Conformational Stability and Disassembly of Norwalk Virus-like Particles

EFFECT OF pH AND TEMPERATURE

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Salvador F. Ausar, Thomas R. Foubert, Mary H. Hudson, Thomas S. Vedvick, and C. Russell Middaugh

From the Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas 66047 and LigoCyte Pharmaceuticals, Inc., Bozeman, Montana 59718

Greater than 99% of the Norwalk virus (NV) capsid consists of 180 copies of a single 58-kDa protein. Recombinantly expressed monomers self-assemble into virus-like particles (VLPs) with a well defined icosahedral structure. NV-VLPs are an appropriate vaccine antigen since the antigenic determinants of the parent virion are preserved. They also constitute very simple models to study the mechanisms of assembly and disassembly of viral capsids. This work examines the inherent stability of NV-VLPs over a range of pH and temperature values and provides detailed insight into structural perturbations that accompany disassembly. The NV-VLP structure was monitored using a variety of biophysical techniques including intrinsic and extrinsic fluorescence, high resolution second-derivative UV absorption spectroscopy, circular dichroism (CD), dynamic light scattering, differential scanning calorimetry, and direct observation employing transmission electron microscopy. The data demonstrate that NV-VLPs are highly stable over a pH range of 3–7 and up to 55 °C. At pH 8, however, reversible capsid dissociation was correlated with increased solvent exposure of tyrosine residues and subtle changes in secondary structure. Above 60 °C NV-VLPs undergo distinct phase transitions arising from secondary-, tertiary-, and quaternary-level protein structural perturbations. By combining the spectroscopic data employing a multidimensional eigenvector phase space approach, an empirical phase diagram for NV-VLP was constructed. This strategy of visualization provides a comprehensive description of the physical stability of NV-VLP over a broad range of pH and temperature. Complementary, differential scanning calorimetric analyses suggest that the two domains of VP1 unfold independently in a pH-dependent manner.

Norwalk virus (NV) and Norwalk-like viruses are responsible for more than 95% of acute nonbacterial gastroenteritis with 23 million reported cases per year in the United States alone (1). These viruses are members of the family Caliciviridae and the recently designated genus Norovirus, which comprises genogroups I and II. They are highly contagious, requiring low numbers of viruses for infection and appear to be stable outside the host for several days. Transmission of NV occurs primarily via the fecal-oral route with several outbreaks of waterborne and food-borne gastroenteritis documented on cruise ships, in schools, nursing homes, residential institutions, and areas devastated by natural disasters (1, 2). NV outbreaks have also been documented in the United States military population and in different areas of the world where crowded living conditions facilitated person to person spread (3, 4). NV and other members of the Caliciviridae family have recently been designated as category B biodefense pathogens (National Institutes of Health Publication 03-5315). The gastroenteritis associated with NV has an average incubation period of 12–48 h, with symptoms characterized by acute onset of nausea, vomiting, and diarrhea. Although the illness is frequently self-limiting and mild, the consequent severe dehydration can be fatal in susceptible populations (e.g. elderly, immune-suppressed patients) due to secondary complications (5).

The genome of NV consists of a single-stranded, positive sense RNA of about 7.6 kilobases that is organized into three open reading frames. These open reading frames encode a non-structural polyprotein, the major capsid protein (VP1), and the minor basic capsid protein (VP2) (6). The study of NV pathology and immunology has been hindered by the lack of growth supporting cell culture systems. Recombinant expression of the VP1 in an insect cell system, however, results in the self-assembly of empty, noninfectious virus-like particles (NV-VLPs) that are morphologically similar to the infective virion (7). Elucidation of the structural and immunological features of NV has been accomplished primarily through studies of these NV-VLPs. The three-dimensional structure of the NV-VLP has been resolved through the use of electron cryomicroscopy and computer analysis techniques (8) and x-ray crystallography (9). These studies reveal that NV-VLPs have a diameter of ∼38 nm and exhibit a T = 3 icosahedral symmetry. They are composed almost entirely of 180 molecules of the VP1 capsid protein organized into 90 dimers with a few copies (<1%) of VP2 per intact VLP. The NV-VLP has a continuous protein shell with prominent protrusions at all the local and strict icosahedral 2-fold axes, leaving cup-like depressions at the icosahedral 5- and 3-fold axes. VP1 consists of two principal domains; a shell...
(S) and a protruding (P) domain, linked together by a flexible hinge region (9). The N-terminal 225 residue S domain is involved in formation of the icosahedral contacts between VP1 dimers. Residues 50–225 fold into an 8-stranded antiparallel β sandwich arrangement, which is a commonly observed structure of other viral capsids. The C-terminal P domain is further divided into two subdomains, P1 and P2. Both subdomains are rich in β-strand structures that are involved in coordinating dimer formation, with an extensive interfacial contact area of about 2000 Å² (9).

