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Structural Requirements for Low-pH-Induced Rearrangements in the Envelope Glycoprotein of Tick-Borne Encephalitis Virus

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The exposure of the flavivirus tick-borne encephalitis (TBE) virus to an acidic pH is necessary for virus-induced membrane fusion and leads to a quantitative and irreversible conversion of the envelope protein E dimers to trimers. To study the structural requirements for this oligomeric rearrangement, the effect of low-pH treatment on the oligomeric state of different isolated forms of protein E was investigated. Full-length E dimers obtained by solubilization of virus with the detergent Triton X-100 formed trimers at low pH, whereas truncated E dimers lacking the stem-anchor region underwent a reversible dissociation into monomers without forming trimers. These data suggest that the low-pH-induced rearrangement in virions is a two-step process involving a reversible dissociation of the E dimers followed by an irreversible formation of trimers, a process which requires the stem-anchor portion of the protein. This region contains potential amphipathic α-helical and conserved structural elements whose interactions may contribute to the rearrangements which initiate the fusion process.

The entry of enveloped viruses into cells is mediated by homo- or heterooligomeric glycoproteins, which induce the fusion of the viral membrane with cellular membranes (for reviews, see references 22, 23, and 26). In the case of viruses taken up by receptor-mediated endocytosis, the acidic pH in endosomes triggers conformational changes and/or oligomeric rearrangements which are necessary for initiating fusion with the endosomal membrane (9, 19, 31). So far, influenza virus hemagglutinin (HA) represents the best-studied example of these processes because the crystal structure of soluble forms of this protein has been determined both at neutral pH and at the pH of fusion (3, 32).

Recently, the structure of a soluble, dimeric form of the envelope glycoprotein (protein E) from a flavivirus, tick-borne encephalitis (TBE) virus, was determined at a resolution of 2 Å (0.2 nm) (27). Although this protein is functionally analogous to the influenza virus HA, its structure is fundamentally different. Rather than forming a spike-like projection, the protein E dimer is a slightly curved, rod-like structure that is oriented parallel to the viral membrane (27). Cross-linking studies (12) suggest that the virion surface consists of a network of closely packed protein E dimers.

Incubation of virions at an acidic pH leads to irreversible conformational changes in protein E associated with an increase in hydrophobicity (17) and the exposure of the putative fusion sequence (28, 29) as well as oligomeric rearrangements resulting in the formation of protein E trimers (1). The fact that the dimer-trimer transition is quantitative (1) suggests that the envelope proteins have a regular organization. This view is supported by preliminary image reconstructions from cryoelectron micrographs and modelling studies, which are consistent with an icosahedral symmetry and a triangulation number (T) of 3 (6a, 27a). The quantitative dimer-trimer transition at an acidic pH also implies that the dimer contacts revealed in the X-ray structure at neutral pH have to be broken to allow the formation of trimers.

To obtain further insights into the structural requirements for these transitions, which so far have been studied only with whole virions, we investigated the effect of acidic pH on different soluble and solubilized forms of protein E from TBE virus strain Neudoerfl (15, 25). These included (i) full-length protein E dimers obtained by Triton X-100 solubilization of virions (13), (ii) protein E rosettes obtained by reassociation of solubilized protein E dimers (14), and (iii) the dimeric soluble and crystallizable form (sE dimers) used previously for structure determination by X-ray crystallography (27). sE dimers, which lack the carboxy-terminal 20% of the protein containing the membrane anchor and stem region, were obtained by limited trypsin digestion of virions (16).

The data presented in this report suggest that (i) the low-pH-induced dimer-trimer transition is a two-step process involving a reversible dissociation of protein E dimers into monomers followed by the irreversible formation of trimers and that (ii) the stem-anchor portion of protein E, which contains predicted α-helical structural elements, is essential for the formation of trimers.

To investigate irreversible changes occurring at low pH, the various forms of protein E in TAN buffer (0.05 M triethanolamine, 0.1 M NaCl, 0.1% bovine serum albumin [BSA] [pH 8.0]) were diluted with a low-pH buffer containing 0.05 M morpholineethanesulfonic acid (MES), 0.1 M NaCl, and 0.1% BSA to yield a final pH of 6.0. The experiments were performed at final protein E concentrations ranging from 7 to 70 μg/ml during the low-pH incubation step. The samples were incubated for 10 min at 37°C and readjusted to pH 7.6 with a buffer consisting of 0.1 M triethanolamine, 0.1 M NaCl, and 0.1% BSA. For mock incubation, TAN buffer (pH 8.0) was substituted for the pH 6.0 buffer. Experiments involving full-length E dimers were carried out in the presence of 0.5% Triton X-100 to prevent aggregation.

