt24b and was grown in the E. coli strain (BL21pLysS). In this construct the VP22 sequences are flanked at the N-terminus by an extra 16 residues comprising the T7 epitope tag from the vector and at the C-terminus by 6 histidine residues to facilitate purification by nickel chelating chromatography.

 Cultures were grown overnight in L Broth plus kanamycin and chloramphenicol, diluted the next morning by 1:10 and incubated at 37°C until the OD 600 had reached ~ 0.4. IPTG was then added to a final concentration of 0.1mM and incubation continued for a further 4 hours. The cultures were cooled on ice and centrifuged at 6,000rpm for 20 minutes at 4°C. The pellet were frozen and stored at -20°C overnight. Thawed pellets were then resuspended in ~30ml cold ‘Lysis’ buffer, lysozyme was added to 1 mg/ml and the sample was incubated for 30 minutes on ice with occasional shaking. Lysis was completed by sonication (3 x 10-second bursts on ice). NP40 was added to 0.1% followed by DNase1 and RNase to 10µg/ml and the sample was incubated on ice for a further 20 minutes with occasional mixing. The lysate was then drawn through a 21G1½ needle using a 60mL syringe 3 times and centrifuged at 14,000rpm at 4°C for 30 minutes.

 The supernatant was added to 3.5ml DEAE Agarose (50% slurry equilibrated in 'Lysis' buffer) and incubated for 30 minutes at 4°C, rotating throughout. The agarose was pelleted (3,000rpm at 4°C for 5 minutes) and the unbound supernatant added to a column of ~5ml Ni-NTA beads (50% slurry equilibrated in lysis buffer plus 0.1%NP40). The unbound fraction was collected and the column washed at 12ml/hr with ‘Wash’ buffer. VP22.C1 was then eluted in 20 mL employing a 40 to 500mM Imidazole gradient. One mL fractions were collected, frozen and stored at -70°C, with VP22 eluting at around 180mM Imidazole.
The Lysis buffer consisted of 50mM sodium phosphate, pH 8.0, 300mM NaCl, 5mM imidazole, 5mM β-mercaptoethanol, 0.5mM PMSF, and 1µg/ml Leupeptin and Aprotinin (alternatively, Protease Inhibitor Cocktail tablets, Complete™ EDTA-free, Roche, were used at 1x concentration). The Wash buffer was prepared as the lysis buffer with 10% glycerol and 40mM Imidazole. The Elution buffer was prepared as lysis buffer with 10% glycerol and 500mM Imidazole.

The protein was then further purified by cation exchange chromatography (Mono S®, Pharmacia). Samples from the Ni-NTA purification were diluted 1:3 in buffer A containing 100mM NaCl, applied to a Mono S HR5/5 column equilibrated in the buffer A containing 100mM NaCl, and eluted with a salt gradient (100mM to 1M NaCl in 15mL). VP22.C1 eluted at approximately 250 mM NaCl. The peak fractions were divided into aliquots where necessary and stored at -70°C. Buffer A: 50mM sodium phosphate, pH 8.0, 100mM NaCl, 5mM β-mercaptoethanol, 0.5mM PMSF, 1µg/ml Leupeptin and Aprotinin (alternatively, Protease Inhibitor Cocktail tablets, Complete™ EDTA-free, Roche, were used at 0.1x concentration) and 10% glycerol. Buffer B was prepared as buffer A with 1M NaCl.

Transport Activity of VP22.C1 - Cells were grown on coverslips in 6 well dishes in Dulbecco’s Modified Minimal Essential Medium (DMEM) containing 10% newborn calf serum (NCS). Prior to the import assay (1 hour) the medium was changed and the cells incubated with 1ml of fresh DMEM containing 1% NCS.

Aliquots of VP22.C1 (200ng) as indicated (clarificated by centrifugation at 12,000 rpm at 4°C for 10 minutes) were added to pre-warmed medium containing 1% NCS with a final volume of 1ml. The medium was then aspirated from the cell cultures, replaced with the medium containing protein, and the cells incubated at 37°C for 30 minutes. The medium was removed,
the cells washed gently with PBS, and fixed in methanol at RT for 15 minutes. The coverslips were then blocked in PBS containing 10% NCS and probed with a rabbit polyclonal antibody (AGV600 1:100) directed against a GST fusion protein containing the C-terminal residues 257-302 of VP22. The secondary antibody was FITC-conjugated goat anti-rabbit antiserum.

*VP22.C1 Sample Preparation* - Protein samples were stored at -80°C in the original elution buffer until used. Solutions of VP22.C1 were dialyzed against 10mM phosphate containing 200mM NaCl at pH 7.4. Metal cation interaction studies were conducted in 10mM MOPS, 200mM NaCl, at pH 7.4. The presence of high salt concentration was necessary to prevent protein precipitation. Buffer exchange was conducted at 4°C using Pierce Slide-A-Lyzer® MINI-Dialysis units (cellulose membrane, 7K MWCO), with a buffer to sample ratio of 10,000:1 v:v, over a period of 6 hours. In some cases, solutions were centrifuged at 10,000 rpm for 60sec to remove trace quantities of aggregated protein prior to concentration determinations and subsequent experimental procedures.

