Structural Analysis of the Extracellular Domain of Vaccinia Virus Envelope Protein, A27L, by NMR and CD Spectroscopy*

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This study presents the molecular structure of the extracellular domain of vaccinia virus envelope protein, A27L, determined by NMR and CD spectroscopy. A recombinant protein, eA27L-aa, containing this domain in which cysteines 71 and 72 were replaced with alanine, was constructed to prevent self-assembly due to intermolecular disulfide bonds between these two cysteines. The soluble eA27L-aa protein forms an oligomer resembling that of A27L on vaccinia virions. Heteronuclear correlation NMR spectroscopy was carried out on eA27L-aa in the presence or absence of urea to determine backbone resonance assignments. Chemical shift index (CSI) propensity analysis showed that eA27L-aa has two distinct structural domains, a relatively flexible 22-amino acid random coil in the N-terminal region and a fairly rigid α-helix structure in the remainder of the structure. Binding interaction studies using isothermal titration calorimetry suggest that a 12-amino acid lysine/arginine-rich segment in the N-terminal region is responsible for glycosaminoglycan binding. The rigid α-helix portion of eA27L-aa is probably involved in the intrinsic self-assembly, and CSI propensity analysis suggests that region N37-E49, with a residual α-helix tendency, is probably the self-assembly core. Self-assembly was ascribed to three hydrophobic leucine residues (Leu42, Leu45, and Leu48) in this segment. The folding mechanism of eA27L-aa was analyzed by CD spectroscopy, which revealed a two-step transition with a Gibbs free energy of 2.5 kcal/mol in the absence of urea. Based on these NMR and CD studies, a residue-specific molecular model of the extracellular domain of A27L is proposed. These studies on the molecular structure of eA27L-aa will help in understanding how vaccinia virus enters cells.

The process involved in the entry of a virus into its host cell is complicated and often involves multiple stages which require viral and cellular factors (1, 2). It is generally accepted that enveloped viruses bind to a cell surface receptor then triggers a conformational change in the virus-receptor complex (3). Co-receptors or fusion factors interact with the virus and initiate fusion of the virion envelope and the plasma membrane (4).

Vaccinia virus has a wide host range and infects many different cell types. It is conceivable that viral proteins mediate virus entry by binding to components that are ubiquitously expressed on cell surfaces. Previous studies suggest that recombinant vaccinia virus envelope protein A27L binds to heparin sulfate on BSC40 cells (5). In addition, when BSC40 cells are treated with sodium chlorate to block sulfation of glycosaminoglycan (GAG)1 side chains, the cells become more resistant to vaccinia virus infection (5). These results suggest that A27L interacts with negatively charged sulfates of GAGs and that this interaction may facilitate vaccinia virus entry into BSC40 cells. The A27L amino acid sequence includes a stretch of 12 amino acids, residues 21–32 (STKAAKKPEAKR), that is rich in lysine and arginine and is necessary for electrostatic interaction with GAG sulfates (15). In addition, this region of A27L binds to the cell surface (5). The amino acid sequence of A27L, deduced from the DNA sequence, indicates that the mature protein starts at serine 21 (6, 7), placing the arginine/lysine region at the N terminus and making it highly accessible for binding interactions. Furthermore, several monoclonal antibodies that recognize overlapping epitopes within this region of A27L prevent vaccinia virus infection (8). Thus, it is likely that this N-terminal region modulates the virus entry functions of A27L. It is clear that A27L is required for fusion of virus-infected cells, but the detailed molecular mechanisms of GAG binding and cell fusion remain unclear.

The molecular structure of A27L is not yet known, but it is expected to be important in understanding the mechanism by which vaccinia virus enters cells. Attempts to determine the structure by x-ray crystallography have been unsuccessful, primarily because of protein self-assembly. A27L has a strong concentration-dependent tendency to trimerize (9). In these high resolution NMR and CD studies, we have analyzed the molecular structure of a truncated A27L mutant, eA27L-aa, consisting of the extracellular domain of A27L in which two cysteine residues, Cys71 and Cys72, have been substituted with alanine, thus resulting in less aggregated than that seen with wild-type A27L. Self-assembly was also intentionally suppressed by the use of 2.5 M urea during NMR spectroscopy. Similar approaches have been recently used to improve spectroscopy results for other proteins showing spectral line-broadening due to molecular self-association (10–14). The NMR and

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CD results presented here were used to develop a residue-specific structural model of eA27L-aa. CD spectroscopy was also used to study the folding/unfolding of eA27L-aa, which involves a two-step process. These results will improve our understanding of vaccinia virus structure and of how the virus enters host cells.