Conformational changes of low-pH-treated and back-neu-

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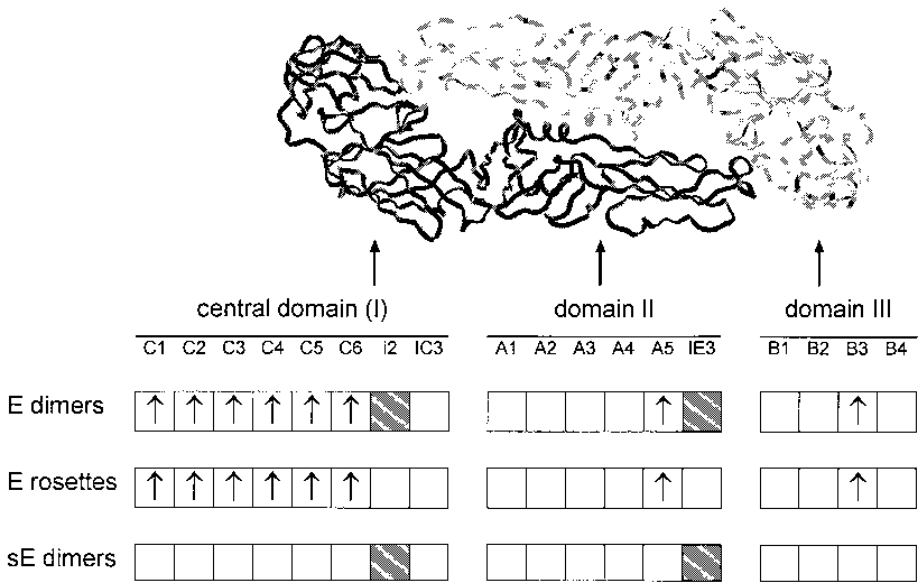

FIG. 1. Changes in MAb reactivity after low-pH treatment of E dimers, E rosettes, and sE dimers. The binding activities of 18 protein E-specific MAbs with low-pH-treated and untreated preparations were compared by four-layer ELISA. The top of the figure shows a ribbon diagram of the sE dimer structure as determined by Rey et al. (27), and the arrows indicate the positions of the three domains to which the antibodies bind. Each subunit is depicted in a different shade of grey. □, unchanged reactivity; ↑, increased reactivity; ▨, no reactivity at either pH.

tralized preparations of protein E were monitored in a four-layer enzyme-linked immunosorbent assay (ELISA) (18) using monoclonal antibodies (MAbs) (10, 21) which are specific for epitopes within each of the three structural domains of protein E. The antigen (0.5 μg/ml) was captured on the solid phase by guinea pig anti-TBE virus immunoglobulin, and titration curves were established with each MAb in order to quantify differences in reactivity. Consistent with earlier observations (11), the dimeric soluble forms and, to a lesser extent, the E rosettes exhibited reduced reactivity at pH 8.0 with MAbs A3, A4, A5, and IE3 (all specific for domain II epitopes) and i2 and and IC3 (both specific for domain I epitopes) relative to the native virions. Exposure of the E dimers and E rosettes to pH 6.0 for 10 min did not result in a further reduction with these MAbs, which recognize epitopes in the viral envelope that are normally sensitive to low pH (17). However, full-length E dimers and E rosettes did exhibit significantly increased reactivities with MAbs C1 to C6 (Fig. 1) after pretreatment at low pH, as has been observed with whole virions (17), and the reactivities with MAbs A5 and B3 were also increased. These MAbs apparently recognize elements which become more exposed by the low-pH-induced alterations. In contrast, when the truncated dimeric form of protein E (the sE dimer) was treated in the same manner, no change in the MAb reactivity profile was detectable (Fig. 1).

