Conformational Lability of Herpesvirus Protein VP22*

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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 Fourier transform infrared analyses indicate a secondary structure consisting of approximately 30% α-helix, 17% β-sheet, and 51% disordered and turn structure. Unfolding studies conducted by CD, differential scanning calorimetry, and fluorescence reveal a series of discrete structural transitions in the range of 20–60 °C. CD and fluorescence studies of interactions between VP22.C1 and divalent cations and model polyanions indicate that Mg2+, Zn2+, oligonucleotides, and heparin interact with the protein, changing 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.

VP22 is a 38-kDa, 301-amino acid residue protein encoded by the UL49 gene of herpes simplex virus type 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 herpes simplex virus type 1 infection process is not presently understood, although it has been shown that the protein is modified by phosphorylation (2) and induces bundling of cytoskeletal microtubules (3). The property that has stimulated the most interest in the protein, however, is the unique transport activity demonstrated in initial function studies (4). 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 (Fig. 1). 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 (4), 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 that utilize nonclassical secretory mechanisms to traverse lipid bilayers (5), including acidic fibroblast growth factor (FGF-1) (6, 7), interleukin-1β (8), the Antennapedia homeodomain (9, 10), and the HIV-1 Tat protein (11). 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 nonclassical export utilizes an ATP-driven protein transporter (12). 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), herpes simplex virus type 1 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. Such experiments have been successfully conducted in multiple cell lines (e.g. COS-1, BHK-21, HeLa, Vero (4)), in

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¶ The abbreviations used are: FGF-1, acidic fibroblast growth factor; FTIR, Fourier transform infrared; NCS, newborn calf serum; MOPS, 3-(N-morpholino)propanesulfonic acid; DSC, differential scanning calorimetry; trans-PA, trans-parinaric acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).
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 20–60 °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 S.E.

**Materials**—Phosphorothioate oligonucleotides (5′-CCC CCA CCA CTT CCC TTC TC-3′) were synthesized by Genset. trans-Parinaric acids were purchased from Molecular Probes, Inc. (Eugene, OR). High molecular weight heparin (M = 12,000–30,000) was purchased from Sigma. Dialysis materials were obtained from Pierce. SDS-polyacrylamide gel electrophoresis buffers, stains, precast 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 Escherichia 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 mid-exponential growth phase had reached ~0.4. Isopropyl β-D-thiogalactoside was then added to a final concentration of 0.1 mM, and incubation continued for a further 4 h. The cultures were cooled on ice and centrifuged at 6,000 rpm for 20 min at 4 °C. The pellets were frozen and stored at −20 °C overnight. Thawed pellets were then resuspended in ~30 ml of cold “lysis buffer,” lysosome was added to 1 mg/ml, and the sample was incubated for 30 min on ice with occasional shaking. Lysis was completed by sonication (three 10-s bursts at 100% power, nonidet P-40 was added to 0.1% followed by DNase I and RNase to 10 μg/ml, and the sample was incubated on ice for a further 20 min with occasional mixing. The lysate was then drawn through a 21-gauge needle using a 60-ml syringe three times and centrifuged at 14,000 rpm at 4 °C for 30 min.

The supernatant was added to 3.5 ml of DEAE-agarose (50% slurry equilibrated in lysis buffer) and incubated for 30 min at 4 °C, rotating throughout. The agarose was pelleted (3,000 rpm at 4 °C for 5 min), and the unbound supernatant was added to a column of ~5 ml Ni2+-nitrilotriacetic acid beads (50% slurry equilibrated in lysis buffer plus 0.1% Nonidet P-40). The unbound fraction was collected, and the column was washed at 12 ml/min with “wash buffer.” VP22.C1 was then eluted in 20 ml employing a 40–500 mM imidazole gradient. One-milliliter fractions were collected, frozen, and stored at −70 °C, with VP22 eluting at around 180 mM imidazole.

The lysis buffer consisted of 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin and apoprotein (alternatively, Protease Inhibitor Mixture tablets, Complete™ EDTA-free (Roche Molecular Biochemicals) were used at 1× concentration).

The wash buffer was prepared as the lysis buffer with 10% glycerol and 40 mM imidazole. The elution buffer was prepared as lysis buffer with 10% glycerol and 500 mM imidazole.

