The Flavivirus Envelope Protein E: Isolation of a Soluble Form from Tick-Borne Encephalitis Virus and Its Crystallization

FRANZ X. HEINZ,[1]* CHRISTIAN W. MANDL,[1] HEIDEMARIE HOLZMANN,[1] CHRISTIAN KUNZ,[1]
BARBARA A. HARRIS,[2] FELIX REY,[2] AND STEPHEN C. HARRISON[2]

Institute of Virology, University of Vienna, Kinderspitalgasse 15, A-1095 Vienna, Austria,[1] and Department of Biochemistry and Molecular Biology, Howard Hughes Medical Institute, Harvard University, Cambridge, Massachussetts 01238[2]

By the use of limited trypsin digestion of purified virions, we generated a membrane anchor-free and crystallizable form of the tick-borne encephalitis virus envelope glycoprotein E. It retained its reactivity with a panel of monoclonal antibodies, and only subtle structural differences from the native protein E were recognized. Treatment with the bifunctional cross-linker dimethylsuberimidate resulted in the formation of a dimer. Crystallization experiments yielded hexagonal rod-shaped crystals suitable for X-ray diffraction analysis.

The envelope protein E of flaviviruses has a molecular weight of 50,000 to 60,000 and represents the major constituent of the virion surface. It is the viral hemagglutinin, and it induces a protective immune response (reviewed in references 1 and 10). It is also thought to be involved in a number of additional viral functions, such as receptor binding and membrane fusion after acid pH-induced conformational changes (2, 28). From the conservation of all cysteines, which have been assigned to six disulfide bridges in the case of West Nile virus (22), and the virtually superimposable hydrophilicity plots, one can assume a common structural organization of flavivirus E proteins.

As deduced from genomic sequence analysis, the tick-borne encephalitis (TBE) virus protein E contains 496 amino acids (20) and carries a single carbohydrate side chain (34). Studies using monoclonal antibodies (MAbs) revealed three nonoverlapping clusters of epitopes (termed antigenic domains A, B, and C) in addition to three isolated epitopes (termed i1, i2, and i3) (3). We have recently proposed a specific arrangement of the polypeptide chain into distinct protein domains (19) which correlate with these MAb-defined antigenic domains and which we believe are involved in different functional activities. In particular, comparative sequence analyses of attenuated flaviviruses such as yellow fever virus strain 17D (4), dengue 2 virus strain S1 (5), host range mutants of Murray Valley encephalitis virus (17), and a MAb escape mutant of TBE virus (14) provide evidence that sequence elements within domain B represent an important determinant of virulence. Domain A contains the most highly conserved sequence of protein E and also undergoes conformational changes upon exposure to low pH (3, 25, 26). These observations have led to the proposal that the acid pH-dependent fusogenic activity of flaviviruses (2, 23) may reside within this domain (25, 26). For a further understanding of the structure and functions of protein E, determination of its three-dimensional structure is important. Although protein E can be isolated in biologically active form by detergent solubilization and density gradient centrifugation (12), the aggregates formed do not appear to be suitable for crystallization. We have therefore generated a soluble and

crystallizable form of protein E for X-ray diffraction analysis.

In a first series of experiments, purified TBE virus (9) was subjected to digestion with trypsin (tolylsulfonyl phenylalanyl chloromethyl ketone treated; Worthington) under different conditions, including various protease-substrate ratios for different times and temperatures ranging from 4 to 37°C. Before analysis of the cleavage products, the reaction was stopped by the addition of trypsin inhibitor (from bovine lung; Serva). As revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), digestion at 37°C resulted in the generation of a series of smaller protein fragments (data not shown), including a 9-kDa immune-reactive fragment corresponding to antigenic domain B (19).
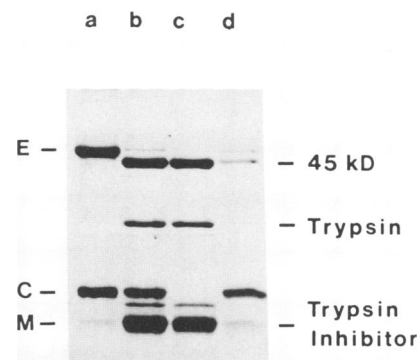