EXPERIMENTAL PROCEDURES

Sample Preparation—eA27L, a recombinant protein consisting of the extracellular domain (amino acids 21–84) of the vaccinia viral protein, A27L, was expressed in bacteria and purified as described previously (15). A second recombinant protein, eA27L-aa, with an identical sequence to eA27L except that two cysteines, Cys71 and Cys72, were substituted with alanine residues, a change that did not affect the biological functions of the protein (9), was also expressed and purified, as was D-A27L, an N-terminal truncated derivative of eA27L (residues 33–84) lacking the GAG binding domain (residues 21–32) (5). All three proteins have a 14-aminoc acid T7-tag at the N terminus and a 13-aminoc acid hexahistidine tag (His-tag) at the C terminus; these tag sequences do not interfere with the function of A27L (9). eA27L-aa contains 91 amino acids and has a molecular mass of 10.3 kDa. In this study, the transformed bacteria were grown at 37 °C, then harvested, and proteins were purified with a nickel-NTA affinity column as described previously (15). For NMR studies, the 15N-labeled or 13C/15N-labeled proteins were dialyzed against 50 mM sodium phosphate, pH 5.0 (unless otherwise indicated) in 90% H2O and 10% D2O, and the protein samples (0.8–1.0 mM) were transferred to Shigemi NMR tubes (5 mm outer diameter). 2,2-Dimethyl-2-silapentane-5-sulfonic acid was used as the internal chemical shift standard (16, 17).

NMR Spectroscopy—All three-dimensional NMR spectra were recorded at 23 °C on a Bruker Advance 500 MHz spectrometer equipped with a 5-mm inverse triple resonance (1H/13C/BB) z axis gradient probe. Linear prediction was used in the 13C and 15N dimensions to improve digital resolution. Spectra were processed using XWINNMR and displayed using the AURELIA software package (Bruker, Karlsruhe, Germany). Backbone sequential assignments were based on the following pairwise three-dimensional NMR experiments: HNCO, HN(CA)CO, CBCA(CO)NH, CBCA(CO)NH (18, 19). A Fortran program was developed to search the connectivity of the sequence semi-automatically. The effect at low temperature was examined at 15 °C.

CD Spectroscopy—CD spectra were recorded on a Jasco J-720 spectrometer equipped with a water bath for temperature control. CD spectra were collected at 25 °C (except temperature-dependent studies) using either a quartz cuvette with a 1-mm path length and a protein concentration of 30 μM or a quartz cuvette with a 0.2-mm path length and a protein concentration of 120 μM; the latter combination was used to optimize sample conditions for NMR measurements. The step size was 0.2 nm with a 1.0-nm bandwidth at a scan speed of 50 nm/min. Each spectrum shown represents the average for five measurements. In thermal denaturation and renaturation experiments, the temperature was increased from 5 to 75 °C with an equilibration time of about 5 min at a scan rate of 20 °C/min. All spectra were collected in 50 mM potassium phosphate buffer with background buffer correction.

Isothermal Titration Calorimetry (ITC)—ITC experiments were performed using a MCS-ITC titration microcalorimeter (MicroCal, Northampton, MA) calibrated with electrically generated heat pulses, as recommended by the manufacturer. A digitally controlled water bath (model RTE-111, Thermo NESLAB, Portsmouth, NH) was used to maintain the operating temperature at 28 °C. Studies were carried out on three vaccinia virus proteins in the presence of a 15-fold molar excess of heparin: eA27L (0.15 mM)/heparin (2.2 mM), eA27L-aa (0.3 mM)/heparin (4.33 mM), and D-A27L (0.18 mM)/heparin (2.8 mM). Both the protein and the heparin were dissolved in 50 mM sodium phosphate buffer containing 50 mM sodium chloride. Heparin, with an average molecular mass of 3 kDa, was purchased from Sigma and was used without further purification. In pH studies, the binding of heparin to eA27L-aa (0.077–0.1 mM) was assessed at three different pH values, 6.5, 7.0, and 7.5. The heparin binding effect at low temperature was examined at 15 °C. All solutions were passed through a 0.45-μm filter and degassed prior to use. Stock solutions were diluted with a single batch of buffer to minimize artifacts due to differences in buffer composition. To measure the equilibrium binding constant, K_a, a
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RESULTS