The oligomeric state of the low-pH-treated samples was analyzed by rate zonal sucrose density gradient centrifugation, which allows differentiation between monomeric, dimeric, and trimeric forms (1). As observed previously (1), protein E in the virion underwent a quantitative irreversible conversion from the dimeric to the trimeric form (Fig. 2A), independent of the concentration of protein E during the low-pH incubation step (data not shown). The E dimers (Fig. 2C and D) and the E rosettes (Fig. 2E and F) also underwent a partial dimer-trimer transition at an acidic pH. In the case of the E dimers, this trimerization was significantly concentration dependent and was not detectable at the lower concentration of 7 μg/ml (Fig. 2C). These data show that the dimers are intrinsically capable of forming trimers at low pH, not only in the context of the whole virion but also in the detergent-solubilized form. The dimer-to-trimer transition appears to be facilitated in E rosettes (Fig. 2E and F), presumably because the aggregation of protein E to form these complexes (14) brings the relevant structural elements into close contact.

Surprisingly, no changes were observed in the oligomeric state of the truncated sE dimer after low-pH treatment and back neutralization (Fig. 2B). Neither a formation of trimers nor a dissociation into monomers, which had been predicted to be necessary for the oligomeric rearrangements (1), was observed. These data suggest that interactions in the membrane anchor-stem region of protein E are necessary for the formation of trimers at low pH.

In the previous set of experiments only irreversible alterations were analyzed. To investigate possible reversible changes, sE dimers and full-length E dimers were treated with MES buffer to yield a final pH of 6.0 and a concentration of 40 μg/ml during the 10-min incubation step at 37°C as described above. In contrast to the previous experiments, however, the samples were not back neutralized, and the subsequent sedimentation analysis was carried out at pH 6.0 (Fig. 3C and D). Under these conditions, in contrast to the experiment of Fig. 2D, in which the incubation at low pH lasted only 10 min, the low-pH-treated full-length E proteins were almost exclusively trimeric (Fig. 3C). This is most likely due to the extended time of exposure to acidic pH during the detergent treatment and sedimentation analysis. Low-pH-treated sE proteins analyzed under acidic conditions sedimented as monomers, showing that the sE dimers dissociate but do not trimerize at low pH (Fig. 3D).

It was also possible with full-length protein E to detect small amounts of free monomers in addition to the trimers when low protein concentrations (≤7 μg/ml) were used in such experiments (data not shown). This suggests that full-length E dimers