*UV-Vis Spectroscopy* - Protein concentrations were determined at room temperature by UV absorbance measurement at 280nm (ε = 11214, calculated(25)) on a Hewlett-Packard 8453 UV-Visible spectrophotometer fitted with a Peltier temperature controller. Temperature perturbation studies were conducted at a protein concentration of 150µg/mL, with a 5 minute equilibration time included before collection of each spectrum. Spectral analysis was conducted using UV-Visible Chemstation software (Hewlett-Packard). Fourth derivative spectra were calculated employing a nine point data filter and fifth degree Savitzky-Golay polynomial, and subsequently fitted to a cubic function, with 99 interpolated points per raw data point, permitting 0.01nm resolution. Peak positions were then determined from the interpolated curves.
Circular Dichroism - CD spectra were recorded on a Jasco J-720 spectrophotometer equipped with a Peltier temperature controller. Far-UV spectra (between 198 and 290nm) were collected using a 1mm pathlength cuvette, sealed with a Teflon stopper. A resolution of 0.1nm and scanning speed of 10nm/min with a 2 second response time were employed. Spectra presented are an average of three consecutive spectra. In some cases, spectra were recorded at 10°C intervals employing a thermostated cuvette holder. Equilibration times of 5 minutes were included at each temperature interval. Alternatively, the molar ellipticity ([θ]m) at 215nm was collected at 0.1°C intervals, using a 15°C/hr temperature ramp rate. The reversibility of observed transitions was evaluated by monitoring the return of heated samples to the original (low temperature) CD spectrum. A protein concentration of 200µg/mL was employed in all studies. Noise reduction and data analysis was performed using Standard Analysis and Temperature/Wavelength Analysis programs (Jasco) and MicroCal Origin™ 5.0 software. Midpoints of temperature curves were determined by derivative analysis. Secondary structure content was estimated using the CONTIN(26), SELCON(27), and Neural Network(28) analysis programs provided with the SoftSec™ conversion program (Softwood).

Differential Scanning Calorimetry - DSC thermograms were collected with a Calorimetry Sciences Nano-DSC calorimeter. Scans were obtained over consecutive heating and cooling cycles, between 0°C and 100°C under 3 atm pressure. A temperature scanning rate of 1°C/min was used in all cases. An equilibration time of 10 minutes was included at the beginning and end of each cycle. Sample and reference buffers were degassed prior to use and a protein concentration of 500µg/mL was employed. A buffer baseline was subtracted from each scan and the data was converted to molar heat capacity (MHC). The excess MHC was then deconvoluted.
with CpCalc (Applied Thermodynamics, Inc.) and MicroCal Origin™ 5.0 software to analyze the underlying components.

*Fourier Transform Infrared Spectroscopy* - FTIR spectra were collected on a Nicolet Magna-IR 560ESP fitted with an MCT detector and continuous dry air purge. Dry protein samples were analyzed by placing 25µL of a 1mg/mL solution on polyethylene cards and drying under vacuum for 1 hour. Spectra were recorded at room temperature, with a resolution of 4cm⁻¹ and 256 consecutive scans collected for each sample. Buffer and card spectra were then subtracted. Solution studies were performed using a 5.3µm pathlength transmission cell (BioCell™, Biotools, Inc.). Ten microliters of a 3.75mg/mL protein solution were used for each sample. Collection parameters were as above. Buffer spectra were collected following each protein spectra, using the water association band at 2300cm⁻¹ as a reference for pathlength matching, and were then subtracted from the protein spectra. Subtractions, smoothing, and Fourier self deconvolution (FSD) of the Amide I band of all protein samples were performed using Omnic software (Nicolet). FSD bandwidth and enhancement parameters were 21 and 2.5, respectively. Final deconvolution into underlying components was performed with Galactic Peaksolve, using the FSD trace and second derivative spectra as guides for initial parameters and a mixed Gaussian-Lorentzian peak shape.

*Fluorescence* - Steady state fluorescence emission spectra were recorded using a PTI QuantaMaster spectrophotometer with a thermostated cuvette holder. The intrinsic fluorescence spectrum of tryptophan was monitored, using an excitation wavelength of 280nm, an emission range of 300nm to 450nm, and a data collection rate of 1nm/sec. For thermal perturbation studies, excitation and emission slits were set at 3nm and 2nm, respectively. The protein concentration employed was 50µg/mL, and a 1cm pathlength cuvette with a Teflon cap was used
in all experiments. Temperature control was measured directly from the thermostated holder. A 10 minute equilibration time at each temperature point was used, during which the sample was stirred continuously. Studies of cation and polyanion interactions employed excitation and emission slits set at 3nm. Protein concentration was 30µg/mL, and a 1cm pathlength cuvette was employed. For all experiments, data was corrected internally for lamp fluctuations and the wavelength dependence of intensity. Tryptophan peak positions were determined by first derivative analysis. Midpoints of thermal transitions were determined by fits to model sigmoidal functions. Data analysis was performed using Felix (PTI) and MicroCal Origin™ 5.0 software.