eA27L-aa Oligomerization Probed by NMR and CD Spectroscopy—NMR spectroscopic analysis of eA27L-aa in solution is difficult because of poor spectral resolution due to self-assembly. In the two-dimensional $^{1}H/^{15}N$ HSQC spectrum of eA27L-aa at pH 5.0 in the native state, only one-third of the expected resonances were seen and the line widths were rather broad, the remaining two-thirds of the resonances being absent due to rapid $T_{2}$ relaxation decay (Fig. 1A). It was reported that the degree of self-assembly is sample concentration-dependent (9). To analyze the effect of concentration upon the formation of oligomerization, eA27L-aa was diluted systematically in a relatively low concentration range from 200 to 10 $\mu$m. The corresponding two-dimensional $^{1}H/^{15}N$ HSQC spectra revealed consistently the absence of a majority of resonances described above. And yet, significantly, the relative positions of individual resonances are virtually unchanged (Fig. 1, B and C); the data imply that the samples of this concentration range share similar structural features. Technically, it is rather difficult to specify the state of self-association of A27L-aa protein due to NMR limitation. In a separated sedimentation equilibrium analysis (9), A27L-aa exists as a trimer at 20 $\mu$m concentration, which is within our studied concentration range. Thus, based on NMR and sedimentation analysis results, we hereby suggest that A27L-aa maintains a trimeric form for these concentrations.

eA27L-aa samples at different pH values and urea concentrations have been carefully examined by far-UV CD spectroscopy. As a high molar protein concentration is required for NMR, a combination of a high protein concentration (120 $\mu$m) and small quartz cuvette (0.2 mm path length) was used for this approach. The residual $\alpha$-helical content of eA27L-aa in the absence of urea was monitored by varying the pH from 3.0 to 6.0 (Fig. 2A), the overall CD intensity decreased substantially at pH 4.5 and decreased further at lower pH values, consistent with observations reported previously (9). At pH 5.0, the partially denatured sample retained considerable residual conformation, suggesting that this pH would be suitable for NMR structural analysis. To further optimize the urea concentration, CD studies were carried out on eA27L-aa at pH 5.0 at urea concentrations ranging from 0.5 to 4.0 $\mu$m (Fig. 2B). Due to interference by urea in the CD absorption below 210 nm, the CD intensity in this region is not shown. The overall trend indicated a systematic decrease in secondary structure as the urea concentration increased. At 2.5 $\mu$m urea and pH 5.0, the protein was partially unfolded but contained considerable residual $\alpha$-helix content.

In the presence of urea, the aggregated molecules dissociated to form monomers. eA27L-aa in 2.5 $\mu$m urea was partially unfolded, with the protein possessing considerably residual $\alpha$-helical content. At urea concentrations of 4.0 $\mu$m or higher, a comparable amount of resonances appears in the spectrum. As seen, NMR spectra under these conditions (Fig. 3, A and B) have significant improvement in spectral resolution such that the entire 90 resonances were detected available for backbone assignments. It was indicated by the CD results (see Fig. 2B) that the eA27L-aa protein at urea 2.5 $\mu$m retains considerable residual secondary structure, but the one at 4.0 $\mu$m reaches an unfolded state. Thus, it was found, using the conditions of pH 5.0 and 2.5 $\mu$m urea, that the protein showed moderate chemical shift dispersion and retained residual secondary structure and was therefore suitable for NMR structural analysis. The effect of low temperature on residual structure was also examined by acquiring two-dimensional HSQC spectra of eA27L-aa in the present of urea at 15 °C (Fig. 3C) and was found to be negligible over this temperature range. All subsequent NMR measurements were performed at the ambient temperature of 23 °C.