The protein was then further purified by cation exchange chromatography (Mono S®; Amersham Pharmacia Biotech). Samples from the NaCl/nitrilotriacetic acid purification were diluted from buffer A containing 100 mM NaCl, applied to a Mono S HR5/5 column equilibrated in buffer A containing 100 mM NaCl, and eluted with a salt gradient (100 mM to 1 M NaCl in 15 ml). VP22.C1 eluted at approximately 250 mM NaCl. The peak fractions were divided into aliquots where necessary and stored at −70 °C. Buffer A contained 50 mM sodium phosphate, plus 100 mM NaCl, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and apoprotein (alternatively, Protease Inhibitor Mixture tablets, Complete™ EDTA-free (Roche Molecular Biochemicals) were used at 0.1× concentration) and 10% glycerol. Buffer B was prepared as buffer A with 1 M NaCl.

**Transport Activity of VP22.C1**—Cells were grown on coverslips in six-well dishes in Dulbecco’s modified minimal essential medium containing 10% newborn calf serum (NCS). Prior to the import assay (1 h), the medium was changed, and the cells were incubated with 1 ml of fresh Dulbecco’s modified minimal essential medium containing 1% NCS.

Aliquots of VP22.C1 (200 ng) as indicated (clarified by centrifugation at 12,000 rpm at 4 °C for 10 min) were added to prewarmed medium containing 10% NCS with a final volume of 1 ml. The medium was then aspirated from the cell cultures and replaced with the medium containing protein, and the cells were incubated at 37 °C for 30 min. The medium was then removed, and the cells were washed gently with PBS and fixed in methanol at room temperature for 15 min. The coverslips were then blocked in phosphate-buffered saline containing 10% NCS and probed with a rabbit polyclonal antibody (AQB600 1:100) directed against a glutathione S-transferase fusion protein containing the C-terminal residues 257–301 of VP22. The secondary antibody was fluorescein isothiocyanate-conjugated goat anti-rabbit antisemum.

**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 10 mM phosphate containing 200 mM NaCl at pH 4. Metal cation interaction studies were conducted in 10 mM MOPS, 200 mM NaCl, at pH 7.4. The presence of a 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; molecular weight cut-off, 7,000), with a buffer to sample ratio of 10,000:1 (v/v), over a period of 6 h. In some cases, solutions were centrifuged at 10,000 rpm for 60 s to remove traces of aggregated protein prior to concentration determinations and subsequent experimental procedures.

**UV-Visible Spectroscopy**—Protein concentrations were determined at room temperature by UV absorbance measurement at 280 nm (ε = 11,214, 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 180 μg/ml in 5 min 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 0.01 nm 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 290 nm) were collected using a 1-mm path length cuvette, sealed with a Teflon stopper. A resolution of 0.1 nm and scanning speed of 10 nm/min with a 2-s response time were employed. Spectra were presented as an average of three consecutive spectra. In some cases, spectra were recorded at 10 °C intervals employing a thermostatted cuvette holder. Equilibrium times of 5 min were included at each temperature interval. Alternatively, the molar ellipticity ([θ]θ) at 215 nm was collected at 0.1 °C intervals, using a 15 °C/h 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 240 μg/ml was employed in all experiments. Nase reduction and data analysis was achieved using Standard Analysis and Temperature/WaveLength Analysis programs (Jasco) and MicroCal OriginTM 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 and 100 °C under 3 atmospheres of pressure. A temperature scanning rate of 1 °C/min was used in all cases. An equilibration time of 10 min was included at the beginning and end of each cycle. Sample and reference buffers were degassed prior to use, and a protein concentration of 600 μg/ml was employed. A buffer base line was subtracted from each scan, and the data were converted to molar heat capacity. The excess molar heat capacity 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 spectrophotometer fitted with an MCT detector and continuous dry air purge. Dry protein samples were analyzed by placing 25 μl of a 1 mg/ml solution on polyethylene cards and drying under vacuum for 1 h. Spectra were recorded at room temperature, with a resolution of 4 cm⁻¹, and 256 consecutive scans were collected for each sample. Buffer and card spectra were then subtracted. Solution studies were performed using a 5.3-μm path length transmission cell (BioCell™, Biotools, Inc.). Ten microliters of a 4.5 mg/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 2300 cm⁻¹ as a reference for path length matching, and were then subtracted from the protein spectra. Subtractions, smoothing, and Fourier self-deconvolution of the amide I band of all protein samples were performed using Omnic software (Nicolet). Fourier self-deconvolution bandwidth and enhancement parameters were 21 and 2.5, respectively. Final deconvolution into underlying components was performed with Galactic Peptide (Felix (PTI) and MicroCal Origin® 5.0 software).