At present only a few studies have been initiated to examine the environmental stability of NV. In experiments involving human volunteers, NV retained infectivity, producing gastroenteritis even after exposure to pH 2.7 for 3 h and after heating to 60 °C for 30 min (10). Another study that used feline calicivirus as an NV surrogate (11) showed that the particles were stable at room temperature for between 21 and 28 days and up to 60 days at 4 °C when dried onto a surface. Infectious virus titers were found even after exposure of feline calicivirus to 70 °C temperatures for up to 3 min. Because much of the current knowledge of Norwalk virus has been gained from studies of NV-VLPs, a comprehensive biophysical analysis of their stability with respect to temperature- and pH-induced structural changes is of significant value for several reasons. A better understanding of the life cycle of the virus, especially assembly/disassembly mechanisms, could contribute to the design of drugs and inactivation strategies aimed to disrupt the viral capsid. In addition, because the NV-VLP has been proposed as a promising vaccine candidate to combat NV infection (12, 13), such studies might provide a basis for rational vaccine development and formulation conditions. Last, NV-VLPs constitute an attractive and simple model to study assembly, stability, and unfolding of icosahedral viral capsids. In this work we have examined the effects of pH and temperature on intact NV-VLPs using a combination of several spectroscopic methods as well as calorimetric and microscopic analyses. Synthesis of the spectroscopic data, employing a multidimensional eigenvector phase space approach (14), provides a comprehensive description of the physical stability of the NV-VLP. Complementary, calorimetric analysis revealed that the two different domains of VP1 unfold independently in a pH-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Norwalk Virus-like Particles Preparation**—Recombinant Norwalk virus capsid monomers were expressed in a baculovirus-transformed *Spodoptera frugiperda* (Sf9) ovarian cell line as described previously (7). NV-VLPs were purified from the insect cell medium 5–7 days post-infection using four steps of centrifugation as described previously with later modifications (7, 15). The Sf9 cells were pelleted by centrifugation at 500 × g for 5 min at room temperature. NV-VLPs were concentrated from the resulting supernatant fraction by ultracentrifugation for 2 h in a Beckman SW28 rotor at 26,000 rpm (89,450 × g) at 4 °C. The NV-VLP pellet was then resuspended in sterile water and sedimented through CsCl (0.39 g/ml) using a Beckman SW55 Ti rotor at 35,000 rpm (116,000 × g) for 18 h at 4 °C. The band containing the NV-VLPs was collected and diluted in sterile water, and the NV-VLPs were pelleted by centrifugation again for 2 h in a Beckman SW28 rotor at 26,000 rpm (89,450 × g) at 4 °C. The samples were decanted, and the pellet was resuspended in sterile water and stored at 4 °C until further use. Purity was assessed by SDS-PAGE and Coomassie staining. Purified NV-VLPs were dialyzed against 20 mM citrate/phosphate buffer at the desired pH (3–8). Protein concentrations (monomer based) were determined by measuring the absorbance at 280 nm corrected for any light scattering contribution of the NV-VLPs (16) employing an extinction coefficient, ε_{280 nm} of 0.852 ml mg⁻¹ cm⁻¹ (17).

**Far UV Circular Dichroism Spectroscopy**—Far UV CD spectra were collected using a Jasco 810 spectropolarimeter equipped with a Peltier temperature controller (Jasco Inc., Easton, MD) and a six-position cuvette holder. NV-VLP solutions were prepared at a protein concentration of 0.22 mg/ml and placed in 0.1-cm path length stopped quartz cuvettes. Spectra were acquired using a resolution of 0.5 nm and a scanning speed of 20 nm/min with a response time of 2 s and a bandwidth of 1 nm. Sample temperature was monitored by a thermocouple located in the cuvette holder block. Spectra presented are an average of three consecutive measurements. Secondary structure content was estimated using the Dichroweb software package (18), which permits the analysis of secondary structure by CONTIN (19), SELCON (20), and CDSSTR (21) algorithms. The thermal unfolding of NV-VLPs was followed by monitoring the ellipticity at 205 and 222 nm over the temperature range of 10–90 °C, with a resolution of 0.5 °C, at a heating rate of 15 °C/h. CD signals were converted to mean residue molar ellipticities [θ]_{θ}, and the thermal transitions were analyzed using the Jasco Spectral Manager™ and derivative analyses employing Micrcoral Origin™ 7.0 software.