NMR Spectra of Native eA27L-aa Are Exclusively Due to 33 Residues, Met4–Asp36, in the N-terminal Region, Which Contains the Heparin Binding Domain—Three-dimensional NMR experiments (HNCA, HNCOCA, CBCANH, CBCACONH, HCNO, and HNCACO) (18, 19) were used to determine the sequential assignments for $^{13}C/^{15}N$ double-labeled eA27L-aa in the partially denatured state in 2.5 $\mu$m urea and at pH 5.0. Based on the inter- and intrarresidue correlations (Fig. 4), with the exception of residues Lys$^{50}$, Lys$^{58}$, Gln$^{61}$, Arg$^{65}$, and Glu$^{71}$, which did not appear in the three-dimensional spectra, the backbone resonances were successfully assigned. The full resonance assignments of partially denatured eA27L-aa (Fig. 5B) provide unique $^{13}C$ chemical shift information valuable for structural analysis.

Employing a two-dimensional mapping approach, the resonance assignments for eA27L-aa in the native state were determined based on those for the partially denatured state (Fig. 5B). Interestingly, the resulting intensities for eA27L-aa in the

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**FIG. 2.** Secondary structure of eA27L-aa examined by CD spectroscopy at the high protein concentration of 120 $\mu$m. A, CD spectra in the absence of urea at pH 3.0 (○), 4.0 (▲), 5.0 (■), 5.5 (▲), and 6.0 (△). B, CD spectra at pH 5.0 and urea concentrations of 0.5 M (○), 1.0 M (▲), 2.0 M (■), 2.5 M (solid line), 3.0 M (△), and 4.0 M (▲); for comparison, the CD spectrum of native eA27L-aa is shown (+). Note that the partially denatured eA27L-aa at 2.5 M urea and pH 5.0 is suitable for NMR structural measurements, as it retains considerable residual $\alpha$-helical content.
native state arose exclusively from 33 residues, Met4–Asp36, in the N-terminal region (Fig. 5 A), which includes the GAG binding domain, Ser15–Arg26. To check these assignments, the three-dimensional correlation NMR methods described above were applied to the uniformly 13C/15N-double-labeled eA27L-aa sample and the backbone resonance assignments determined independently from the three-dimensional correlation analysis were found to be in good agreement with those deduced from the two-dimensional mapping approach. Thus, it was confirmed that the NMR measurements for eA27L-aa in the native conformation were exclusively due to Met4–Asp36.

eA27L-aa Contains a Structureless Flexible Chain and a Rigid /H9251-Helix Coiled-coil—It is generally accepted that the three-dimensional structure of a protein can be determined by NMR spectroscopy relying principally on proton-proton NOE measurements. Unfortunately, in the case of eA27L-aa in the partially unfolded state, the NOE signals detected by three-dimensional NOESY-HSQC (20–23) were too weak to be analyzed, due to the rapid exchange of amide protons. Thus, instead of the NOE approach, the 1H, 13C, and 13CO chemical shifts were employed to determine the residual secondary structure (24). In previously published work, deviations of 1H, 13C, and 13CO chemical shifts from their random coil values have been used to measure secondary structure in folded proteins (16, 25, 26) and conformational preferences in unstructured domains (27). On average, for α-helices, the 13C and 13CO chemical shift values are shifted downfield, respectively, by 2.6 and 1.7 ppm, and the 1H chemical shift values are shifted upfield by 0.38 ppm. When the 1H, 13C, and 13CO chemical shift index (CSI) was calculated for partially denatured eA27L-aa, the results showed that most of the backbone chemical shifts deviated less than 0.5 ppm from the random coil chemical shifts, the actual values being <0.5 ppm for the 13CO shifts, <0.5 ppm for the 13C shifts, and <0.3 ppm for the 1H shifts. Thus, the overall secondary structure in the denatured state was far below the average level for the α-helix motif, suggesting a generally structureless random coil character.

The residual secondary structure in partially denatured eA27L-aa was then determined using the CSI propensity approach. The α-helical character was individually quantified for the 13C, 13CO, and 1H resonances for each residue by comparing their chemical shifts to the values reported for a random coil (16). The structural propensity index was defined as $(\delta_{\text{random}} - \delta_{\alpha\text{-helix}})$, where $\delta$ is the experimental chemical shift, and $\delta_{\text{random}}$ and $\delta_{\alpha\text{-helix}}$ the chemical shift values reported in the literature for random coil and α-helix, respectively (16). A residue is considered to be α-helical if the propensity index falls between 0.8 and 1 and to be random coil if it is less than, or equal to, 0.2, while for indices between 0.3 and 0.8, the relative tendency to form an α-helix is proportional to the propensity index. This analysis is particularly useful in locating regions in partially denatured proteins with limited residual secondary structure. For example, in the case of eA27L-aa in 2.5M urea and at pH 5.0, most of the 13C chemical shift propensity indices were less than 0.3, but in a certain group of residues (Asn37–Glu49), they fell between 0.4 and 0.8 (Fig. 6), suggesting the residual unsuppressed α-helical character of this region. Although 13CO and 1H chemical shift propensities were less sensitive, they also showed an unsuppressed α-helical character in this region. These results demonstrate that partially denatured eA27L-aa (pH 5.0 and 2.5 M urea) reveals a residual α-helical molecular structure that can be identified using CSI propensity analysis.

