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Acidic pH enhances structure and structural stability of the capsid protein of hepatitis E virus

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Abstract

Hepatitis E virus (HEV) is enterically transmitted and endemic to tropical areas of the world. The major capsid protein of HEV is pORF2 (~74 kDa), encoded by open reading frame 2 (ORF2). When expressed in insect cells, it is processed into a ~55 kDa form (n-pORF2). We also generated a mutant, m-pORF2, lacking a C-terminal hydrophobic region shown earlier to be required for its homo-oligomerization. Circular dichroism was used to measure the secondary structure and stability of these proteins as a function of pH and temperature. With decreasing pH both proteins acquired increasing α-helicity and thermal stability in terms of midpoint of denaturation and the Gibbs energy change.

Keywords: Hepatitis E virus; ORF2 protein; Secondary structure; Denaturation; pH-stability

Hepatitis E virus (HEV) is the etiological agent of hepatitis E, an acute and self-limited form of viral hepatitis prevalent in the developing world. The virus is transmitted feco-orally and is associated with rampant sporadic infections and large epidemics [1,2]. Though HEV infection is not associated with chronicity, a fraction of patients progress to fulminant hepatitis [3,4], the most severe form of acute hepatitis. A curious feature of hepatitis E is the unusually high rates of mortality (20–30%) that are observed in pregnant women as a result of fulminant liver disease [5,6]. The lack of a reliable cell culture system has severely restricted studies on the biology and pathogenesis of HEV. The hepatitis E virion is a spherical particle with a diameter of 27–34 nm, first shown by immuno-electron microscopy [7]. Subsequent studies have revealed that HEV is a non-enveloped icosahedral particle with indentations on its surface. The fact that HEV can survive in the intestinal tract suggests that the virus is relatively stable to acid and mildly alkaline conditions [8]. The viral genome is a positive-stranded RNA of ~7.2 kb and encodes at least three proteins designated pORF1, pORF2, and pORF3 [9], the products of open reading frames (ORFs) 1, 2, and 3, respectively.

The ORF2 of HEV has been expressed using various expression systems including *Escherichia coli* [10,11], insect cells using baculoviruses [12–14], and animal cells using transfection [15] or infection with recombinant vaccinia virus [16] or αviruses [17]. Expression in insect cells infected with recombinant baculovirus has revealed multiple forms of pORF2 ranging in size from 72 to 50 kDa, of which the 50–53 kDa forms are secreted as virus-like particles (VLPs) [13]. This protein lacks 111 N-terminal and 51–59 C-terminal amino acids. The VLPs carry antigenic epitopes similar to those present on HEV particles and are able to raise systemic and mucosal immune responses when orally administered to mice [18]. In earlier studies, we have observed ORF2 to express an approximately 74–88 kDa protein in transfected animal cells, one form of it being N-glycosylated [15]. The glycosylation has been mapped to asparagine residues at positions 137, 310, and 562 [19]. We have

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further shown that pORF2 carries an N-terminal signal sequence that translocates it across the endoplasmic reticulum (ER) membrane; the ER also appears to be the major site of pORF2 glycosylation and accumulation [19]. When expressed in vitro, or in transfected cells, pORF2 assembled as dimers, trimers, and higher order forms [20]. While N-terminal deletions up to 111 amino acids had no effect, the deletion of amino acids 585–610 led to reduced homo-oligomerization of pORF2 [20]. The homo-oligomerization of pORF2 has also been demonstrated through a yeast two-hybrid analysis [21]. Multiple immunodominant B cell epitopes have been identified on pORF2. The protein contains a highly basic N-terminal half with about 10% arginine residues, presumably to neutralize the negative charge on the genomic RNA backbone. These observations support the premise that ORF2 encodes the HEV capsid protein.