**UV Absorption Spectroscopy**—High resolution absorbance spectra were collected on a Hewlett Packard 8453 UV-visible diode-array spectrophotometer (Agilent, Palo Alto, CA) equipped with a Peltier temperature controller. A protein concentration of 0.055 mg/ml was used. Spectra were analyzed between 200 and 400 nm in 1.0-nm increments over a temperature range of 10–90 °C at 2.5 °C increments. A 5-min temperature equilibration time was also incorporated before collection of each spectrum. Second derivative spectra were generated using a 9 data-point filter that was then fit to a cubic spline function with 99 interpolated points per raw data point, which permitted an effective 0.01-nm resolution. Spectral analyses were conducted as previously reported (14) using Micrcoral Origin™ 7.0 software.

**Fluorescence Spectroscopy**—Intrinsic fluorescence emission spectra of NV-VLPs were obtained in 2.5 °C increments from 10–90 °C employing a QuantaMaster™ spectrophotometer (Photon Technology International, Inc., Birmingham, NJ) equipped with a four-position cell holder and Peltier temperature control device. A 5-min equilibration time was again used at each temperature before data acquisition. The intrinsic fluorescence spectra were collected at a protein concentration of 0.055 mg/ml by exciting samples at 280 nm, whereas the emission was monitored between 305 and 450 nm using excitation and emission slit widths of 4 nm. Spectra were smoothed using the software included with the instrument, and the wavelength
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of emission maximum ($\lambda_{\text{max}}$) for each spectrum was determined by derivative analysis using Microcal Origin™ 7.0.

To further investigate the stability of the NV-VLPs, samples (0.055 mg/ml protein) in the presence of 40 $\mu$M 8-anilino-1-naphthalene sulfonate (ANS) were excited at 385 nm, and the emission spectrum of the dye was recorded from 400 to 580 nm as a function of temperature. Excitation and emission slits were set at 4 nm, and a 5-min hold time was used at each temperature before data collection. Emission spectra of 40 $\mu$M ANS in buffer alone were subtracted from sample spectra at each temperature. Values are presented as the mean with accompanying standard errors ($n = 3$).

Dynamic Light Scattering (DLS) — The size of NV-VLPs as a function of temperature and pH was monitored by dynamic light scattering. Stock solutions of NV-VLPs were diluted to 0.75 mg/ml in 20 mM citrate-phosphate buffer at the appropriate pH and filtered through 0.45-0.22-μm filters. Measurements were recorded every 2.5 °C from 10 to 90 °C at 15 °C/h in a light scattering instrument (Brookhaven Instrument Corp., Holtsville, NY) equipped with a 50-milliwatt diode-pumped laser ($\lambda = 532$ nm). The scattered light was monitored at 90° to the incident beam, and the autocorrelation function was generated via a digital generator (BI-9000AT). The effective hydrodynamic diameter was calculated from the diffusion coefficient by the Stokes-Einstein equation using the method of cumulants (22). In some experiments the data were fit to a curve using a non-negative constrained least squares algorithm to yield multimodal distributions. Data were collected over five 30-s intervals for each sample and plotted as the mean hydrodynamic diameter with accompanying standard error ($n = 5$).

Transmission Electron Microscopy (TEM) — A JEOL JEM-1200EXII (JEOL-USA, Inc., Peabody, MA) transmission electron microscope was used to examine the morphology of NV-VLPs under different combinations of pH and temperature. Purified NV-VLPs suspended at 0.35 mg/ml were dialyzed overnight against the appropriate buffer (20 mM citrate/phosphate buffer, pH 3–8). NV-VLP samples were placed on a length of Parafilm™ for staining. Carbon-coated Formvar membranes were layered with the filmed side contacting the NV-VLP samples for 2 min. The grids were then washed by placing them onto two separate drops of nanopure water for 10 s each and stained for 45 s with 1% ammonium molybdate. The pH of the staining solution was previously adjusted to the pH of the individual samples. Excess stain was removed with the edge of a filter paper, and the grids were allowed to air dry. For the temperature studies, the NV-VLP samples at pH 7 were incubated in a water bath at 25 °C, and the temperature was increased to 90 °C at the same heating rate used for the DLS experiments (15 °C/h). Ten-microliter samples were taken at different temperatures and stained at room temperature. The NV-VLP sizes were measured from digital electron micrographs using UTHSCSA ImageTool software version 3.0.

Empirical Phase Diagram (EPD) — The EPD was generated using data points at 2.5 °C intervals from 10–90 °C. The data used to construct the EPD were all 6-s derivative UV peaks, CD signals at 222 nm, intrinsic fluorescence peak position maxima, intrinsic fluorescence intensity at 330 nm, and ANS fluorescence intensity at 485 nm. The data generated from the various biophysical techniques were initially ordered by the parameter measured (i.e. pH and temperature) in Microsoft Excel® and copied into MatLab® Version 4.2 (Wolfram Research, Inc., Champaign, IL) for phase diagram preparation. The discrete parameters are analogous to coordinates that are associated with a set of variables from each technique employed. These variables represent unit vectors that define an $n$-dimensional vector space with dimensions equal to the number of variables included in the data set. Individual unit vector projectors were calculated and summed to obtain a density matrix. The eigenvalues and eigenvectors of the density matrix were calculated, and the three most influential components were each assigned a single color in an RGB-based (red, green, or blue) scheme. Finally, the three components were summed to yield a final color that represents a physical state of the capsids under those conditions (i.e. temperature and pH). A more detailed description of this method has been previously described (14). Note that the result is not an equilibrium (thermodynamic) phase diagram but merely a graphical representation of different structural states and that the final colors are arbitrary (the same color in different regions of the diagram may reflect different states since there are multiple ways of producing the same color through RGB additions).