The region Met4–Asp36 can be divided into two segments,
Met⁴–Ser¹⁴ and Ser¹⁵–Asp³⁶. The former, i.e. the N-terminal T7-tag, is unique to the recombinant proteins and is presumably irrelevant to the protein native structure. While the Ser¹⁵–Asp³⁶ region is equivalent to 21–42 in the wild-type A27L sequence, which has an important biological function, since it contains a lysine/arginine-rich domain, Ser¹⁵–Arg²⁶, which binds heparin via electrostatic interactions (15). Interestingly, in native eA27L-aa, this heparin binding domain has a random coil secondary structure with considerable conformational disorder, as shown by the ¹³C/H and the ¹³CO chemical shifts (Table I). The same chemical shift values were obtained for this domain in partially denatured eA27L-aa, indicating that the highly flexible random coil structure was unaffected after denaturation.

CSI propensity analysis showed that eA27L-aa contained two fairly distinct structures, a flexible random coil and a rigid α-helical motif. A different approach, measurement of the $T_2$ relaxation time, can also provide dynamic information on the protein structure. The NMR spectra for eA27L-aa showed slow molecular dynamics due to sample self-assembly. In particular, the resonances of residues directly involved in self-assembly were absent from the HSQC spectra (Fig. 1A), and a fast $T_2$ relaxation mechanism was estimated (i.e. time scale smaller than 1 ms), which led to rapid decay prior to data acquisition. For residues not directly involved in self-assembly, a relatively long $T_2$ relaxation was estimated (i.e. >50 ms) based on the $¹H$ line width in the two-dimensional $¹H/¹⁵N$ HSQC spectrum. Thus, the $T_2$ relaxation phenomena differed by 2 orders of magnitude in time scale,indicating dramatic dissimilarities in molecular structure and dynamics within eA27L-aa. Although these NMR data provide unique structural information about eA27L-aa, such slow tumbling molecules with $T_2$ relaxation times less than 1 ms are beyond the analytical capability of solution NMR, and the detailed characterization of the rigid α-helical region of eA27L-aa was therefore performed by CD spectroscopy, as discussed below.

**CD Studies of the Effects of Heparin, Temperature, and pH on eA27L-aa Secondary Structure—**The CD ellipticity seen at 222 nm was indicative of a helical structure in eA27L-aa. Thermal denaturation and renaturation processes in the absence of urea were monitored by the 222 nm ellipticity, which showed two almost identical sigmoidal curves, indicating a
highly cooperative, reversible two-step folding/unfolding structural transition and a melting temperature of about 50 °C (Fig. 7A). When the same experiment was performed on a mixture of eA27L-aa (30 μM) and heparin (15 μM), a similar reversible thermal denaturation process with the same melting temperature of 50 °C was seen (Fig. 7B), suggesting that the overall thermal stability and secondary structure of eA27L-aa were unaffected by heparin binding. In pH titration studies, the ω-helical secondary structure content decreased with decreasing pH, indicating an equilibrium point at pH 4.3 (Fig. 7C) and that the protein was fully unfolded at pH values of 3.0 or lower. The pH denaturation curve was identical to that for A27L with a point mutation (9).