**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 280 nm, an emission range of 300–450 nm, and a data collection rate of 1 nm/s. For thermal perturbation studies, excitation and emission slits were set at 3 nm. For all experiments, data were corrected internally for lamp fluctuations and the wavelength dependence of intensity. Tryptophan peak positions were determined by first solving, using the Fourier self-deconvolution trace and second derivative solution into underlying components was performed with Galactic Peak

**Steady State Fluorescence—**Excitation and emission spectra were recorded using a spectrofluorimeter (PTI) equipped with a thermostated cuvette holder. Temperature was maintained constant over the course of each titration point was used, during which the sample was stirred continuously. Excitation and emission spectra were recorded using 1-cm path length cuvettes. For all experiments, data were corrected internally for lamp fluctuations and the wavelength dependence of intensity. Tryptophan peak positions were determined by first solving, using the Fourier self-deconvolution trace and second derivative solution into underlying components was performed with Galactic Peak

**Molecular Weight Determination—**Molecular weight of protein in solution was determined by SDS-polyacrylamide gel electrophoresis gel electrophoresis, using the Novex NuPage™ 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 nonreduced conditions. Protein bands were detected using Coomassie Blue staining.

**RESULTS**

Analysis of purified VP22.C1 by nonreducing SDS-polyacrylamide gel electrophoresis indicates the presence of a single band corresponding to the monomeric protein (18.5 kDa) 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). The presence of reducing agents (10 mM dithiothreitol) 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.
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 284.0 nm with a distinct shoulder at 291.2 nm, 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 330 nm, 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 206 and 223 nm, suggesting a significant α-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 (pH 7.4), the protein consists of 30 ± 3% α-helix, 17 ± 3% β-sheet, and 51 ± 1% random and/or turn structure (n = 16). The amount of β-turn structure was predicted to be ~25% 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 ± 1% α-helix, 39 ± 2% β-sheet, 14 ± 1% β-turn, and 15 ± 2% disordered structure (n = 6) were predicted. Additional analysis of the protein by FTIR was performed with 1.2 mg/ml solutions dried on polyethylene cards. Secondary structure of the dried protein was estimated to be 40 ± 3% α-helix, 32 ± 1% β-sheet, 14 ± 1% turn, and 15 ± 3% 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 are complex (Fig. 4). A main transition is observed from 40 to 55 °C, having a midpoint near 48.0 ± 0.7 °C (n = 2). This is accompanied by a second major transition between 20 and 40 °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 Fig. 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 ($\chi^2 = 4.9$).

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 223 nm 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 215 nm versus temperature (Fig. 9). A single transition occurs between 35 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 pretransition (10–30 °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 330 nm decreases dramatically between 35 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 330 to 342 nm over this temperature range. A sharp transition is observed between 30 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 ~280 nm. An approximately 0.6-nm blue shift of the tryptophan peaks at 274, 280, 284, and 291 nm was observed in the range of 25–50 °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 264 nm representing phenylalanine exposure also exhibit shifts, with changes occurring as low as 25 °C (Fig. 7).

Interaction of VP22.C1 with trans-Parinaric Acid—VP22.C1 possesses unique, nonclassical 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).

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 trans-PA were performed at temperatures from 20 to 60 °C to examine the effects of protein conformational state upon any interaction. Fluorescence emission intensities of the parinaric acids were measured at 410 nm and 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 than that of the control (parinaric acid in buffer). Saturation type
binding isotherms were observed at 40, 45, and 50 °C, but not at temperatures of ≤35 and ≥55 °C (results not illustrated). Binding constants and stoichiometries could not be reliably extracted from the data, however, due to concurrent shifts in base line 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 and 45 °C, however, it appears that the interaction has a stoichiometry of 1–2 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. The addition of both polyanions resulted in an overall increase of tryptophan exposure at 20 °C, suggesting some type of limited stabilization of the native protein under these conditions.

No significant change in secondary structure content is detectable at pretransition temperatures (20 °C) upon the addition of heparin (Fig. 10a, Table I). 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 with the native protein (Table I). 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).

The 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 ~335 nm upon reaching 60 °C. Compared with the shift to ~341 nm 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 the addition of the oligonucleotide to protein solution, we observed an ~1-nm 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 ~333 nm at 60 °C (not illustrated).