Parinaric Acid Titrations - VP22.C1 and parinaric acid binding experiments were performed using a protocol adapted from Sklar and Hudson(29). To prevent probe degradation (oxidation) due to light and atmosphere exposure, all trans-parinaric acid (transPA) solutions were stored in light protective containers under nitrogen. For long term storage, transPA solutions were stored at -20°C. To minimize transPA degradation, 20µg/mL solutions of VP22.C1 solutions were placed in a 1cm pathlength sealed cuvette under nitrogen atmosphere. Equivolume (4 or 8 microliter) additions of transPA in EtOH were then made to the protein solution through a self-sealing septum. The solutions were stirred continuously for a 5 minute equilibration period, and transPA emission spectra were then recorded from 350nm to 500nm, with excitation at 314nm to minimize contribution of tryptophan emission. Excitation and emission slits were set at 3nm and 10nm, respectively. Temperature was maintained constant over the course of each titration using a Peltier temperature control unit cooled with a circulating water pump with temperature measured directly from the thermostated cuvette holder. Titrations were conducted at temperatures ranging from 20°C to 60°C. Total transPA concentrations ranged from 0µM to 3.9µM, corresponding to [transPA] to [VP22.C1] ratios of 0:1 to 3:1 in each
experiment. Concentration of transPA in EtOH was calculated by UV absorbance at 298nm ($\varepsilon=85000$)\(^{(30)}\). Total dilution of protein solutions was <5% during each titration. Total EtOH was kept to less than 4% of total volume.

*Sulfhydryl Quantitation* – Total and exposed free sulfhydryl content of the protein was determined using a modification of the protocol previously developed by Habeeb\(^{(31)}\). To measure total free sulfhydryl content, protein samples were denatured in 2% SDS at 60°C for 40 minutes. DTNB was added in 50-fold excess, and absorbance at 412nm was collected for 30 minutes. The concentration of free sulfhydryl residues was then calculated using $\varepsilon=13,600$ at 412nm. Degree of exposure of the free sulfhydryl residues was estimated by similar means, in the absence of SDS and denaturing conditions. Protein concentration was 75$\mu$g/mL for all experiments.

*Molecular Weight Determination* – Molecular weight of protein in solution was determined by SDS-PAGE gel electrophoresis, using the Novex NuPage\(^{TM}\) system. Protein samples were separated in 4-12% bis-tris polyacrylamide or 10-20% Tris-glycine gels in the presence of SDS, under both reduced and non-reduced conditions. Protein bands were detected using Coomassie Blue staining.

**Results**

Analysis of purified VP22.C1 by non-reducing SDS-PAGE indicates the presence of a single band corresponding to the monomeric protein accompanied by a band of varying intensity at a dimeric position. The intensity of the dimer band is low, consisting of approximately 5% of the total protein. However, for one protein preparation the dimer does account for approximately 30% of the total protein, which may be the result of uncontrollable variations in dialysis.
conditions (results not illustrated). Presence of reducing agents (10mM DTT) converts the dimer band completely to monomer, consistent with the presence of a disulfide linked dimer.

Analysis of the exposure of the protein's two cysteine residues employing DTNB finds ~0.7 free sulfhydryl groups under both native and unfolding conditions for analysis of the sample with high dimer percentage. This variability between protein preparations further supports the possibility of a small amount of disulfide linked dimer present in the sample material. All of the following studies were performed with this material.

Results of immunofluorescence studies [Fig. 1] show that VP22.C1 can be detected in cells after application in media, exhibiting pronounced accumulation in the nucleus although a significant fraction of the protein can be observed in the cytoplasm. These results indicate that purified VP22.C1 retains the import property, which has been previously shown only with crude preparations of native VP22.

**INSERT FIGURE 1**

*Spectroscopic Characterization* - No data currently exists on the structure of VP22. We therefore examined VP22.C1 with a variety of spectroscopic techniques to gain some insight into its native structure. The UV absorbance spectrum at room temperature [Fig. 2] exhibits a maximum at 284.0nm with a distinct shoulder at 291.2nm, indicative of the presence of tryptophan but no tyrosine residues, which is consistent with the known amino-acid sequence. The fluorescence emission spectrum of VP22.C1 [Fig. 2] consists of a broad peak near 330nm, suggesting that both tryptophan residues are only partially solvent-exposed under neutral solution conditions.
The Far-UV CD spectrum of the protein at room temperature exhibits strong negative ellipticity at 206nm and 223nm, suggesting a significant $\alpha$-helix content [Fig. 3a]. Secondary structure analysis of VP22.C1 by CONTIN, SELCON, and a Neural Network prediction program indicate that under these conditions at physiological pH (7.4), the protein consists of $30\pm3\%$ $\alpha$ helix, $17\pm3\%$ $\beta$ sheet, and $51\pm1\%$ random and/or turn structure ($n=16$). The amount of $\beta$ turn structure was predicted to be $\sim25\%$ by the SELCON program. Additional secondary structure information was obtained by solution FTIR spectroscopy. The Amide I spectrum of VP22.C1 was deconvoluted into underlying components based on initial Fourier self deconvolution of the zero-order spectrum and second derivative analysis [Fig. 3b]. The resulting peaks were then assigned to secondary structure elements using the assignments of Susi and Byler(32). Based on this approach, $32\pm1\%$ $\alpha$ helix, $39\pm2\%$ $\beta$ sheet, $14\pm1\%$ $\beta$ turn, and $15\pm2\%$ disordered structure ($n=6$) were predicted. Additional analysis of the protein by FTIR was performed with $1\text{mg/mL}$ solutions dried on polyethylene cards. Secondary structure of the dried protein was estimated to be $40\pm3\%$ $\alpha$ helix, $32\pm1\%$ $\beta$ sheet, $14\pm1\%$ turn, and $15\pm3\%$ disordered structure ($n=6$) based on this method.