FIG. 1. Limited digestion of TBE virus with trypsin. One part of purified TBE virus (2 mg/ml) in 0.05 M triethanolamine–0.05 M NaCl, pH 8.0, was treated with one part of tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (1 mg/ml; 209 U/mg) for 1 h at 0°C. The reaction was stopped by the addition of a 1.5 times excess of trypsin inhibitor, and residual particles were pelleted by ultracentrifugation at 45,000 rpm for 3 h in a Beckman TI 70.1 rotor. Samples were analyzed by SDS-PAGE in 15% acrylamide gels according to the method of Laemmli and Favre (15). Shown is Coomassie blue staining. Lanes: a, undigested virus control; b, virus after digestion with trypsin and addition of trypsin inhibitor; c, supernatant after ultracentrifugation of sample b; d, pellet after ultracentrifugation of sample b.
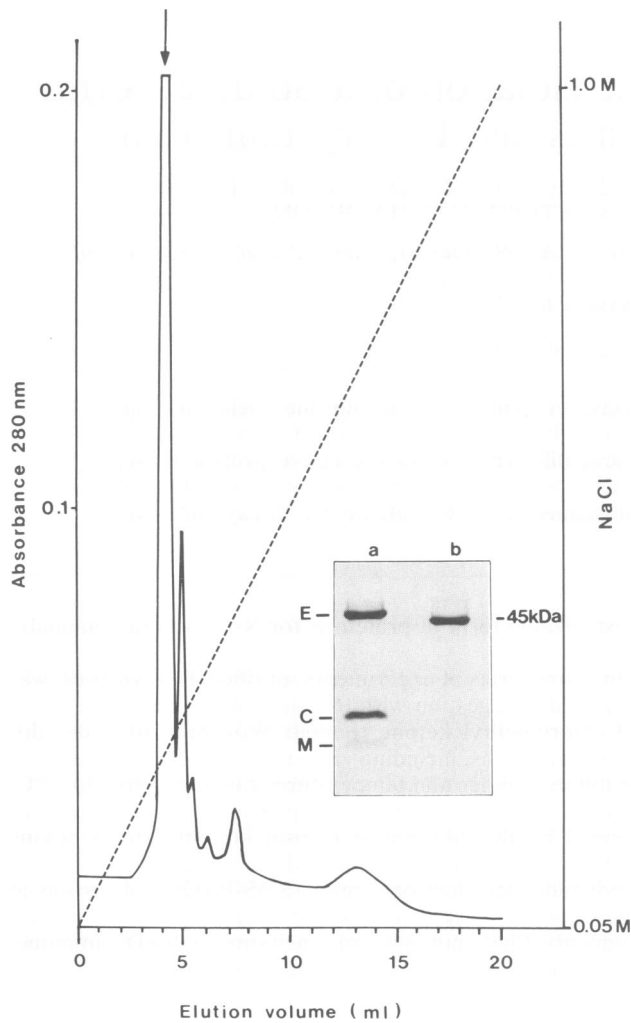
* Corresponding author.

At 0°C, however, cleavage was limited and led to the quantitative conversion of protein E into a 45-kDa species (Fig. 1, lane b) which contained the authentic N terminus (Ser-Arg-X-Thr-His-Leu-Glu). After ultracentrifugation of the digestion products, the 45-kDa fragment remained, together with trypsin and trypsin inhibitor, in the supernatant (Fig. 1, lane c). Analysis of the residual particles in the pellet revealed proteins C and M and small amounts of both protein E and the 45-kDa fragment (Fig. 1, lane d).

Purification of the 45-kDa fragment was performed by anion-exchange chromatography (Fig. 2). Before application to the column, the supernatant from the ultracentrifugation step was adjusted to 1.0% n-octyl-β-glucopyranoside (nOG; Pierce) and allowed to stand for 1 h at room temperature in order to inactivate any residual infectivity. Specific elution of the bound 45-kDa fragment was performed with a linear NaCl gradient (Fig. 2). As revealed by SDS-PAGE (inset in Fig. 2), the major peak eluting at about 0.2 M NaCl consisted of the 45-kDa species. Some microheterogeneity of the starting material was apparent from one or two smaller peaks eluting at slightly higher molarity. By SDS-PAGE, however, the protein contained in these peaks was indistinguishable from the major 45-kDa species.