The ORF2 capsid protein would be expected to interact with receptors on host cells and is a promising candidate for a recombinant subunit vaccine for hepatitis E [22]. Structural characterization of pORF2 would shed light on the basic biology of HEV, viral entry into host cells, its survival in the environment, and the pathogenesis. Sufficient quantity of HEV particles is however not readily available for structural or functional analysis, due to lack of an in vitro culture system for HEV. While the capsid protein (pORF2) has been expressed in several expression systems [10,11,15–17], none of these have produced large amounts of the proteins suitable for structural or functional characterization. Only when expressed in (Tn5) insect cells, by use of recombinant baculoviruses sufficient amounts of the protein are expressed. However, these proteins are not full-length (~72 kDa) but are processed forms of pORF2 some with the ability to assemble into virus-like particles (VLPs) [13]. Because of this spontaneous intracellular processing, sufficient quantities of the full-length ORF2 protein are not available for structural analysis.

In an attempt to structurally characterize the HEV capsid protein we have generated recombinant baculoviruses to express the naturally processed form of pORF2 (n-pORF2) as well as a mutant form (m-pORF2). The latter lacked 111 N-terminal amino acid as well as a C-terminal hydrophobic region encompassing amino acids 585–610 that we have shown earlier to be important for homo-oligomerization of pORF2 in vitro [20]. Here, we describe efficient expression and purification of these HEV proteins. The purified proteins were subsequently used for structural studies using circular dichroism (CD) measurements. We report increased secondary structure and thermal stability at low pH. This is the first report on such biophysical characterization of the HEV capsid protein.

Materials and methods

Preparation of ORF2 proteins. The recombinant baculovirus expressing a mutant ORF2 protein (m-pORF2) lacking 111 amino acid at its N-terminus and a hydrophobic region encompassing amino acids 585–610 was constructed as described earlier [14]. A recombinant baculovirus expressing the full-length ORF2 protein was similarly constructed. The high five cell line (BT1-TN-5B1-4 cells; Tn5) originating from the ovarian cells of the insect *Tricoplusia ni* (Tni; cabbage looper) was used for expressing the ORF2 protein following infection with the respective baculoviruses. Tn5 cells were infected with recombinant baculoviruses at a multiplicity of infection (moi) of 10. Five days post-infection cells from 1 L of suspension culture were harvested and resuspended in 15 ml of 50 mM Tris–HCl, 2 mM EDTA, and 0.5% sodium deoxycholate, and vortexed for 1 min, followed by incubation on ice for 15 min. The cells were sonicated six times for 30 s each and then centrifuged at 10,000*g* (SA 600 rotor, Sorvall) at 4 °C. The pellet was resuspended in 15 ml of 4 M urea in 50 mM Tris–HCl, 2 mM EDTA, and centrifuged as above. The supernatant was collected in a fresh 50 ml Falcon tube and the pellet was further suspended in 15 ml of the same buffer. The pellet and supernatant were then analyzed for pORF2 on 10% SDS–PAGE; both m-pORF2 and n-pORF2 were found in the supernatant. Urea was removed by using protein desalting PD-10 columns (Amersham/Pharmacia) according to supplier's protocol. The fractions containing ORF2 proteins were pooled, dialyzed against 50 mM phosphate buffer, pH 7.5, and concentrated by using a 10 kDa cutoff Centricon cartridge (Millipore). The protein preparations were analyzed for purity by SDS–PAGE. Western blotting was carried out with polyclonal antibodies generated against the full-length ORF2 protein expressed in *E. coli* [10].

Circular dichroism measurements. CD measurements were carried out in a Jasco J-715 spectropolarimeter equipped with a Peltier-type temperature controller (PTC-348 WI). Far-UV CD spectra of the proteins were recorded in the wavelength range 240–200 nm. The raw CD data at a given wavelength (λ) were converted into mean residue ellipticity, $[\theta]_\lambda$ (in deg cm^2 dmol^{-1}) using the relation

$$[\theta]_\lambda = \frac{\theta_\lambda M_0}{10lc}, \quad (1)$$

where θ_λ is the observed ellipticity in millidegrees at wavelength λ, M_0 is the mean residue weight of the protein, ($M_0 = 110$), c is the protein concentration (mg/cm^3), and l is the path length (cm). In thermal denaturation studies protein samples were heated at the rate of 1 °C/min and the changes in $[\theta]_{222}$ were measured in the temperature range of 20–85 °C. About 650 data points were collected. After denaturation, the protein sample was immediately cooled to measure the reversibility of the reaction [23].