Effects of Temperature on VP22.C1 - Although of relatively small size, the 159-301 fragment of VP22 appears to possess significant conformational lability. This is readily observed upon temperature perturbation of the protein. Differential scanning calorimetry thermograms of the protein at $0.5\text{mg/mL}$ are complex [Fig. 4]. A main transition is observed from $40^\circ\text{C}$ to $55^\circ\text{C}$, having a midpoint near $48.0 \pm/\ -0.7^\circ\text{C}$ ($n=2$). This is accompanied by a second major transition between $20^\circ\text{C}$ and $40^\circ\text{C}$. This lower temperature transition is not as reproducible as the higher temperature endotherm between individual protein preparations (data
not shown). Furthermore, both transitions consist of multiple components, implying that neither of these two conformational transitions can be described by a simple two-state model. For example, in Figure 4 six underlying peaks provided the best fit to the representative data, at 26.2, 31.5, 37.5, 41.9, 46.1, and 49.8°C ($\chi^2 = 4.9$). Since this series of transitions is not reversible upon reaching high temperature (e.g. 100°C) (data not shown), thermodynamic analysis of the transitions was not undertaken.

**INSERT FIGURES 4 AND 5**

To further characterize the nature of these structural transitions, CD, UV, and intrinsic fluorescence spectra were obtained as a function of temperature. As temperature increases, a loss of secondary structure is observed in the far-UV CD spectrum [Fig. 5], with a substantial loss of ellipticity at 223nm evident upon reaching 60°C. Secondary structure estimations based on the 60°C spectrum support this, predicting 19±3% helix, 36±6% β sheet, and 46±4% random and/or turn structure at the higher temperature (n=9). (It should be noted that noise in the spectral data at higher temperatures introduces a greater error into the secondary structure predictions, which is reflected in the wide range given to the β sheet and random structure values.) The pattern of this change in secondary structure content is more clearly observed in a plot of ellipticity at 215nm versus temperature [Fig. 9]. A single transition occurs between 35°C and 55°C, with a midpoint of 46.1 ± 0.9°C (n=4). Additional variation in ellipticity is observed at high and low temperatures, but no clear transitions are evident. The pre-transition (10°C to 30°C) data for the native protein is less reproducible than post transition data (data not shown), for reasons that are currently unclear.

Changes in the fluorescence spectrum of the protein are also observed with increasing temperature [Fig. 6]. The intensity of the tryptophan peak at 330nm decreases dramatically
between 35°C and 50°C, with a midpoint of 40.9 ± 0.8°C (n=3). Interestingly, deviation from linearity is evident at temperatures as low as 25°C. Additionally, the tryptophan emission peak undergoes a red shift from 330nm to ~342nm over this temperature range. A sharp transition is observed between 30°C and 55°C, with a midpoint occurring at 46.0 ± 1.3°C (n=5).

Derivative UV spectroscopy can be employed to examine exposure of phenylalanine, tyrosine, and tryptophan residues under different conditions(33). Here we use fourth derivative spectroscopy, specifically to further resolve a shoulder appearing in the second derivative spectrum at ~280nm. An approximately 0.6 nm blue shift of the tryptophan peaks at 274, 280, 284, and 291nm was observed in the range of 25°C to 50°C, indicating the tryptophan residues are in a more polar environment at higher temperatures [Fig. 7]. However, the degree of the shift suggests that the tryptophans are not fully exposed to solvent, which is in agreement with the fluorescence data. Peaks at 253, 258, and 264nm representing phenylalanine exposure also exhibit shifts, with changes occurring as low as 25°C [Fig. 7].

**INSERT FIGURES 6 AND 7**

*Interaction of VP22.C1 with trans Parinaric Acid* - VP22.C1 possesses unique, non-classical transport properties. This could involve direct interaction of the protein with the lipid bilayer of membranes. We therefore examined the interaction of the protein with a model lipid. One particularly convenient approach employs parinaric acids, fluorescence polyenes that mimic fatty acids in structure [Fig. 8].

**INSERT FIGURE 8**

The fluorescent properties of parinaric acids often alter significantly upon insertion into an apolar environment(34), typically exhibiting an increase in intensity (30). They have been used previously as probes of lipid membranes(29) as well as of the binding sites of fatty acid binding
proteins(35), making them candidates for general probes of lipid-protein interactions. Titrations of constant concentration protein solution with transPA were performed at temperatures from 20°C to 60°C to examine the effects of protein conformational state upon any interaction. Fluorescence emission intensities of the parinaric acids were measured at 410nm, then normalized by subtraction of the intensity of parinaric acid in the absence of protein at each concentration.

At all temperatures examined, the net fluorescence intensity of parinaric acid in the presence of protein was greater that that of the control (parinaric acid in buffer). Saturation type binding isotherms were observed at 40°C, 45°C, and 50°C, but not at temperatures of ≤ 35°C and ≥ 55°C (results not illustrated). Binding constants and stoichiometries could not be reliably extracted from the data, however, due to concurrent shifts in baseline not present in the controls that arose from simultaneous alterations in the intensity of the Rayleigh light scattering peak. Unfortunately, these two sources of emission intensity could not be unambiguously separated. Based on the well defined signal saturation seen at 40°C and 45°C, however, it appears that the interaction has a stoichiometry of 1 to 2 parinaric acid molecules per protein molecule. EtOH itself had no effect on the peak position of the VP22.C1 spectrum, indicating no significant induced structural change. A significant decrease in VP22.C1 fluorescence was observed, however, at intermediate and high (2 and 4%) EtOH concentrations, with such effects more pronounced at higher (37°C) temperatures. Based on this, the stoichiometry of the reaction may be underestimated here, but could perhaps be more accurately resolved using alternate methods.