To obtain information on the oligomeric structure of the soluble form as compared with the native form of protein E, cross-linking experiments with the bifunctional cross-linker dimethylsuberimidate (DMS; Pierce) were performed. Purified virus and also the isolated 45-kDa protein were treated with increasing concentrations of DMS in the absence or in
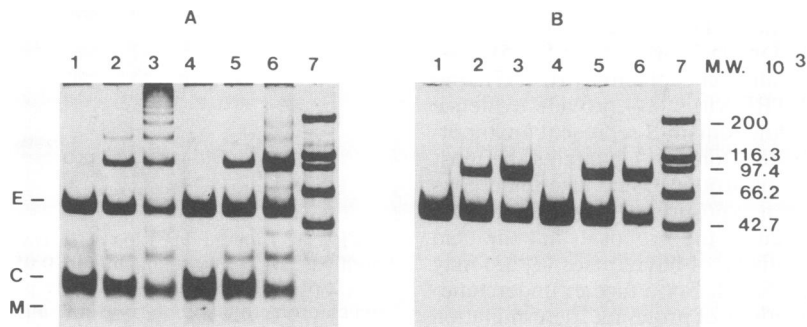

FIG. 3. DMS cross-linking of TBE virus (A) and the isolated 45-kDa protein E fragment (B) in the absence (lanes 1 to 3) and in the presence (lanes 4 to 6) of TX-100. Purified virus and the isolated protein E fragment (both at a protein concentration of 100 μg/ml in 0.05 triethanolamine–0.1 M NaCl, pH 8.0) were cross-linked by the addition of 2 or 10 mM DMS in the same buffer. After 30 min at room temperature, the reaction was stopped by the addition of ethanolamine to a final concentration of 10 mM. For cross-linking in the presence of TX-100, the samples were adjusted to 0.5% final concentration of the detergent and allowed to stand for 30 min at room temperature prior to the addition of DMS. Analysis was by SDS-PAGE in 5% acrylamide gels using a continuous phosphate-buffered system (18). Shown is Coomassie blue staining. Lanes 1 and 4, no cross-linker added; lanes 2 and 5, 2 mM DMS; lanes 3 and 6, 10 mM DMS; lane 7, molecular weight markers.
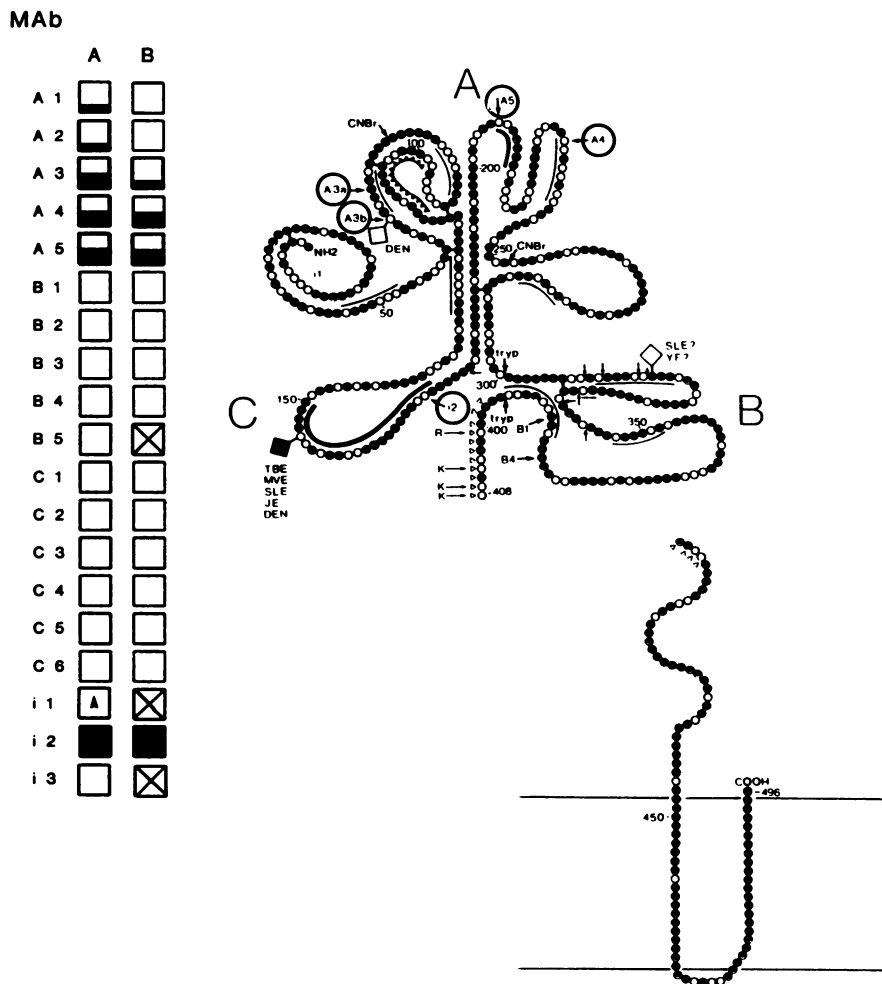
FIG. 4. (Left panel) Antigenic reactivity of purified 45-kDa protein E fragment compared with that of protein E contained in the virion with a panel of MAbs (3). Column A, three-layer ELISA; column B, four-layer ELISA. □, unchanged reactivity; ▣, slightly reduced reactivity; ▨, strongly reduced reactivity; ■, no reactivity; ▲, increased reactivity; ⊠, MAbs B5, i1, and i2 do not react in the four-layer ELISA. (Right panel) Schematic diagram of protein E (adapted from reference 19 with permission of the publisher) showing the cleavage into a 45-kDa soluble fragment (presumably at Lys-408) and a residual membrane anchor. Underlined sequences represent the most variable parts of protein E. Potential N-glycosylation sites in different flaviviruses are marked by solid and open diamonds (MVE, Murray Valley encephalitis virus; SLE, Saint Louis encephalitis virus; JE, Japanese encephalitis virus; DEN, dengue virus). Arrows together with the designations of neutralizing MAbs indicate the location of amino acid substitutions in the corresponding MAb escape mutants. Circles depict sites which are changed in the 45-kDa fragment.