When the effect of heparin binding of eA27L-aa was investigated at heparin concentrations from 0 to 49 μM, the CD ellipticity systematically decreased as the heparin concentration increased (Fig. 7D). Such a substantial change in CD intensity was ascribed to heparin either unfolding or precipitating eA27L-aa. The former interpretation implies that heparin binds to the flexible random coil region and unwinds the ω-helical secondary structure of eA27L-aa. If this were the case, we would expect to detect a lower melting temperature for heparin-bound eA27L-aa than for eA27L-aa alone; however, the melting temperature of 50 °C for the complex was the same as that described above for eA27L-aa alone, thus suggesting that the secondary structure was unaffected by heparin binding. In agreement with the latter interpretation, the CD data for the heparin studies indicated that the typical ω-helical character remained basically unchanged over the heparin concentration range of 0–30 μM (see Fig. 7D). Consistent with this, a parallel heparin binding NMR study showed that the overall intensities decreased as the heparin concentration increased without any...
significant change in their chemical shifts (data not shown). These results therefore lead us to conclude that the decay in overall CD absorption in the presence of heparin was ascribed to protein precipitation due to the multivalent nature of the heparin molecules. It should be noted that, in a control experiment, heparin alone contributed no CD intensity, only noise. We have further confirmed the formation of eA27L-aa protein precipitation by differential centrifugation experiment (data not shown). The fine precipitate formed between eA27L-aa protein (30 μM) and heparin at the same concentration could be removed from solution by centrifuge at 15,000 × g for 60 min, whereas the eA27L-aa alone did not precipitate at all under the same condition.

eA27L-aa Exhibits a Two-step Folding/Unfolding Transition—In the urea-induced denaturation process, the folding/unfolding transition curve (Fig. 7F) could be fitted on a two-step model (28). The Gibbs free energy can be expressed by the following equation:
\[ \Delta G = -RT \ln K \]
where \( R \) is the gas constant (1.987 cal °C⁻¹ mol⁻¹), and \( T \) the absolute temperature and the equilibrium constant, \( K \), can be obtained from the unfolded and folded fraction of the population determined by CD spectroscopy (29). The measurement is expected to be more accurate near the midpoint of the denaturation curve. The Gibbs free energy can then be calculated from the denaturation transition curve for urea-induced denaturation (Fig. 7G).

By extrapolating to zero urea concentration, the Gibbs free energy for native eA27L-aa in the absence of urea, \( \Delta G_{H_2O} \), could be determined from \( \Delta G = \Delta G_{H_2O} - m \text{[urea]} \) (2). This calculation assumes that the Gibbs free energy is linearly proportional to the denaturant concentration, with a proportionality of \( m \), which is a measure of the sensitivity of the transition to denaturant. The intercept on the \( y \) axis in the linear plot of Gibbs free energy versus denaturant concentration therefore yields the value for \( \Delta G_{H_2O} \). The \( \Delta G_{H_2O} \) values calculated for eA27L-aa at concentrations of 30 and 120 μM were 2.45 ± 0.20 and 2.56 ± 0.20 kcal/mol, respectively, the average value being 2.51 kcal/mol.

eA27L-aa/Heparin Binding Affinity Studied by ITC—ITC, which has been shown to be suitable for investigating low affinity binding, such as heparin-peptide binding interactions (30–32), was used to characterize the interaction between eA27L-aa and heparin. Heparin is a polyelectrolyte co-polymer of idouronic acid and glucosamine with a high degree of sulfation. Wild-type A27L has been shown to interact with the...
Fig. 7. Effects of heparin, pH, temperature, and urea on the far-UV CD spectra. A, thermal denaturation (△) and renaturation (×) studies on eA27L-aa (30 μM) monitored by the CD intensity at 222 nm. B, thermal denaturation (△) and renaturation (×) studies on a mixture of 30 μM eA27L-aa and 15 μM heparin. C, pH denaturation of eA27L-aa (30 μM). D, far-UV CD spectra of eA27L-aa (at 30 μM and pH 5.0) as a function of heparin concentration; the numbers on the right encoded by different colors refer to the heparin concentration (μM). Note that the characteristics of the α-helical pattern remain mostly unchanged from 0 to 30 μM. E, urea denaturation of eA27L-aa at 30 μM (△) and 120 μM (○) in 10 mM sodium phosphate buffer at pH 5.0. F, Gibbs free energy versus urea concentration for eA27L-aa at 30 μM (△) and 120 μM (○). By extrapolating to zero urea concentration, Gibbs free energy values in the absence of urea of 2.45 ± 0.20 kcal/mol and 2.56 ± 0.20 kcal/mol were determined for 30 and 120 μM, respectively, with a mean of 2.51 ± 0.20 kcal/mol.
negatively charged sulfates on GAGs (15). A putative heparin binding site in A27L is the 12-amino acid region in the N terminus, STKAAKKPEAKR, which is rich in positively charged lysine and arginine residues. The role of this sequence in heparin binding was demonstrated using wild-type eA27L, eA27L-aa, and an eA27L mutant lacking this 12-amino acid stretch, denoted as D-A27L (Fig. 8). An iterative nonlinear fitting calculation was used to determine the thermodynamic parameters of ΔH (enthalpy of binding), K_a (binding constant), and n (stoichiometry); the fitting parameters are tabulated in Table II. For eA27L and eA27L-aa, the respective ΔH values were −2.98 and −2.15 kcal/mol and the stoichiometry values 0.96 and 0.99, strongly suggesting that eA27L and eA27L-aa have similar, if not identical, heparin binding activity with a one-to-one binding mechanism. In contrast, D-A27L had a low stoichiometry value of 0.09, suggesting that it did not bind heparin.