Results

Expression and purification of ORF2 proteins

Recombinant baculoviruses carrying genes for full-length and mutant ORF2 (m-pORF2) were used to infect Tn5 cells for protein expression and purification. The infecting dose and time of harvest were optimized so as to give maximum yield of the recombinant proteins. The full-length pORF2 was quantitatively processed into the ~55 kDa naturally processed form of the protein (n-pORF2). Infection at a multiplicity of infection (moi) of 10 and harvest on day 5 gave the best results, with the mutant or naturally processed form of pORF2 being the dominant cellular proteins (Fig. 1A).

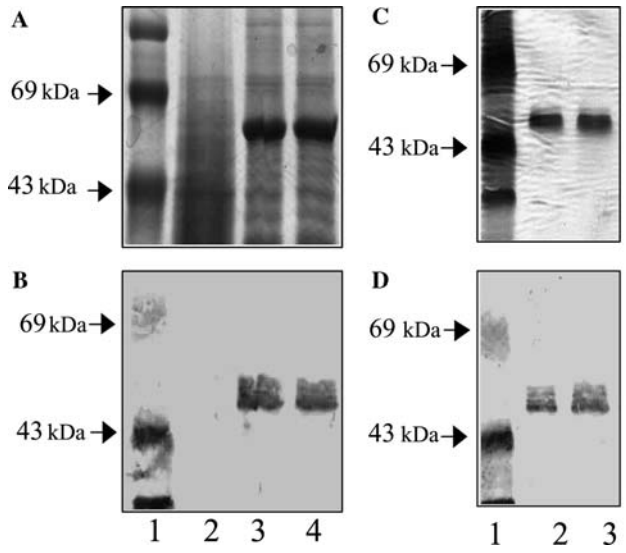

Fig. 1. Expression and purification of m-pORF2 and n-pORF2. Lysates prepared from Tn5 cells infected with wild type (lane 2), recombinant m-pORF2 (lane 3) or recombinant full-length pORF2 (lane 4) baculoviruses were subjected to SDS–10% PAGE, followed by staining with Coomassie blue (A) or Western blotting (B). The purified proteins m-pORF2 (lane 2) and n-pORF2 (lane 3) were simultaneously separated and the gels are stained (C) or Western blotted (D). Lane 1 in each panel shows molecular weight markers.

Unlike an earlier study [13], we did not find any secreted forms of pORF2 or VLPs following infection of Tn5 cells with baculoviruses expressing either the full-length or mutant versions of the protein. The deduced amino sequences from the ORF2 gene sequences of our HEV isolate and the isolate used to produce VLPs [13] did not reveal any significant differences (data not shown). Further, our recombinant ORF2 baculovirus also did not produce VLPs using the culture conditions and infrastructure used in the earlier report (N. Takeda, personal communication). Since our aim was to carry out biophysical studies on the monomeric proteins, we restricted our studies to ORF2 proteins that were purified to apparent homogeneity (Fig. 1C) from cell lysates as described under Materials and methods. The identity of these proteins in crude cell lysates as well as in a purified form was confirmed by Western blotting with pORF2-specific antibodies (Figs. 1B and D).

Far-UV CD spectra of m-pORF2 and n-pORF2 at different pH values

The far-UV CD spectra of m-pORF2 (A) and n-pORF2 (B) at different pH values are shown in Fig. 2. Each CD spectrum was analyzed for the content of secondary structure using the software developed by Yang et al. [24]. The results of this analysis are shown in Table 1. Near neutral pH, the mutant m-pORF2 contained 38% α-helix and 12% β-sheet, while the naturally processed form, n-pORF2 contained 20% α-helix and 46% β-sheet. On lowering the pH to 2.5, both proteins acquired more helical structures with a corresponding decrease in β sheets (Table 1). Fig. 2 also shows CD spectrum of the urea-denatured proteins at pH 2.0 and 20 °C and that of the heat-denatured proteins at pH 2.0 and 85 °C. These spectra showed that compared to heating, urea treatment led to more extensive denaturation of the proteins.