VP22.C1 contains two cysteine and two histidine residues, suggesting that it could possess a metal binding site (37).
therefore examined the interaction of VP22.C1 with magnesium and zinc cations employing CD and fluorescence spectroscopy with the hypothesis that any such change might involve a conformational change of the protein. Comparing the ellipticity at 215 nm 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, 46.9 ± 0.8, and 43.7 ± 1.2 °C (native protein, with the addition of zinc and magnesium, respectively). However, the transition between 35 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 the addition of either cation, a significant change in secondary structure content is detectable at pretransition temperatures (20 °C), consisting of an increase in helix content and loss of β-sheets (Table I). These differences are subtle enough that they are not readily apparent in the spectra themselves (Fig. 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 the addition of magnesium or the native VP22.C1 is evident (Fig. 10b). The difference centers around 222 nm, possibly indicating a greater amount of α-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 the addition of the polyaniions (results not illustrated). The 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 with the emission of the protein alone. Aggregation of the protein sample was observed at 60 °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 herpesvirus 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 159–301. 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 and 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 λ<sub>max</sub> of 284.0 nm at room temperature. The fluorescence spectrum of VP22.C1 shows a single peak centered at 330 nm. 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 (≈340 nm) suggests that one of the tryptophan residues may be partially exposed.

The far UV CD spectrum of VP22.C1 manifests strong negative ellipticity at 206 and 223 nm, indicating substantial α-helix content, while the distinct plateau at 215 nm suggests the presence of β-sheet. Secondary structure estimates obtained from three different algorithms give values of 30 ± 3% α-helix, 17 ± 3% β-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 β-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<sup>−1</sup> is consistent with this possibility (38). Another possibility is that there is significant intrinsic error in the CD secondary structure estimation methods for β-sheet content. An analysis of various programs’ ability to correctly estimate different types of secondary structure in comparison with known crystal structures was recently performed by Greenfield (39). The study demonstrated that the ability of such programs to estimate β-sheet and β-turn content is greatly reduced in comparison with that of α-helix. It is also possible that we have misassigned the disordered peak usually found at 1641–1647 cm<sup>−1</sup>, which may not have been completely resolved from the peak at 1635–1640 cm<sup>−1</sup>, normally assigned to β-sheet (32). This would result in an overestimation of β-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 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 dem-

| Sample            | Temperature | α-Helix (%) (S.E.) | β-Sheet (%) (S.E.) | Disordered/ Turn (%) (S.E.) |
|------------------|-------------|-------------------|-------------------|-----------------------------|
| VP22.C1          | 20          | 31 (4)            | 16 (3)            | 53 (2)                      |
| VP22.C1 + 5 mM Mg<sup>2+</sup> | 20          | 37 (4)            | 11 (3)            | 50 (3)                      |
| VP22.C1 + 5 mM Zn<sup>2+</sup> | 20          | 42 (5)            | 10 (4)            | 48 (2)                      |
| 1:1 [VP22.C1]: [heparin] | 20          | 30 (1)            | 15 (1)            | 55 (2)                      |
| 1:1 [VP22.C1]: [oligonucleotide] | 60          | 12 (1)            | 37 (2)            | 51 (2)                      |
onstrate a large change in ellipticity at 215 nm. This change corresponds to a decrease in helical content, with a concurrent increase in β-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 330 to −342 nm, with a midpoint at 41 °C. This shift to 342 nm again indicates that this protein is not extensively unfolded, in which case a peak position of 350–355 nm 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 confirm this partial unfolding, since 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 (−1.2-nm) 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 with their native states thought to facilitate, in some ill defined way, their interactions with membranes or perhaps molecular chaperones. Herpesvirus 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, the protein appears to exist 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-PA between 20 and 60 °C. Binding of the trans-PA to the protein was observed in the range of 40–55 °C, with an apparent stoichiometry of 1–2 parinaric molecules binding per protein molecule. We cannot, however, rule out some nonspecific binding at higher and lower temperatures, since (a) a 3-fold increase in fluorescence over control was observed at each temperature examined, and (b) an increase in baseline was also observed that could not be completely subtracted from the emission spectrum. Thus, at least at higher temperatures, this linear increase in base line 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 polynucleotide, 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 the 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 polynucleotides 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. 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 2 cysteine and 2 histidine residues held in a stereochromically 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 the 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 nonspecific protein-cation interaction. This possibility 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

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2 P. O’Hare, unpublished data.
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 exhibit nonclassical transport processes. Although the physical basis of nonclassical transport remains to be better defined, we will focus on three proteins, limiting our discussion to the similarities between them. VP22, FGF-1, and the HIV-1 Tat protein exhibit nonclassical transport processes. Although the physical basis of nonclassical transport remains to be better defined, we will focus on three proteins, limiting our discussion to the similarities between them. VP22, FGF-1, and the HIV-1 Tat protein exhibit nonclassical transport processes.

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