Differential Scanning Calorimetry (DSC) — Calorimetric experiments were performed with a VP-DSC MicroCal high throughput capillary differential scanning calorimeter (Northampton, MA). DSC thermograms were obtained from 10 to 115 °C at a scan rate of 1 °C/min. A protein concentration of 0.5 to 0.75 mg/ml in 20 mM citrate/phosphate buffer at the appropriate pH was employed. A base-line correction was performed by subtracting the result of a sample versus buffer thermogram under identical conditions. After the first scan the samples were cooled and re-scanned to check the reversibility of any unfolding process. Melting temperatures ($T_m$) were calculated using MicroCal Origin 7.0 (Origin-Lab Corp., Northampton, MA) software assuming a non-two-state unfolding model.

Trypsin Digests — Trypsin from bovine pancreas tissue covalently attached to agarose beads (Sigma) was used to obtain the C-terminal fragment of VP1, which contains the entire P domain. Digestion was performed by incubating 1 mg of capsids with 2 IU of trypsin for 8 h at 37 °C in a digestion buffer (pH 8.5) containing 140 mM NaCl, 5 mM KCl, 400 μM Na₂HPO₄, 400 μM KH₂PO₄, 220 mM glucose, and 180 mM NaHCO₃. After incubation, the enzyme was removed by low speed centrifugation, and the supernatant containing digested capsid was dialyzed against 20 mM citrate/phosphate buffer at the appropriate pH. After dialysis, the protein concentration was determined by measuring the absorbance at 280 nm employing an extinction coefficient $\epsilon$ of 1.092 ml mg⁻¹ cm⁻¹ (17). The purity of the C-terminal fragment was found to be greater than 95% as analyzed by SDS polyacrylamide gel electrophoresis (using 12% gels) and Coomassie Blue staining (not shown).
from native NV-VLPs at 10 °C and pH 7.0 showed a minimum near 205 nm, a shoulder at 220 nm, and some weak positive maxima around 190 and 233 nm (Fig. 1). These spectral features, which somewhat resemble those of unfolded proteins, have been assigned to a group of β-sheet-rich proteins containing short segments of β-strands (classified as β-II proteins) (23). The secondary structure analysis using the program CDSSSTR (21) revealed a high percentage of β-sheet (36–37%), which is in good agreement with the crystallographic data available for VP1 (9). Because the data obtained at each pH value were similar, significant alterations of the secondary structure of the protein did not appear to be induced by changes in pH alone. At pH 8, however, there was a small decrease in α-helix (from 11 to 8%) and a corresponding increase in disordered structure from 32 to 34%. The CD spectrum observed at pH 8 also differed from that seen at other pH values with a shift in the minima from 205 to 202 nm, concomitant with a decrease in the overall intensity of the CD signal (Fig. 1). Temperature-induced changes in the secondary structure of the NV-VLP were also monitored at 222 nm (Fig. 2A). At pH 7, a sigmoidal increase in the ellipticity was observed as the temperature was increased, with a transition midpoint at ~63 °C. A separate experiment (not shown) revealed that the thermally induced changes were irreversible. At pH 4–6, the increase in the ellipticity at 222 nm was followed by a sharp transition characterized by a significant decrease in the CD signal. This last event was found to be due to extensive aggregation, making estimation of the transition midpoint unreliable. Further efforts to avoid capsid aggregation by the addition of up to 2 M guanidinium chloride were unsuccessful. At pH 8, the NV-VLPs displayed two well defined transitions at around 45 and 58 °C. The NV-VLPs also displayed two transitions at pH 3 near 65 and 80 °C, although the second transition was not well resolved (Fig. 2). Similar transition temperatures were observed for all pH experiments when the CD signal was monitored at 205 nm (not shown). To further characterize the conformational changes occurring in NV-VLP secondary structure upon heating, the far UV spectra were recorded as a function of temperature and deconvoluted using CDSSSTR. As the temperature was increased at pH 7, a shoulder began to appear around 220 nm, and the spectrum became broader (Fig. 2B). Analysis of the secondary structure distribution indicated a decrease in the β-sheet structure concomitant with a moderate increase in β-turn and α-helix (Fig. 2C). NV-VLPs at pH 3 and 8 displayed similar results (not shown). Solute interference, however, hampered data collection below 200 nm and, thus, prevented spectra deconvolutions at pH 4–6.