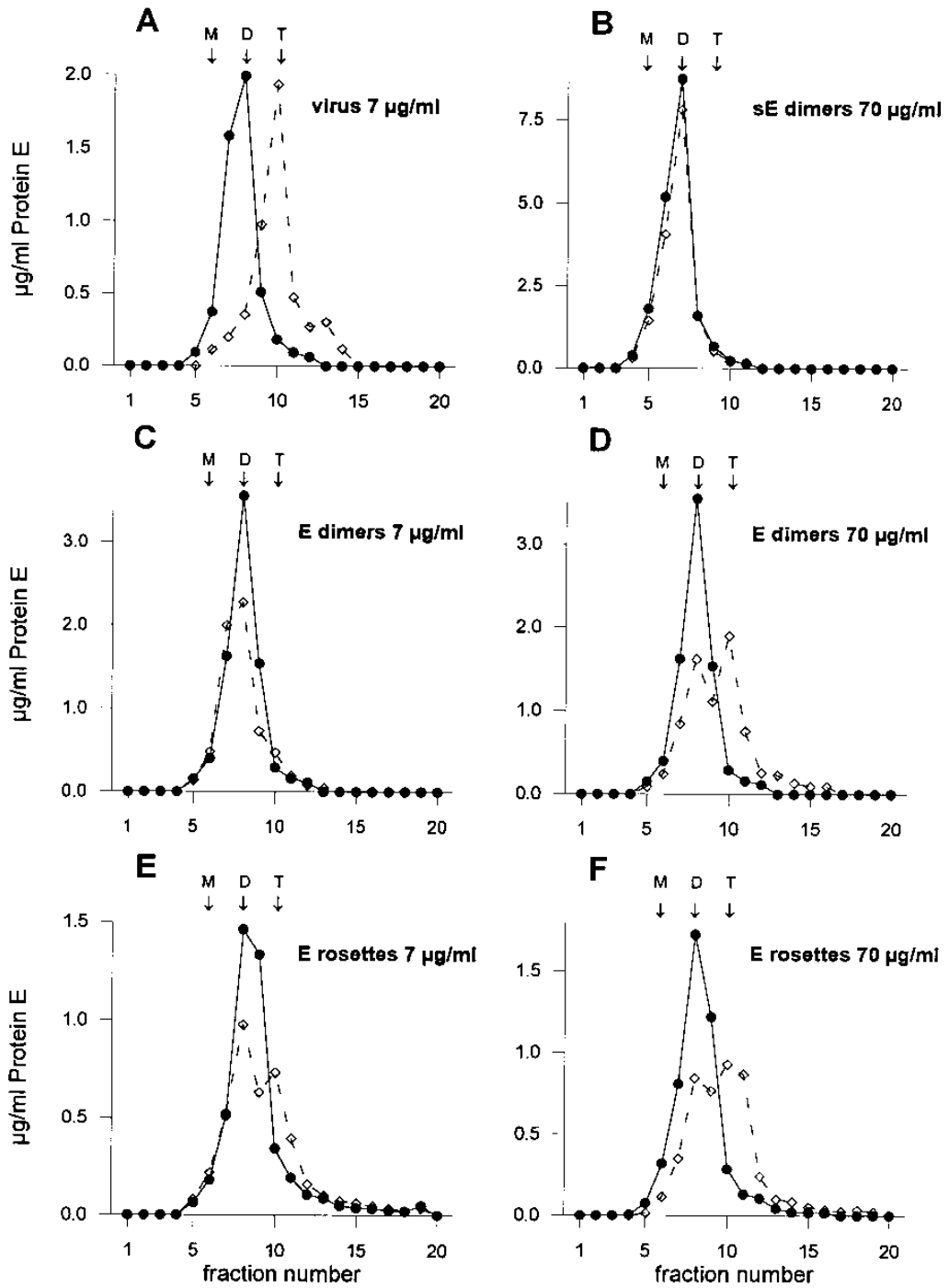

FIG. 2. Sedimentation analysis of Triton X-100-solubilized protein E complexes at pH 8.0 after pretreatment at pH 6.0. Preparations were incubated at pH 8.0 (●) or pH 6.0 (◇) at a concentration of 7 μg/ml (A, C, and E) or 70 μg/ml (B, D, and F), back neutralized, and solubilized with 0.5% Triton X-100. Aliquots of protein E were then analyzed by sedimentation in 7 to 20% sucrose gradients containing 0.1% Triton X-100, and protein E in the fractions was quantitated by four-layer ELISA (16). The positions of E monomer (M), E dimer (D), and E trimer (T) are indicated. (A) Virus; (B) sE dimers; (C and D) E dimers; (E and F) E rosettes.

also undergo low-pH-induced dissociation before trimer formation occurs.

To test the reversibility of these changes, the peak fractions containing the E trimers and sE monomers were back neutralized and analyzed by sucrose density gradient centrifugation at pH 8.0 (Fig. 3E and F). The oligomeric structure of the full-length E trimers, as expected, remained unchanged (Fig. 3E), whereas the sE monomers reassociated at pH 8.0 to form dimers (Fig. 3F).

sE dimers are thus capable of a low-pH-induced dissociation in the absence of the membrane anchor-stem region, but elements within this portion of protein E appear to be required for the formation of trimers. A closer examination of the stem region, which links the crystallizable portion of the ectodomain