![Image](image_url)

**Fig. 8.** ITC interaction studies of the binding of heparin to eA27L-aa and D-A27L. ITC titration studies of D-A27L (A and B) or eA27L-aa (C and D) with a 15-fold molar excess of heparin in 50 mM phosphate buffer, pH 7.0, containing 50 mM NaCl at 26 °C. A, raw data for D-A27L (0.18 mM) titrated with heparin (2.8 mM). B, integrated data for A. The fitted data (solid line) indicate that D-A27L does not bind heparin. C, raw data for eA27L-aa (0.3 mM) titrated with heparin (4.33 mM). D, integrated data for C. Peak areas in C were fitted using an iterative nonlinear least squares algorithm that varies ΔH, K_a, and n. The fitted data (solid line) describe a binding interaction with the following parameters: enthalpy of binding interaction (ΔH) of −2.15 kcal/mol, a binding constant (K_a) of 8.9 × 10^4 M⁻¹, and a stoichiometry (n) of 1.0 mol of heparin/mol of eA27L-aa.

**TABLE II**

| Sample  | K_a × 10^4 | n      | ΔH kcal mol⁻¹ |
|---------|------------|--------|---------------|
| eA27L   | 3.0 ± 0.2  | 0.96 ± 0.07 | -2.98 ± 0.31  |
| eA27L-aa| 8.9 ± 1.6  | 0.99 ± 0.02 | -2.15 ± 0.05  |
| D-A27L  | 0.4 ± 0.1  | 0.09 ± 0.05 | -0.31 ± 0.31  |

**TABLE III**

| pH    | K_a × 10^4 | n      | ΔH kcal mol⁻¹ |
|-------|------------|--------|---------------|
| 6.5   | 9.2 ± 0.6  | 0.95 ± 0.07 | -1.37 ± 0.32  |
| 7.0   | 1.7 ± 0.7  | 1.11 ± 0.12 | -0.99 ± 0.08  |
| 7.5   | 7.6 ± 0.4  | 1.00 ± 0.07 | -1.60 ± 0.35  |
| 7.0°a | 8.6 ± 0.3  | 1.13 ± 0.15 | -0.93 ± 0.25  |

* Experiment carried out at low temperature (16 °C).
any indication of a structural or conformational intermediate. It is believed that the two-step folding/unfolding process coincides with a self-assembled/disassembled conformational transition. Presumably, if the denaturation process were a multistep process, the eA27L-aa oligomers would form intermediate(s) during disassociation. In this case, the degree of dissociation would depend on the urea and sample concentrations, where the Gibbs free energy, $\Delta G_{M,0}$, is then made up of two components, $\Delta G_{\text{Dis},M}$ and $\Delta G_{\text{Unfolding},M}$, the latter being the Gibbs free energy of protein unfolding (concentration-independent) and the former the Gibbs free energy associated with molecular disassociation (concentration-dependent). In the case of a two-step folding/unfolding mechanism, $\Delta G_{M,0}$ is simply equal to $\Delta G_{\text{Unfolding}}$ ($\Delta G_{\text{Dis},M} = 0$) and is thus concentration-independent. The type of folding/unfolding mechanism can therefore be determined on the basis of the concentration-dependence of the Gibbs free energy.