Analysis of pH- and Temperature-induced Changes in the Tertiary Structure of NV-VLPs—Temperature- and pH-induced changes in the tertiary structure of the VP1 protein were evaluated by UV-second derivative absorption and intrinsic and extrinsic fluorescence spectroscopies. VP1 possesses a substantial number of aromatic residues: 27 Phe, 11 Tyr, and 6 Trp. Changes in the microenvironment of these residues induces changes in their spectral characteristics, thus providing a means to monitor tertiary structure.

FIGURE 1. CD spectra of NV-VLPs at various pH values. CD spectra were recorded from 190 to 260 nm at each of the indicated pH values. The spectra presented are an average of three consecutive scans.

FIGURE 2. The effects of temperature on the secondary structure of NV-VLPs at different pH values. A, mean residue molar ellipticity at 222 nm as a function of temperature over a range of pH 3–8 (n = 3). The CD spectra at pH 7.0 were collected at the indicated temperatures (β), and the distribution of secondary structure was estimated by the program CDSSSTR (C). The spectra presented are an average of three consecutive scans.
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A number of these residues coordinate interactions between the quasi-equivalent subunits, which form the icosahedral capsid (9). Thus, UV and fluorescence spectroscopies might also provide information pertaining to the disassembly of the quaternary icosahedral structure. The second derivative spectra of the NV-VLPs at 10 °C and pH 7.0 showed six negative peaks with the following assignments: Phe (~253 and 259 nm), Tyr (~269 and 279 nm), and an overlapping of Tyr/Trp signal (285 nm) and Trp (292 nm) (Fig. 3A). In general, exposure of aromatic amino acid side chains to a more polar environment causes a blue shift in the absorbance minimum, whereas shifts to longer wavelengths suggest that the residues are on average incorporated into a more buried, less polar environment (24). Significant changes in the environment of the NV-VLP aromatic side chains were observed when the pH was increased from 7 to 8 (Fig. 3B–D). The changes were largest for the Tyr peak, with an ~1-nm blue shift, which is consistent with increased solvent exposure of this residue (Fig. 3B). The extent of Tyr shift, however, was not complete, suggesting that these residues were not fully solvent-exposed but remained partially buried. The small shift observed in the Trp peak was to a longer wavelength, suggesting that these residues were moved to a more apolar environment. Thermally induced changes in the local environment of the aromatic amino acid residues at physiological pH are illustrated in Fig. 4. All six minima exhibited thermal transitions between 55 and 60 °C. In general, the Phe minima displayed an overall red shift, changing to a blue shift near the transition at around 56 °C. The Tyr and Trp peaks displayed a sigmoidal decrease in the absorption wavelength minima with a transition temperature around 55 °C, suggesting transitions to a more polar environment at higher temperatures (Fig. 4). In contrast, at pH 4–6, increasing temperature corresponded to a red shift in the absorption minima (not shown), suggesting that the aromatic residues became less solvent-exposed. This change correlated with the extensive aggregation observed at these intermediate pH values.

Intrinsic fluorescence spectroscopy was employed to further investigate the stability of the tertiary structure of the NV-VLPs. The Trp fluorescence emission spectrum of the NV-VLPs at pH 7 manifested a maximum at about 333 nm, in support of the extensive burial of most of the 6 NV-VLP Trps (9). At 10 °C, the emission maximum was identical over the range of pH studied (not shown), indicating no significant changes in the environment of the indole side chains by variation in pH alone. Similar to the results obtained by second derivative UV absorbance spectroscopy, changes in the peak position and intensity at 330 nm were also observed with increasing temperature (Fig. 5). At pH 3, 7, and 8, conditions under which no temperature-dependent aggregation was detected, the shift in the emission maxima were to longer wavelengths, indicating that the Trps on average became more solvent-
exposed as the temperature was increased. Although there is some suggestion of the minor transition previously noted, only the event at higher temperatures was well resolved at pH 8. A blue shift was seen in NV-VLP samples at pH 4 and 5. At pH 6, however, the blue shift was preceded by an abrupt change to a longer wavelength at around 65 °C. The changes in fluorescence peak position agreed well with the changes in Trp fluorescence intensity at 330 nm (Fig. 5).