the presence of Triton X-100 (TX-100) and then subjected to SDS-PAGE (Fig. 3). As described previously (8) cross-linking of the whole virion resulted in the formation of a series of oligomeric complexes of both the C protein and the E protein (Fig. 3A, lanes 2 and 3). After solubilization with TX-100, the dimer represented the major oligomeric species and only faint bands of trimers and tetramers were detectable (Fig. 3A, lanes 5 and 6). The soluble form of protein E yielded dimers both in the absence and in the presence of TX-100 (Fig. 3B), and no higher-molecular-weight complexes were observed.

The antigenic structure of the TBE virus protein E has been characterized previously by the use of 19 MAbs (3, 19), each recognizing a different antigenic site in distinct structural elements of the protein. We have now used these MAbs to study possible structural changes in the soluble 45-kDa

species as compared with the protein as part of the virion. These analyses were performed in a three-layer enzyme-linked immunosorbent assay (ELISA) (7) using either the 45-kDa protein or the purified virus adsorbed to the solid phase and a four-layer ELISA (11) using guinea pig anti-TBE immunoglobulin for antigen capturing. The results are shown in the left panel of Fig. 4 and are also included in the structural model of protein E, which was adapted from the work of Mandl et al. (19) to indicate the liberation of the 45-kDa fragment by trypsin cleavage (right panel). Changes in antigenic reactivity affected epitopes within antigenic domain A and especially epitope i2, which was not recognized by the corresponding antibody on the 45-kDa protein. MAb i2 also had a strongly reduced reactivity with the rosettelike aggregates of the whole protein E obtained after solubilization with TX-100 (12) (data not shown). Alterations
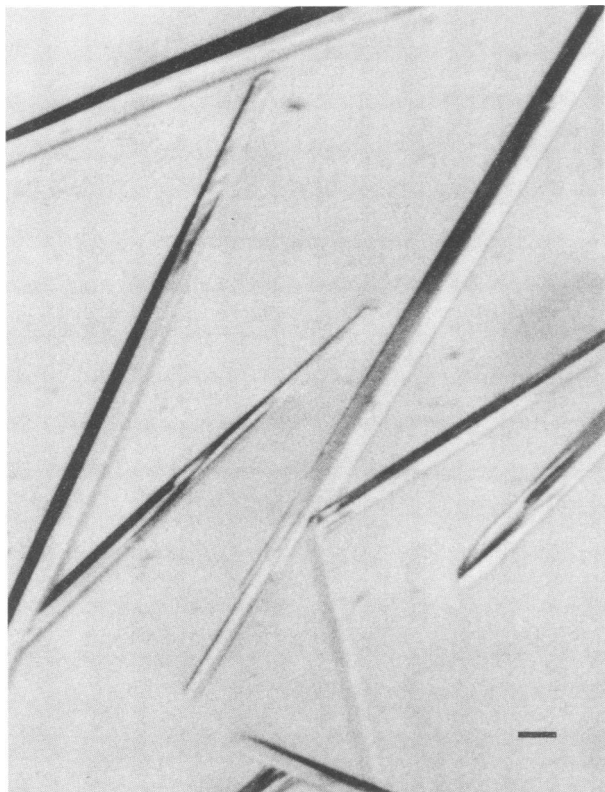
FIG. 5. Photograph of crystals of soluble fragment of protein E. Bar, 0.1 mm.