Heat-induced denaturation of ORF2 proteins

The heat-induced denaturation of m-pORF2 (Fig. 3A) and that of the n-pORF2 (Fig. 3B) were evaluated as a function of pH. This denaturation of m-pORF2 was found to be reversible at pH $\leqslant 3.0$. The denaturation data were analyzed using the relation

$$y(T) = \frac{y_N(T) + y_D(T)\exp[-\Delta H_m/R(1/T - 1/T_m)]}{1 + \exp[-\Delta H_m/R(1/T - 1/T_m)]}, \quad (2)$$

where $y(T)$ is the experimentally observed far-UV CD property of the protein at T Kelvin (T K); $y_N(T)$ and $y_D(T)$ are the CD properties of the native and denatured molecules at T K, respectively; and ΔH_m represents the van't Hoff enthalpy change at T_m, the midpoint of denaturation. It should be noted that the non-linear analysis of the heat-induced denaturation curve according to Eq. (2) assumes that the temperature dependence of $y_N(T)$ and $y_D(T)$ is described by a parabolic function [24] (i.e., $y_N(T) = a_N + b_N T + c_N T^2$ and $y_D(T) = a_D + b_D T + c_D T^2$, where a, b, and c are temperature-independent constants and subscripts N and D represent the native and denatured state, respectively). Analysis of the reversible heat denaturation curve at pH 1.0, 2.0, 2.5, and 3.0 according to Eq. (2) gave values of ΔH_m and T_m which are plotted in the inset of Fig. 3A. A linear least-squares analysis of the ΔH_m versus T_m plot gave a value of 1.67 kcal K^{-1} mol^{-1} for ΔC_p, the constant-pressure heat capacity change. Using this value of ΔC_p and ΔH_m and T_m values (see Fig. 3A) in the Gibbs–Helmoltz equation

$$\Delta G_D(T) = \Delta H_m\{1 - (T/T_m)\} - \Delta C_p\{(T_m - T) + T\ln(T/T_m)\}. \quad (3)$$

We obtained values of ΔG_D at 20 °C (ΔG_D^0) at different pH values for m-pORF2, which are given in Fig. 3A. The effect of temperature on $[\theta]_{222}$ of n-pORF2 is shown in Fig. 3B. It was observed that the protein underwent irreversible thermal denaturation at pH 7.5. Further, there appeared to be no change in its secondary structure on heating n-pORF2 at lower pH values (Fig. 3B). While it was not possible to calculate the thermodynamic parameters for this protein, it appeared to be highly stable at low pH.