Interaction of VP22.C1 with Cations and Polyanions - The 159-301 fragment of VP22 contains 25 positively charged residues at neutral pH, with an overall net charge of +12. Based on this, it seemed possible that the protein will interact with polyanions in some manner, as is
commonly observed for many basic and nuclear localizing proteins (36). We therefore examined the interaction of VP22.C1 with both high molecular weight heparin and small oligonucleotides at an approximate 1:1 molar ratio using circular dichroism and fluorescence spectroscopy. Addition of both polyanions resulted in an overall decrease in signal intensity at both low [Fig. 10a] and high [Fig. 10b] temperatures. The midpoints of the CD thermal melting transitions are not, however, significantly different from those of the native protein (45.5 ± 0.4°C and 47.7 ± 0.6°C in the presence of heparin and oligonucleotide, respectively) [Fig. 9], although the transition observed upon addition of the oligonucleotide does appear to be somewhat broadened. In the presence of both polyanions a linear decrease in ellipticity is observed from 10°C to the initiation of the thermal transition (30-50°C), in contrast to the native fragment. Additionally, the presence of either polyanion appears to eliminate the variability between experiments observed at low temperatures, suggesting some type of limited stabilization of the native protein under these conditions.

**INSERT FIGURES 9, 10, AND TABLE 1**

No significant change in secondary structure content is detectable at pre-transition temperatures (20°C) upon addition of heparin [Fig. 10a, Table 1]. In contrast, the presence of the oligonucleotide induces a significant increase in the amount of β sheet content with a corresponding loss of α helix content compared to the native protein [Table 1]. The low precision of β sheet determination for the native protein (36±6%) hindered interpretation of changes in β sheet content at post-transition (60°C) temperatures, but the amount of α helix retained post-transition drops from 19±3% in the native protein to 12±1% in the presence of both polyanions [Fig. 10b].

Addition of heparin to VP22.C1 results in no detectable alteration of the tryptophan
emission at 20°C. However, upon heating the sample, it is observed that the peak position shifts to ~335nm upon reaching 60°C. Compared to the shift to ~341nm observed upon heating VP22.C1 alone, this is a dramatic difference in tryptophan exposure at higher temperatures. This difference is even more evident in the presence of a 1:1 molar ratio of oligonucleotide. Immediately upon addition of the oligonucleotide to protein solution, we observed an ~1nm blue shift of the tryptophan spectrum (1.0 ± 0.1, n=3), which is accompanied by a dramatic decrease in peak intensity. Upon heating the protein, the tryptophan peak shifts only slightly to ~333nm at 60°C (not illustrated).

VP22.C1 contains two cysteine and two histidine residues, suggesting it could possess a metal binding site(37). We therefore examined the interaction of VP22.C1 with magnesium and zinc cations employing CD and fluorescence spectroscopies with the hypothesis that any such change might involve a conformational change of the protein. Comparing the ellipticity at 215nm as a function of temperature in both the presence and absence of excess cation [Fig. 9], we observed no significant differences in the estimated transition midpoints of 46.1 ± 0.9°C, 46.9 ± 0.8°C, and 43.7 ± 1.2°C (native protein, with addition of zinc and magnesium, respectively). However, the transition between 35°C and 55°C seen in the native protein is extended to 60°C in the presence of zinc and slight alterations in the profile at high and low temperatures are also evident. Upon addition of either cation a significant change in secondary structure content is detectable at pre-transition temperatures (20°C), consisting of an increase in helix content and loss of β-sheet [Table 1]. These differences are subtle enough that they are not readily apparent in the spectra themselves [Figure 10a]. Quantitative analysis of post-transition (60°C) spectra could not be conducted, but a difference in the spectrum of the protein in the presence of zinc versus the protein upon addition of magnesium or the native VP22.C1 is evident [Fig. 10b].
difference centers around 222nm, possibly indicating a greater amount of \( \alpha \) helix retained in the post-transition protein. Additionally, the presence of either cation eliminated the variability in ellipticity observed at lower temperatures in the native protein, as also seen upon additions of the polyanions (results not illustrated). Addition of either cation to VP22.C1 did not alter the tryptophan emission spectrum. Upon heating the protein, no significant differences were observed when compared the emission of the protein alone. Aggregation of the protein sample was observed at 60\( ^\circ \)C, however, in the presence of excess zinc. This effect was not observed in the presence of magnesium, and, most interestingly, was not observed in the circular dichroism studies. (Fluorescence results not illustrated.) The implications of this finding are not clear at this time, however, they do appear to confirm some type of interaction between VP22.C1 and zinc cations.

**Discussion**

A major barrier for any macromolecule to cellular entry and exit is the cell membrane. Most proteins appear to negotiate this barrier by specialized protein-based systems such as receptor-mediated endocytosis and signal-peptide based secretion. In contrast, VP22 appears to cross the cell membrane by an unknown mechanism. Whatever the nature of this process, this herpes virus protein can tow along with it quite large auxiliary proteins that cannot normally traverse the lipid bilayer. In this work, we have examined some basic structural features of the protein, with the idea that such information may provide a clue to the physical mechanism underlying VP22 transport.