were not apparent in epitopes of domains B and C, and the reactivity of epitope i1, which is located within the first 22 amino terminal residues (13a), was significantly enhanced.

Experiments to crystallize the soluble form of protein E were performed at 20°C using hanging-drop (3 µl) vapor diffusion (21). The samples had a protein concentration of 6 mg/ml and contained 0.5% nOG (Sigma). Hexagonal rod-shaped crystals were obtained by using polyethylene glycol 8000 (Sigma), purified by the method of Ray and Puvathingal (24), in 1.4 to 1.8 M KCl and 50 mM Tris, pH 8.2 to 8.5. Although not required for stability of the 45-kDa species, the presence of 0.5% nOG appeared to be essential for crystallization. The crystals can be grown to over 1.5 mm in length and 0.1 mm in thickness (Fig. 5). They diffract X rays to spacings of about 0.3 nm, and they are therefore suitable for a three-dimensional structure analysis of the E protein fragment.

Such a structure has so far been determined for only two viral membrane glycoproteins: the influenza virus hemagglutinin (33) and neuraminidase (29). In both cases, soluble membrane anchor-free forms of the proteins were generated by limited proteolytic cleavage from purified virions. Similarly, the digestion procedure described in this report leads to the preferential formation of a 45-kDa fragment from the TBE virus protein E without further degradation. Only a particular site in the native protein appears to be susceptible to tryptic cleavage under the conditions used; the site probably corresponds to one of the two accessible sites described by Wengler et al. (31) for West Nile virus.

The elution profile from the anion-exchange column revealed a major peak with two or three satellite peaks. Each

of these contained a 45-kDa fragment which was indistinguishable from the others by SDS-PAGE. The different elution properties are therefore likely to be due to charge heterogeneities. Inspection of the respective sequence reveals four potential tryptic cleavage sites at amino acid positions 401 (Arg), 405 (Lys), 407 (Lys), and 408 (Lys) (Fig. 5). Since the fragment with the highest number of positive charges is expected to elute first from the anion-exchange column, we assume that the major peak in Fig. 2 represents the species formed by cleavage after residue 408.

The differences in MAb reactivity observed concerned antigenic sites within domain A and the isolated epitopes i1 and i2. The reduced binding of MAbs to epitopes of domain A indicates some alterations in that part of the protein, which is also susceptible to low pH-induced conformational changes (3, 25, 26). These structural differences may be due to the removal of approximately 80 amino acids at the carboxy terminus. In contrast, the removal of the membrane anchor does not seem to be responsible for the loss of reactivity with the isolated epitope i2, since a similar result was also obtained with solubilized and aggregated (rosette-like) forms of protein E (unpublished observations). It is also possible that the differences observed represent denaturation effects occurring in the course of the ELISA procedure, especially during coating of the antigen to the solid phase, which may affect the isolated protein more strongly than when it is present on the large virion particle.

Despite these subtle structural differences, the reactivity of most MAbs with the 45-kDa fragment was unchanged. Since it also exhibited the same cross-linking pattern as the whole solubilized protein E, it is reasonable to assume that the X-ray crystallographic analysis will yield a picture which matches closely that of the native protein. Similar to the experience with other viruses (6, 13, 27) and viral glycoproteins (16, 30, 32) the resolution of the three-dimensional structure of protein E will have a great impact on the further elucidation of the functional activities and antigenic properties of flaviviruses.

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