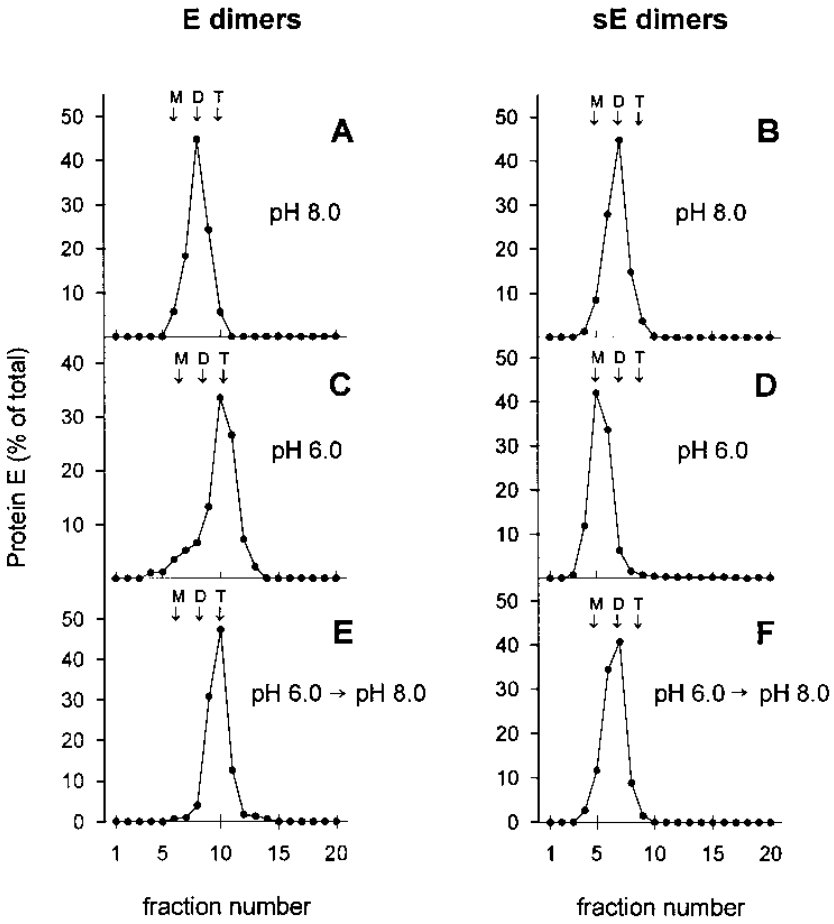

FIG. 3. Sedimentation analysis to assess the reversibility of low-pH-induced changes of full-length E dimers and sE dimers. (A and B) Samples were incubated at pH 8.0 at a protein concentration of 40 μg/ml, solubilized with 0.5% Triton X-100, and centrifuged into 7 to 20% (wt/wt) sucrose gradients in TAN buffer, pH 8.0, containing 0.1% Triton X-100. (C and D) Same as panels A and B, except that incubation with Triton X-100 and centrifugation were carried out in MES buffer at pH 6.0. (E and F) The peak fractions from panels C and D were adjusted to pH 7.6 and analyzed by centrifugation at pH 8.0 as described for panels A and B. The *y* axis shows the amount of protein E in each gradient fraction as a percentage of the total.

(sE) with the membrane anchor, revealed the presence of two potential amphipathic α-helical structures in the region from amino acid residue 400 to 413 and 431 to 449, which were predicted from a multiple alignment (30) of flavivirus sequences. The sequence from amino acid 448 to 474 was predicted to form a transmembrane α-helix by using the computer program TMpred (20). Since this overlaps with the second helix of the stem structure, it is likely that this whole stretch

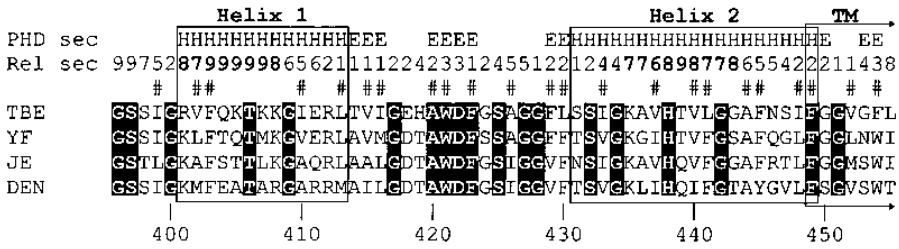

FIG. 4. Multiple-sequence alignment and predicted secondary (sec) structure of the stem region for representative sequences of the four major flavivirus serocomplexes. Secondary-structure prediction was carried out with the PHD-Server (30) at the European Molecular Biology Laboratory, Heidelberg, Germany. The first line gives the prediction of secondary structure (H, α-helix; E, β-sheet) and the second line gives the reliability for the secondary-structure prediction, as reported by the program. Parts of the multiple alignment corresponding to the predicted α-helices are enclosed by rectangles (helix 1 and helix 2). The predicted start of the membrane-spanning helix is indicated (TM) (for the prediction of transmembrane regions, the TMpred computer program was used; see reference 20). Pound signs indicate positions found to be conserved with respect to hydrophobicity in all flavivirus sequences available in the databases. The four sequences used here are as follows: (i) TBE, Western subtype, strain Neudoerfl, POLG_TBEV (P14336); (ii) YF, yellow fever virus, strain Pasteur 17D-204, POLG_YEFV2 (P19901); (iii) JE, Japanese encephalitis virus, strain JAOARS982, POLG_JAEVJ (P32886); and (iv) DEN, dengue virus type 1, strain AHF 82-80, POLG_DEN1A (P27912). The numbering at the bottom refers to the sequence of protein E from POLG_TBEVW. Residues on a black background are highly conserved in all four of the serocomplexes.