The protein employed in the studies presented here is the C-terminal fragment of VP22, composed of residues 159 to 301. Although this fragment consists of almost half the native
protein, it obviously does not possess the full secondary and tertiary structure of wild type VP22. However, VP22.C1 does possess the full transport activity of native VP22, both the intrinsic transport ability as well as the ability to carry proteins of significant size. This leads to the reasonable assumption that the structural characteristics essential to this novel transport mechanism reside within the VP22.C1 fragment.

The UV absorbance spectrum of VP22.C1 exhibits a characteristic tryptophan spectrum, with a $\lambda_{\text{max}}$ of 284.0 nm at room temperature. The fluorescence spectrum of VP22.C1 shows a single peak centered at 330nm. From this, we conclude that both tryptophan residues are extensively buried within the protein. However, the presence of distinct asymmetry in this peak at higher wavelengths (~340nm) suggests that one of the tryptophan residues may be partially exposed.

The far-UV CD spectrum of VP22.C1 manifests strong negative ellipticity at 206nm and 223nm, indicating substantial $\alpha$ helix content, while the distinct plateau at 215nm suggests the presence of $\beta$ sheet. Secondary structure estimates obtained from three different algorithms give values of 30±3% $\alpha$ helix, 17±3% $\beta$ sheet, and 51±1% random and/or turn structure (n=16). The large disordered content suggested by the CD measurement is of particular note. Whether this is just a property of the fragment resulting from a loss of stabilization by the N terminal half of the native protein or actually reflects a substantial lability of a large portion of VP22 remains to be established, requiring examination of the entire VP22 molecule. Deconvolution of the protein’s FTIR spectrum places the estimated structure at 30% helix, 40% sheet, 15% turn, and 15% random structure, in reasonable agreement with the CD results for helix, but in significant disagreement in $\beta$ sheet content. One possible source of this inconsistency may be the 20-fold higher protein concentration employed in the FTIR experiment, necessary for an adequate signal-
to noise ratio, which may lead to aggregation of the protein. The presence of a component peak near 1620 cm\(^{-1}\) is consistent with this possibility(38). Another possibility is that there is significant intrinsic error in the CD secondary structure estimation methods for \(\beta\) sheet content. An analysis of various programs' ability to correctly estimate different types of secondary structure in comparison to known crystal structures was recently performed by Greenfield(39). The study demonstrated that the ability of such programs to estimate \(\beta\) sheet and \(\beta\) turn content is greatly reduced in comparison to that of \(\alpha\) helix. It is also possible that we have misassigned the disordered peak usually found at 1641-1647 cm\(^{-1}\), which may not have been completely resolved from the peak at 1635-1640 cm\(^{-1}\), normally assigned to \(\beta\) sheet(32). This would result in an overestimation of \(\beta\) sheet content in the FTIR spectrum.

Differential scanning calorimetry was used to examine the effect of temperature on the protein. DSC thermograms exhibited surprisingly complex behavior. Although a main transition is observed at 48°C, it appears to be only one of a series of small transitions exhibited between 20°C and 60°C. These transitions cannot be used to quantify thermodynamic changes since they appear to arise, at least partially, from irreversible events. They do, however, illustrate the unusual temperature lability of VP22 over a broad temperature range. Studies of the protein's circular dichroism as a function of temperature demonstrate a large change in ellipticity at 215nm. This change corresponds to a decrease in helical content, with a concurrent increase in \(\beta\) sheet content of the protein. Thus, the protein retains a large amount of secondary structure, and does not completely unfold at temperatures up to 100°C (data not shown). The midpoint of this transition is 46°C, which is within statistical error of the second DSC transition. This structural change is also accompanied by an alteration in tertiary structure, as shown by temperature-dependent fluorescence and UV studies. The tryptophan peak undergoes a red shift from 330nm
to ~ 342nm, with a midpoint at 41°C. This shift to 342nm again indicates that this protein is not extensively unfolded, in which case a peak position of 350 to 355nm would be expected. This peak shift is accompanied by a dramatic decrease in fluorescence intensity, which is centered again at 46°C. The fourth derivative UV data confirms this partial unfolding, as the tryptophan peaks, although indicating increased exposure to solvent during thermal unfolding with a 0.6 nm shift, do not appear to be fully exposed, where a larger (~1.2nm) shift would be expected (33).

Analysis of the many fourth derivative peaks find them not necessarily changing in concert, suggesting that different regions of the protein are altering differentially in response to temperature [Fig. 7], again consistent with the complex DSC thermogram.

From these results, it is apparent that VP22.C1, despite its relatively small size, is extremely conformationally labile, and that this lability occurs at relatively low temperatures. The protein appears to change from the native state to structurally altered forms in which much secondary structure is retained but tertiary structure is severely disrupted. This new conformational state has some resemblance to that class of conformational states generically known as molten globules, in which large losses of tertiary are not accompanied by major changes in secondary structure. Such states have been proposed to be involved in membrane transport in a number of cases (17,20,40), with their increased apolarity in comparison to their native states thought to facilitate, in some ill-defined way, their interactions with membranes or perhaps molecular chaperones. Herpes virus protein VP22.C1 shares the property of some secondary structure retention, but its tertiary structure is clearly not lost, although it is altered. Since both VP22 and its 159-301 fragment exit and enter cells by an unknown mechanism, accompanied by the fact that the protein appears to exists in altered conformational forms under physiological conditions, some type of interaction of these states with lipid bilayers seems
plausible.