Because Trp intrinsic fluorescence measurement was not able to resolve the double transition observed by CD spectroscopy at pH 8, it seemed probable that this event did not involve changes in the polarity of the Trp environments, but apolar regions of VP1 may still have been exposed. We, thus, studied capsid unfolding in the presence of 1–8 ANS, an extrinsic probe that binds to apolar regions of proteins. The fluorescence of 1–8 ANS is highly quenched in aqueous solution but can increase dramatically in nonpolar environments (25). In addition, the emission maximum of the probe is usually blue-shifted upon binding to apolar regions of proteins. As probed by 1–8 ANS binding, NV-VLPs demonstrated two thermal transitions at pH 8 as previously observed by CD spectroscopy (Fig. 6). At pH 6 and 7, increased 1–8 ANS binding was indicated by an increase in the fluorescence intensity at 485 nm between 55 and 70 °C, after which a decline in the intensity was observed (Fig. 6). More complex behavior in 1–8 ANS binding with the temperature was observed below pH 6. These later results suggest some exposure of apolar regions of the protein complexes at temperatures below the major transition as well as above (Fig. 6).

Analysis of the Quaternary Structure and Disassembly of NV-VLPs—Alterations in the quaternary structure of NV-VLPs were initially examined by DLS. At physiological pH the NV-VLPs showed a hydrodynamic diameter of ~45 nm. This diameter is ~8 nm larger than that determined by electron microscopy. As the pH was increased from 3 to 7, a small increase in the hydrodynamic diameter was observed by DLS. At pH 8, there was a dramatic drop in the size from 45 to 25 nm (Fig. 7A), and the polydispersity index increased from 0.070 to 0.287. To further evaluate the changes induced by pH, we examined the morphology and size of NV-VLPs by TEM as a function of pH. The morphology of NV-VLPs was invariant in the pH range 3–7, showing the expected icosahedral structure with emanating protrusions (Fig. 7B). At pH 8, however, very few intact particles were observed, and those that were seen possessed a smaller size (~24 nm), in good agreement with the DLS results (Fig. 7A). When NV-VLPs at pH 8 were dialyzed back to pH 7, fully assembled capsids were again detected by both DLS and TEM, which indicated that the alkaline pH-induced changes in the quaternary structure were reversible (not shown).

Temperature-induced changes in the quaternary structure of the NV-VLP were also examined by DLS. At pH 4–6, a dra...
matic increase in the hydrodynamic diameter was detected at
~65 °C, which indicated NV-VLP aggregation (Fig. 8A). At
those pH values and above 70 °C, the size of the aggregates
was not further evaluated because it exceeded the size range of
the instrument. At pH 8 the 28-nm particles persisted throughout
the temperature increase from 10 to 40 °C. Above 40 °C, further
particle size reduction became evident, resulting in an effective
particle diameter of ~18 nm (Fig. 8A). At pH 3 and 7 an increase
in hydrodynamic diameter from 45 to 55–60 nm occurred at
around 65 °C. Because the increase in the size was relatively
small and no aggregation was detected at pH 3 and 7, the tem-
perature-induced changes in quaternary structure may be
related to particle swelling. We, therefore, examined the mor-
phology of temperature-perturbed NV-VLPs at pH 7 by TEM
(Fig. 8B). The results indicated that NV-VLPs maintained their
intact native quaternary structure up to 60 °C (not shown). At
65 °C (the temperature at which the thermal transition was
detected by DLS) the particles were irregular in shape with sig-
nificant disruption of their icosahedral structure, and the char-
acteristic protrusions appeared irregular (Fig. 8B). At higher
temperatures, only a small number of particles with highly dis-
rupted capsid structure was observed (Fig. 8B). When the tem-
perature was returned to ambient conditions, no intact capsids
were seen by TEM (not shown), suggesting that the tempera-
ture-induced disassembly was an irreversible process.

Global Analysis of the NV-VLP Stability; an Empirical Phase
Diagram Representation—To provide a more comprehensive
picture of the stability of NV-VLPs when stressed by tempera-
ture and pH, much of the spectroscopic data described in the
previous sections was compiled in the form of an EPD (Fig. 9).
This form of data presentation should not be confused with a
thermodynamic phase diagram in which equilibrium across
phase boundaries is present. The colors observed represent
vectors formed by the sum of colored components (red, green,
and blue) whose magnitudes are defined by normalized values of experimental data (14, 26). Because the EPD is generated from spectroscopic data sensitive to the secondary, tertiary, and quaternary structure of the NV-VLP, regions of continuous color in the EPD represent an identifiable physical (conformational) state, whereas abrupt color changes indicate significant alterations in the physical properties of the NV-VLPs (i.e., “pseudo-phase boundaries”). The EPD for NV-VLPs using the second derivative absorbance, intrinsic and extrinsic fluorescence, and CD data (Fig. 9) clearly shows the multiple alterations in physical stability of NV-VLPs over the pH and temperature range investigated. The green-colored phase (labeled as P1 in Fig. 9) can be identified as the state of maximum stability. At low temperatures, the EPD shows a different phase (P2) for NV-VLPs at pH 8, which represents tertiary/quaternary structural perturbations (Fig. 9). At higher temperatures at pH 3, 7, and 8, the NV-VLPs seem to adopt a similar physical state (P3) reflected by the continuous red regions in the EPD. This phase probably reflects the presence of soluble oligomers of VP1. At intermediate pH (4–6) and higher temperatures, the multicolor region in the phase diagram represents severely aggregated capsids that were clearly identified by DLS. Thus, the EPD provides a fairly intuitive and global picture of the stability of NV-VLPs and suggests that they are optimally stable in the pH range 3–7 with a maximal stability at pH 4–5.