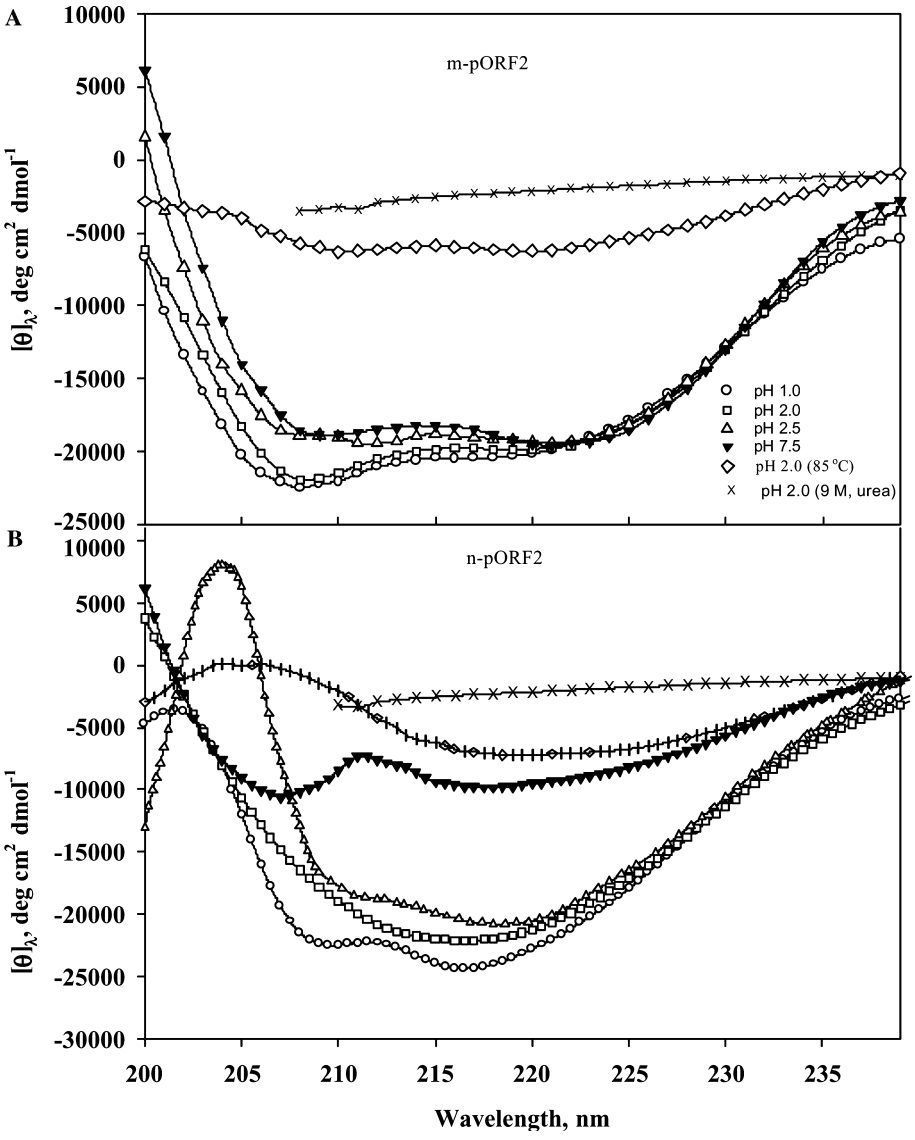

Fig. 2. Far-UV CD spectra of ORF2 proteins. The m-pORF2 (A) and n-pORF2 (B) were subjected to various experimental conditions (pH, temperature, and urea) and the far-UV CD spectra were recorded as described under Materials and methods. Symbols have the same meaning for both proteins. Results are representative of analyses carried out on three independent protein preparations.

Table 1
Elements of secondary structure of ORF2 proteins at different pH values and 20 °C

pH	% α-helix		% β-sheet		% Remaining	
	m-pORF2	n-pORF2	m-pORF2	n-pORF2	m-pORF2	n-pORF2
7.5	38	20	12	46	50	34
2.5	58	68	11	29	31	3
2.0	50	70	0	30	50	0
1.0	51	80	0	20	49	0

Discussion

In this study we have expressed and purified the naturally processed (55 kDa) form of the HEV capsid protein, pORF2, and an engineered mutant protein (57 kDa) lacking a C-terminal hydrophobic domain. This mutant protein was shown in our previous in vitro studies to have reduced ability to form homo-oligomers [20]. The proteins were expressed and purified from Tn5 insect cells infected with recombinant baculoviruses.