As an initial test of this hypothesis, we examined the interaction of VP22.C1 with a model lipid, *trans* parinaric acid (transPA) between 20°C and 60°C. Binding of the transPA to the protein was observed in the range of 40°C to 55°C, with an apparent stoichiometry of 1 to 2 parinaric molecules binding per protein molecule. We cannot, however, rule out some non-specific binding at higher and lower temperatures, since (a) a threefold increase in fluorescence over control was observed at each temperature examined, and (b) an increase in baseline was also observed which could not be completely subtracted from the emission spectrum. Thus, at least at higher temperatures, this linear increase in baseline could partially mask any binding activity present.

The highly basic nature of VP22.C1 suggests the possibility that the protein may interact with polyanions. In fact, evidence for the interaction of both a sulfated polysaccharide and polynucleotide were found. As detected by circular dichroism, the presence of both polyanions alters the CD spectrum of the protein, decreasing its intensity. In the case of the oligonucleotide, the shape of the spectrum and the CD melting profile are perturbed as well. This latter phenomenon corresponds to an increase in the β sheet content of the protein accompanied by a decrease in helix content. The high-temperature spectrum of the protein is also altered upon addition of either polyanion, where a more significant decrease of α helix content is apparent. Somewhat surprisingly, however, no major effect upon the thermal stability of the secondary structure of the protein is apparent in either case, a result often observed upon the binding of heparin and oligonucleotides to many proteins(41). Fluorescence spectroscopic investigation of the interaction, however, does indicate a stabilization of the tertiary structure of the protein with regard to the degree of solvent exposure of the two tryptophan residues. Binding of either
polyanion to the protein greatly retards the degree of unfolding observed at higher temperatures, observed to a greater degree in the presence of the oligonucleotide than heparin. The interaction of VP22.C1 with heparin suggests that it could interact with cell surface proteoglycans. This interaction could be involved in VP22's transport processes, but this remains to be demonstrated. The binding of oligonucleotides to VP22 could reflect the ability of the protein to interact with nuclear DNA, consistent with the nuclear localization of the protein. Interestingly, the indication of a polynucleotide induced conformational change in VP22.C1 does not appear to disrupt its transport activity (O'Hare, unpublished data). This clearly increases the potential use of VP22 as an oligonucleotide delivery vehicle.

A conformational feature found in many DNA binding proteins is the zinc finger structure, many of which consist of two cysteine and two histidine residues held in a stereochemically precise manner(37). VP22.C1 possesses this combination of residues, although they are not near one another in the protein's linear sequence(4). Based on this consideration, the interaction of VP22.C1 with zinc, as well as magnesium, was investigated using circular dichroism and fluorescence spectroscopy. A significant difference in secondary structure was observed initially upon addition of the cations, corresponding to an increase in overall helix content with a corresponding loss in β sheet. Additionally, there were small changes in the melting profile of the protein, which were highly reproducible. The post thermal transition spectrum was altered in the presence of both cations. The differences in the effects of the magnesium and zinc melting profiles, although subtle, indicate that this could be more than a non-specific protein-cation interaction. This is supported by the results of fluorescence studies of the interaction. Although no significant changes in tryptophan emission were observed in the presence of either cation, an aggregation event was observed in the presence of zinc that was not
seen with either magnesium or in the protein by itself. Direct binding studies will be necessary to further clarify the nature of cation/VP22.C1 interactions.

In addition to playing a key role in zinc finger motifs, free thiols are also a common feature of “leaderless” secretory proteins, those proteins secreted without any conventional secretory signal peptide(5). Upon investigation of the state of the cysteine sulfhydryl groups of VP22.C1 with DTNB, we discovered that a small portion of the protein is present in solution as a dimer, joined by an intermolecular disulfide bond. The remaining cysteine residue in the dimer, as well as both cysteines in the monomer, seem to be only partially exposed to the solvent, since the assay under both denaturing and native conditions indicates ~0.7 cysteine side chains are available for reaction with the DTNB. Since the original purification buffer contained β-mercaptoethanol, which was then removed by dialysis prior to performing any structural studies, the extent of dimer in each sample was somewhat variable between samples. If the formation of the dimer has any effect on the secondary or tertiary structure of the protein, one would expect there to be variability between samples in the fluorescence and circular dichroism experiments. No such variation was observed, however, over a course of study of multiple individually dialyzed samples from more than four different productions of the protein. Thus, we do not believe that the presence of a small (≤ 5%) amount of dimer has any major effect on the spectroscopic studies reported here.