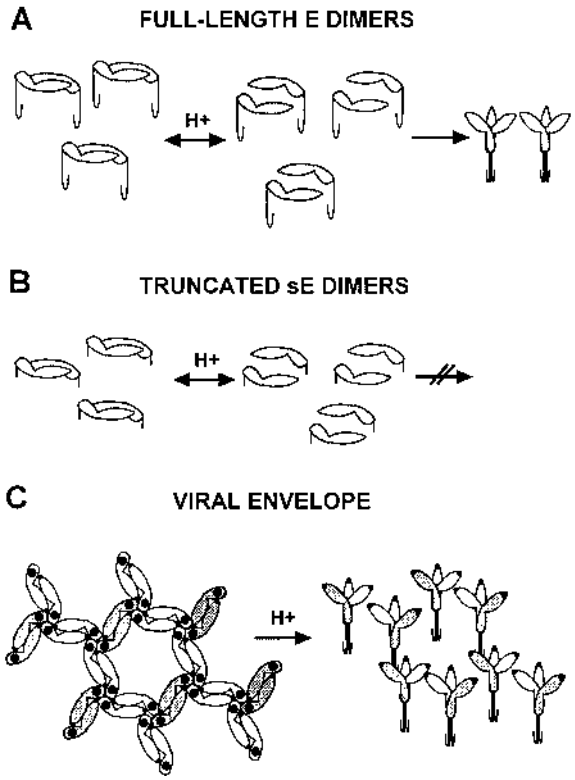

FIG. 5. Schematic representation of low-pH-induced oligomeric rearrangements of protein E dimers in solution (A and B) and in the context of whole virions (C). In this model it is proposed that exposure to low pH first induces a dissociation of the head domains of full-length E dimers, which then leads to the irreversible trimerization step, mediated by the stem-anchor domains (A). The truncated sE dimers, which lack the membrane anchor and stem structure, are able to dissociate at low pH but fail to form trimers (B). In the context of the virion envelope, an arrangement in which stem regions of three different dimers are juxtaposed at putative pseudo-threefold symmetry axes would facilitate a quantitative conversion of dimers to trimers (C). The drawing on the left in panel C shows a top view of a portion of a hypothetical T=3 surface lattice with the positions of the stems indicated by solid circles. After exposure to acidic pH (oblique view, panel C, right), dissociation of dimers and subsequent association of the adjacent stems would result in the formation of trimers composed of subunits from three different dimers. The tip of each subunit is darkened to indicate the position of the putative fusion peptide, which is believed to become exposed after low-pH treatment.

forms a continuous α-helix. As highlighted in Fig. 4, many of the amino acids within the stem region are conserved. Interestingly, the highest degree of conservation is found in the region between the predicted α-helices (amino acid 414 to 430), with 8 of 17 residues absolutely conserved among all flaviviruses. This probably represents a unique structural element which is important for function.

Amphipathic α-helices containing heptad repeats with the potential of forming α-helical coiled coils have been implicated in oligomerization and/or fusion processes in a number of viral envelope glycoproteins, including those of orthomyxoviruses (3, 4), paramyxoviruses (2, 5), retroviruses (8, 24), and coronaviruses (6, 7). This has been shown most directly with the HA2 part of the influenza virus HA, which exhibits dramatic low-pH-induced rearrangements of α-helical coiled coil structures that are apparently necessary for mediating fusion (3, 4). The elucidation of the possible role of interactions between the stem regions during the structural reorganization of the flavivirus envelope remains a challenge for future investigations.

On the basis of the data presented here, we propose a two-step mechanism for the low-pH-induced alterations in the TBE virus protein E dimer (Fig. 5) in which exposure to low pH leads to a reversible dissociation of the head regions corresponding to the sE fragment, followed by an irreversible formation of trimers involving α-helical and conserved sequence elements in the stem regions. This would presumably lead to exposure and possibly an outward projection of the fusion-active sequence as described by Rey et al. (27).

A fast and quantitative low-pH-induced oligomeric rearrangement of protein E from dimer to trimer, which appears to be necessary for the conversion to the fusogenic state of protein E in virions (1), would be facilitated by the proposed icosahedral arrangement of the protein E dimers in the virion envelope (6a, 27a), similar to the hypothetical lattice structure shown in Fig. 5C. A symmetrical arrangement would position the stem structures at putative pseudo-threefold symmetry axes, allowing the rearrangements to occur in a concerted manner.

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