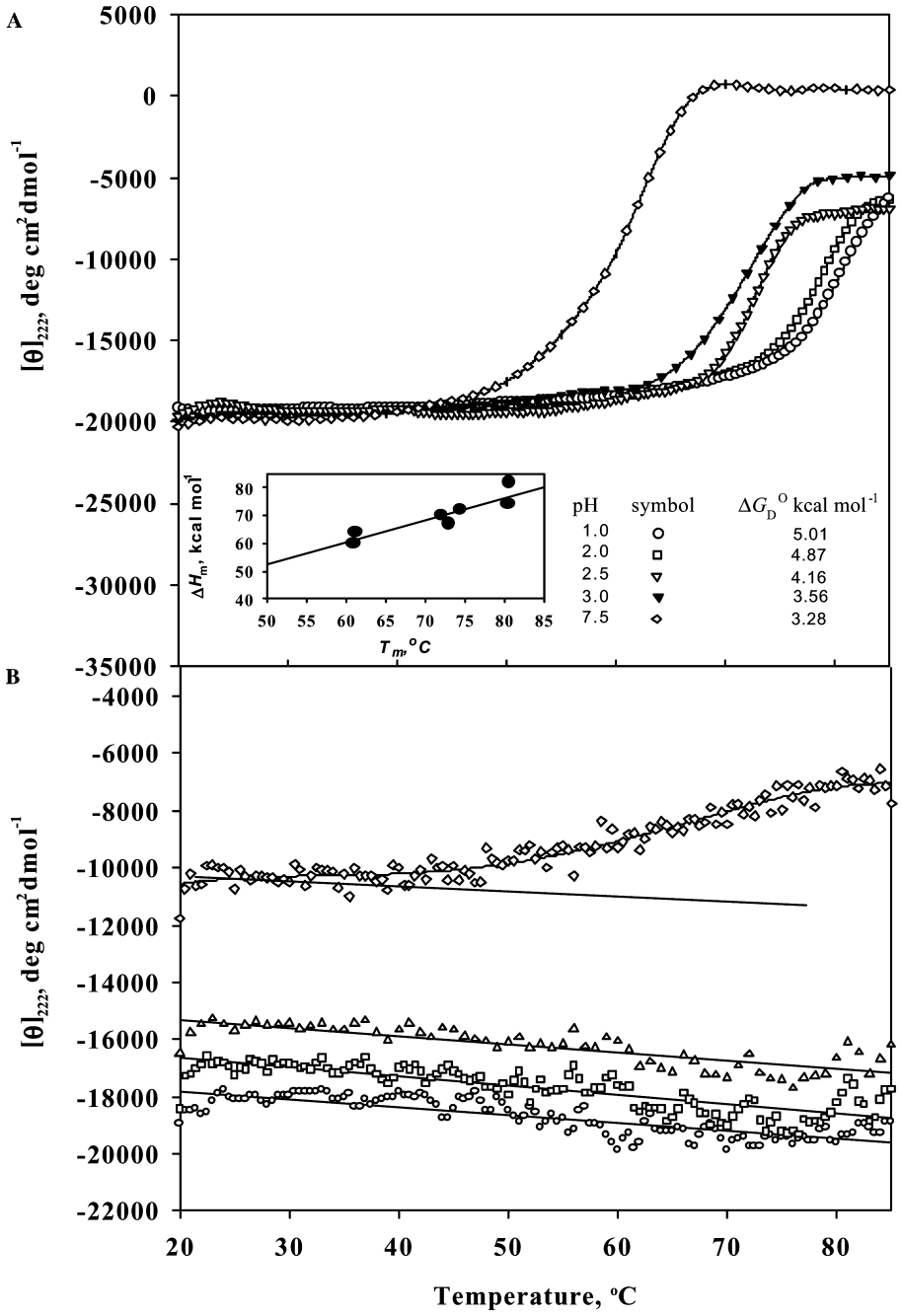

Fig. 3. Heat-induced denaturation of ORF2 proteins. The two proteins, m-pORF2 (A) and n-pORF2 (B), were progressively heated at different pH and the CD spectra were recorded as described under Materials and methods. Symbols have the same meaning for both proteins. The inset shows a plot of ΔH_m versus T_m of m-pORF2 and the estimated values of ΔG_D^0 at different pH values. Results are representative of analyses carried out on three independent protein preparations.