It is worth noting that some of the properties exhibited by VP22.C1 are shared with several other proteins that appear to both enter and exit cells by an unconventional route(5). Here, we will focus on three proteins, limiting our discussion to the similarities between them. VP22, FGF-1 and the HIV-1 Tat protein all exhibit non-classical secretion, uptake, and nuclear localization, as well as the ability to tow attached proteins into cells(42), although VP22 uniquely
displays the ability to move from cell to cell. All three proteins bind polyanions. In the case of both FGF-1 and the Tat protein, polyanion binding appears to prevent translocation activity\(^\text{(43,44)}\). It has been hypothesized for FGF-1 that the binding of polyanions stabilizes the native state, preventing interaction of the molten globule form of the protein with the lipid bilayer\(^\text{(43)}\). All three proteins, as well as many others expressing non-classical secretory activity, possess free thiol moieties\(^\text{(5,45,46)}\). Interestingly, one of the free thiols of FGF-1 also participates in an intermolecular disulfide bond \(^\text{(47)}\). Finally, the presence of partially unfolded states of both FGF-1 and VP22 are necessary for lipid bilayer interaction\(^\text{(20)}\). This has not been demonstrated for the Tat protein per se, but it has been shown that a dramatic increase in the efficiency of Tat fusion protein translocation occurs when the fusion protein has been partially unfolded\(^\text{(48)}\). These similarities between proteins with such unique function suggest that perhaps there is a key structural element(s) to these non-classical transport processes, although the physical basis of VP22's unique transport properties remain to be better defined. Furthermore, this key structural element appears to be more complex than merely a shared native structural fold, as the protein themselves are quite structurally diverse, FGF-1 consisting of primarily β-sheet structure\(^\text{(41)}\), the transport competent peptide of the HIV-1 Tat protein containing an undefined structure, although preliminary reports suggest that the peptide is largely disordered, with a possible amphipathic helix\(^\text{(49,50)}\), and VP22.C1, as illustrated here, containing a mix of helical, sheet and disordered elements. Therefore the relationship between the proteins, if indeed structural in nature, may lie somewhere other than within the "native" state.

**ACKNOWLEDGEMENTS**

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University of Kansas for support and funding. Additional funding was provided by an NIGMS Biotechnology Training Grant to the University of Kansas and a Bristol Myers Squibb Fellowship to Ms. Kueltzo.
Footnotes

1Abbreviations - Herpes Simplex Virus type 1 (HSV-1), acidic Fibroblast Growth Factor (FGF-1), Fourier Transform Infrared (FTIR), Circular Dichroism (CD), isopropyl β-D-thiogalactoside (IPTG), polyethyleneglycol- p-isoctylphenyl ether (NP40), phenylmethysulfonyl fluoride (PMSF), Dulbecco’s Modified Minimal Essential Medium (DMEM), newborn calf serum (NCS), 3-[N-Morpholino]propanesulfonic acid (MOPS), Molar Ellipticity ([θ]m), Differential Scanning Calorimetry (DSC), Molar Heat Capacity (MHC), Fourier Self Deconvolution (FSD), trans Parinaric Acid (transPA), dodecyl sodium sulfate (SDS), 5-5'-dithio-bis(2-nitrobenzoic acid) (DTNB).
References


Figure Legends

Figure 1. Immunofluorescence of import of VP22.C1 after 50µL application to tissue culture. The protein is taken up by the cell, where it then localizes in the nucleus.

Figure 2. UV and fluorescence spectra of VP22.C1. Spectra were collected at room temperature (~22°C). UV absorbance maximum occurs at 284nm. The tryptophan emission maximum (excitation at 280nm) occurs at ~330nm.

Figure 3. CD and FTIR spectra of VP22.C1. a) CD spectrum in solution at RT. Negative ellipticities occur at 206 and 223nm. Secondary structure is estimated at ~30% α helix, 17% β sheet, and 51% random and/or turn structure. b) FTIR spectrum and Amide I deconvolution in solution at RT. Secondary structure estimate: 30% α helix, 40% β sheet, 15% β turn, and 15% random structure.

Figure 4. Deconvolution of representative excess molar heat capacity data from DSC thermogram of VP22.C1. The high temperature transition midpoint is 48°C ± 0.7 (SEM, n=2). Underlying components are present at 26.2, 31.5, 37.5, 41.9, 46.1, and 49.8°C (χ² = 4.9).

Figure 5. Circular dichroism spectra of VP22.C1 as a function of temperature. Loss of ellipticity at 223nm is observed with increasing temperature. Significant secondary structure is retained at higher (60°C) temperatures.
Figure 6. Fluorescence temperature studies of VP22.C1. Representative intensity (●) and peak position (♦) plots, showing transitions in the range of 30°C to 55°C, with transition midpoints of 40.9 ± 0.8°C (SEM, n=3) and 46 ± 1.3°C (SEM, n=5), respectively.

Figure 7. Changes in fourth derivative UV spectral peaks with temperature. Solid and hollow symbols represent tryptophan and phenylalanine peaks, respectively.

Figure 8. trans-Parinaric Acid.

Figure 9. CD temperature studies of VP22.C1. Ellipticity at 215nm as a function of temperature. (□) Native protein. (○) VP22.C1 + 5mM zinc. (♦) VP22.C1 + 5mM magnesium. (+) ~1:1 [VP22.C1]:[heparin]. (▲) 1:1 [VP22.C1]:[oligonucleotide]. Midpoints of the transitions are 46.1 ± 0.9°C, 46.9 ± 0.8°C, 43.7 ± 1.2°C, 45.5 ± 0.4°C, and 47.7 ± 0.6°C, respectively (SEM, n=3).

Figure 10. CD Spectra of VP22.C1 with cations and model polyanions.

(——) VP22.C1. (········) VP22.C1 + 5mM Mg++. (——) VP22.C1 + 5mM Zn++. (—······) 1:1 [VP22.C1]:[heparin]. (————) 1:1 [VP22.C1]:[oligonucleotide]. (a) 20°C. (b) 60°C.
**TABLE I**

*Secondary Structure Estimates for VP22.C1*

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<th>% β-Sheet (SEM)</th>
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*Italicized numbers indicate a P value less than 0.05 as determined by Student's t-test.*
trans-Parinaric Acid

18:4  9t, 11t, 13t, 15t
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