To identify the folding/unfolding mechanism, we measured the Gibbs free energy for eA27L-aa at the concentrations of 30 and 120 $\mu$M. If a multistep process were involved, the Gibbs free energy of the sample at 120 $\mu$M should be higher than that at 30 $\mu$M. In fact, our findings showed that the two $\Delta G_{M,0}$ values (2.45 kcal/mol at 30 $\mu$M and 2.56 kcal/mol at 120 $\mu$M) were almost identical within the experimental error of 0.20 kcal/mol (Fig. 7G), strongly suggesting a two-step folding/unfolding mechanism.

Sedimentation analysis shows that wild-type A27L forms a triple coiled-coil domain (9), and the leucine zipper in A27L may anchor it to vaccinia virus A17L transmembrane protein, thus forming a stable complex (33, 34). The amino acid sequence of wild-type A27L suggests that the protein has four structural regions, a structureless region (residues 1–28), a helical domain (residues 29–37), a coiled-coil helical region (residues 44–72), and a leucine zipper motif (residue 73 to the C terminus) (9). It is noteworthy that A27L has a coiled-coil helical structure similar to those seen in human immunodeficiency virus gp41 (35) and influenza HA2 (36). The NMR and CD spectroscopy data presented here suggest a refined structural model for the extracellular domain of A27L (Fig. 9), consisting of a flexible random coil unstructured chain (21–42 in A27L or Ser$_{15}$–Asp$_{36}$ in eA27L) and an $\alpha$-helical coiled-coil rigid domain (43–84 in A27L or Asp$_{37}$–Asn$_{78}$ in eA27L), involved in protein self-assembly. The flexible random coil contains the GAG-binding region, a 12-amino acid lysine/arginine-rich domain (21–32 in A27L or Ser$_{15}$–Arg$_{26}$ in eA27L). This exposed random coil feature could facilitate the binding of GAGs to cells. It should be noted that the unstructured binding site in A27L is unlike that in foot-and-mouth disease virus, in which GAGs bind to a structured shallow depression on the virion surface (37). In A27L, the trimeric form (Fig. 9C) is a structurally stable unit, the biological function being dependent on the global structure, implying that dissociation or denaturation of the stable self-assembly structural unit might cause failure of virus entry. In heparin binding studies using NMR, it was found that the overall intensities decreased in two-dimensiona1 $^1$H/$^15$N HSQC on heparin binding studies without any significant change in their chemical shifts (data not shown). The observation of a structureless heparin binding domain suggests that the interactions between heparin and eA27L-aa involve a nonspecific multiple-site mechanism via high positive polarity (Fig. 9A).

CSI propensity analysis indicated that, in 2.5 M urea, the Asn$_{37}$–Glu$_{49}$ segment of eA27L-aa had limited residual $\alpha$-helical content. It is probable that the self-assembly of eA27L-aa is intimately correlated with the structural behavior of this rigid domain. We combined the results from CSI and helical wheel analysis.
analyses (9) to identify residues of high hydrophobicity that may potentially form the eA27L-aa self-assembly core. Leucine residues Leu41, Leu45, and Leu48 occupy adjacent a and d positions in the helical repeating sequence (Fig. 9A), which suggests that they interact closely with each other. We believe that the hydrophobic interaction of these residues is the primary driving force in the formation of α-helical bundles and that these residues are probably responsible for the molecular self-assembly of eA27L-aa. A27L mutants with site-specific mutations of these residues may not form aggregations in the native state, and this is currently being tested.

In summary, we have characterized the intrinsic molecular structure of the extracellular domain of the vaccinia virus envelope protein, A27L, using complementary NMR and CD approaches. Under conditions of urea denaturation, the partially denatured state of eA27L-aa led to a large degree of conformational disorder. On the basis of chemical shift propensity analysis, we detected two structurally distinct domains in eA27L-aa, these being a random coil extended chain (residues Ser15–Asp36), containing the heparin binding region in which lysine and arginine residues are thought to bind heparin via charge interactions, and an aggregation-associated α-helical coiled-coil rigid segment (Asn78–Asn258). Combined CSI and helical wheel analyses (9) identified residues of high hydrophobicity in the central core in this α-helical bundle that potentially form the self-assembly core, these being residues Leu41, Leu45, and Leu48. Thus, using NMR and CD studies, we have produced a residue-specific molecular model for A27L that will be a starting point for the detailed structural characterization and the further understanding of the mechanism by which vaccinia virus enters host cells.

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