There are multiple advantages of using this expression strategy for the HEV capsid proteins. Expression of structural proteins of various DNA and RNA viruses through the baculovirus system has been used to produce large amounts of these proteins, often in a form that retains their immunogenic and physico-chemical properties [25]. Many viral pathogens, including HEV, are difficult to grow in vitro and are therefore not easily amenable for the development of live attenuated vaccine strains. For such viruses, recombinant protein expression is a promising approach to develop subunit vaccines. A subunit vaccine consists of a part of the virus, typically a protein capable of generating a protective immune response in immunized individuals. For HEV, the most promising subunit vaccine candidate so far appears to be the ORF2-encoded protein when expressed in insect cells using recombinant baculoviruses [18,22]. Here we have carried out a comparative study of

the naturally processed (n-pORF2) and mutant (m-pORF2) proteins. These proteins were subjected to circular dichroism to analyze their conformation and conformational stability as a function of pH and temperature.

The far-UV CD spectra of m-pORF2 in the pH range of 7.5–1.0 at 20 °C exhibited double minima at 208 and 222 nm (Fig. 2A); this is characteristic of proteins rich in α-helices. Analysis of these spectra showed an increase in α-helical content from 38% at pH 7.5 to 51% at pH 1.0 (Table 1). Analysis of the CD spectra of n-pORF2 under similar conditions (Fig. 2B) showed a more pronounced increase in α-helical content from 20% at pH 7.5 to 80% at pH 1.0 (Table 1). Since HEV is an enterically transmitted non-enveloped virus, transition from a less helical to a more helical structure of the capsid protein with decreasing pH is likely to provide stability and retain viral infectivity in an acidic medium. It is noteworthy that a dramatic transition from non-helical to helical structures in viral fusion proteins is shown to be essential in promoting fusion of the viral and target cell membranes in the case of enveloped viruses [26–29]. Furthermore, it has been reported that hepatitis A virus, another enterically transmitted non-enveloped virus, retained most of its infectivity when subjected to pH 1.0 for up to 5 h [30].

The far-UV CD spectra of randomly coiled proteins in concentrated guanidine hydrochloride solution have been reported [31]. A comparison of these spectra with those of m-pORF2 and n-pORF2 in 9 M urea (Fig. 2) suggests that urea also gives a randomly coiled state of both forms of pORF2. Furthermore, a comparison of CD spectra of the heat-denatured and urea-denatured proteins suggests that the thermally denatured proteins retain significant amounts of secondary structure (Fig. 2). A large body of data exists to suggest that proteins denatured by pH and/or heat retain more secondary structure than the unfolded states obtained with chemical denaturants such as urea or guanidine–hydrochloride (reviewed in [32]).

Heat-induced denaturation of m-pORF2 was found to be reversible only at a pH $\leqslant 3.0$. Our results suggest that m-pORF2 gets stabilized in terms of T_m and ΔG^0_D with a decrease in pH (Fig. 3A). This is an unexpected observation. The estimated p*I* of m-pORF2 being 6.9, it is expected that at pH values below that the protein would be destabilized due to repulsion between positive charges. One possible explanation for our observation is that there exits one or more pair(s) of carboxylate groups on the protein that destabilize it due to repulsion between negative charges near neutral pH. However, as the pH approaches the pK_a values of these groups, they will get protonated resulting in neutralization of the proposed repulsion and increased stability of the protein. Indeed, about 7–8% of the primary amino acid sequences of the two proteins analyzed here are made up of aspartate and glutamate residues, lending support to our hypothesis.

The naturally processed form of pORF2, with an estimated p*I* of 6.1, acquires more secondary structure (Table 1) and increased thermal stability (Fig. 3) than the pORF2 mutant at low pH. A comparison of thermal stabilities of n-pORF2 and m-pORF2 suggests that the hydrophobic region (residues 585–610) imparts stability to the ORF2 protein. This provides support for earlier results on pORF2 homo-oligomerization [20].

We conclude that decrease in pH enhances secondary structure and stability of the ORF2 capsid protein of HEV. This observation has physiological significance, as high stability of the capsid protein to acid and heat is to be expected of a virus that is enterically transmitted and is endemic to tropical areas of the world.

Acknowledgments

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