**Calorimetric Analysis of NV-VLPs—**Heat-induced dissociation/unfolding of the capsids at pH 4–6 at temperatures greater than 60 °C lead to visible protein aggregation, which precluded spectroscopic analyses of any further transitions above this point. In addition, the somewhat restricted temperature range available for most of the spectroscopic techniques prohibited further investigation of conformational behavior at higher temperatures. We thus analyzed the thermal unfolding of the NV-VLP using a capillary cell-based differential scanning calorimeter. This instrument produces much smaller heats of aggregation compared with instruments equipped with either lollipop or pan-shaped cells. Additionally, the use of a pressurized capillary cell allows the evaluation of thermal transitions up to 125 °C. Thermograms of NV-VLPs suspended at different pH values are shown in Fig. 10A. A second heating run across the same temperature range (not shown) found that the thermally induced changes were irreversible, prohibiting a more rigorous thermodynamic analysis of the data. The stability of the NV-VLP was, thus, defined in terms of the temperature of maximal heat capacity difference between the reference and the sample cells or the peak transition temperature \( T_m \). NV-VLPs showed two endothermic transitions at pH 3, one major with a \( T_m \) of 66 °C and one minor at 92 °C. As the pH was increased from 3 to 8, significant changes were observed in the \( T_m \) of these peaks (Fig. 10A). The minor transition was more sensitive to pH change and displayed a progressive destabilization as the pH was increased from 5 to 8. At pH 7, the thermogram appeared to manifest only a single transition. Deconvolution using a non-two-state model, however, shows the presence of both the major and minor transition with similar but distinct \( T_m \) values. At pH 8, the thermogram displayed a trace opposite to those observed under acidic conditions, with the most intense transition now the higher \( T_m \) event (Fig. 10A).

Trypsin treatment of the VP1 protein produces a soluble cleavage product that contains the C terminus (P domain) of the protein, whereas the N terminus (S domain) is completed degraded (15). The thermograms of the isolated P domain produced a single endothermic transition over the entire pH range under study with maximal stability at pH 5 (Fig. 10B). The \( T_m \) values for the isolated P domain were closer to those observed for the major transition of the NV-VLPs and also showed a similar trend in shift with pH (Fig. 11). The results strongly suggest that the major transition detected in the NV-VLP thermograms correspond to the unfolding of the P domain.

**DISCUSSION**

By employing a combination of spectroscopic, microscopic, and calorimetric analyses, we have extensively characterized the solution behavior of NV-VLPs over a wide range of temperature and pH. NV-VLPs were shown to undergo structural changes at the secondary, tertiary, and quaternary levels induced by changes in pH and temperature. The pH conditions under which NV-VLPs were incubated in this study are well within the pH range found in the gastrointestinal tract. In vivo NV needs to overcome drastic changes in pH to reach and ultimately invade the enteric cells. The spectroscopic data revealed significant conformational stability of NV-VLP at neutral and acidic pH. In contrast, significant capsid disruption was observed at alkaline pH. These results are in agreement with the observation of White et al. (27) that native NV-VLPs disassem-
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![Graph showing the pH dependence of the transition temperatures (T_m) obtained by DSC analysis of the NV-VLP and P-domain.](image)

The endothermic transition temperatures (T_m) obtained for full-length NV-VLP (filled circles, T_m1; filled squares, T_m2) and isolated P-domain (open circles) were plotted as a function of pH.

Figure 11. pH dependence of the transition temperatures (T_m) obtained by DSC analysis of the NV-VLP and P-domain. The endothermic transition temperatures (T_m) obtained for full-length NV-VLP (filled circles, T_m1; filled squares, T_m2) and isolated P-domain (open circles) were plotted as a function of pH.

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Temperature-induced changes in the quaternary structure were characterized by a loss of continuity in the icosahedral geometry. At the transition temperature, those icosahedral interfaces involving fewer interactions (i.e., quasi-trimeric ABC and hexameric C-B_2) are probably compromised, but more stable interfaces possessing higher association energies (i.e., dimeric A-B_2 and hexameric B_3-C) remain unaffected (33). Even though no apparent changes in the capsid size were detected by TEM, these misshapen and heterogeneous capsids may have had lower diffusion coefficients, which would explain the small increases in hydrodynamic size detected by DLS.

Changes in the extrinsic fluorescence of ANS revealed that the minor transition detected by CD at pH 8 was accompanied by alterations in the tertiary structure of VP1. This transition temperature, however, occurred 5 °C lower than that observed by CD, suggesting the presence of an intermediate displaying changes at different structural levels of the NV-VLPs into a single graphic image. Previously, the physical stability of several proteins as well as enveloped and non-enveloped viruses was successfully described by this approach (14, 26, 29–31). The EPD generated in this report showed that NV-VLPs maintained a similar (native) conformation at relatively low temperatures over the range of pH 3–7 with maximal thermal stability at pH 4–5 (see PI in Fig. 9). This high stability observed at acidic pH might be a natural adaptation of viruses that infect the gastrointestinal tract and has been reported for poliovirus type 1 as well (32). Similarly, the stability of adenovirus type 5 is significantly enhanced under acidic conditions (30). The structural changes previously discussed at pH 8 are represented by a different empirical phase (P2) in the EPD. At moderately high temperatures, two different physical states were observed, soluble VP1 oligomers at pH 3, 7, and 8 (P3) and severely aggregated capsids in the range of pH 4–6 (P4). An additional pseudo-phase can be visualized at around 40 °C at pH 8 that represents a minor structural transition detected by CD spectroscopy and ANS fluorescence. At pH 4–6, colloidal stability was dramatically altered with increased temperature as reflected by massive aggregation detected by DLS.
molten globule-like behavior. More complex behavior in ANS binding as a function of the temperature was observed at acidic pH. For example, at pH 3 three discrete transitions were detected that might reflect incremental exposure of apolar regions due to changes in tertiary structure or partial disruption of the particles. In this context, Parovirus VLPs are thought to display reversible tertiary structure rearrangement at low temperatures before the dissociation step at higher temperatures (34). The interpretation of ANS binding at acidic pH, however, was complicated by the possibility that the probe could bind to the protein through electrostatic interactions.

Calorimetric analysis of the capsid shows complex pH behavior. DSC analyses of capsids at pH 3–6 revealed a second high temperature but lower intensity transition not detected by spectroscopic methods. The obvious explanation for the presence of the two endotherms is that the two domains of VP1 unfold independently. The more intense lower temperature transition would correspond to the larger and more solvent-exposed P-domain with the less intense higher temperature event observed at acidic pH involving the unfolding of the smaller S-domain. To investigate this hypothesis, a calorimetric analysis over the same range of pH was performed on the isolated P-domain obtained from trypsin digestion of the protein. The single endothermic transition of the isolated P-domain was located about 10 °C lower than the major transition seen for the full-length VP1 in the NV-VLP over the entire range of pH under study. The proximity of the unfolding temperature and the similar behavior with pH strongly suggest that the major transition corresponds to the unfolding of the P-domain of VP1. The reduced $T_m$ and intensity of the transition of the isolated domain suggest that this portion of the protein is stabilized by interactions between the quasi-equivalent subunits in the context of the quaternary structure of the NV-VLP or by domain-domain interactions.

The stability and disassembly of several other VLPs and spherical icosahedral viruses have been studied in the past by thermal, chemical, and hydrostatic pressure perturbation (34–36). In general, they do not seem to follow a common mechanism of unfolding and disassembly. Instead, complex pathways of degradation were described and appear to depend on a number of factors like icosahedral symmetry and intersubunit contact energies. Hepatitis B virus capsids, for instance, have been shown to dissociate into subunits without unfolding of the capsid protein (35). In contrast, a concerted mechanism of disassembly and unfolding has been demonstrated for mature Flock House virus VLPs (36). This last mechanism seems to be similar to the dissociation of NV-VLP at pH 7, in which capsid disassembly (monitored by TEM and DLS) and unfolding of VP1 (monitored by CD and Trp fluorescence) were detected at similar temperatures.

Although the structural resolution of the various techniques used here is low, when used in combination they are powerful tools to study viral structure over a wide range of experimental conditions under which the use of higher resolution techniques are difficult or impossible. The strategy of visualization of the most favorable conditions for stabilizing VLPs by employing the EPD approach provides a number of practical advantages. The conditions at the pseudo-phase boundaries in the EPD could be used to develop potential therapeutic agents and disinfectants that disrupt the integrity of virus capsids. Given the high sensitivity of NV-VLPs to slightly alkaline pH, cationic peptides that have been reported to possess antiviral activity (37, 38) are plausible candidates to induce destabilization of the Norwalk virus capsid. Because NV-VLPs have been proposed as candidates for vaccination against NV gastroenteritis (13), the approach reported here could be used to select the best conditions for maintaining the long term stability of the antigen, support its large scale purification, and aid in screening for stabilizers.

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