Characterization of a Disulphide Bridge-stabilized Antigenic Domain of Tick-borne Encephalitis Virus Structural Glycoprotein

By G. WINKLER,† F. X. HEINZ* AND C. KUNZ
Institute of Virology, University of Vienna, Kinderspitalgasse 15, A-1095 Vienna, Austria

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SUMMARY
Proteolytic digestion of purified whole tick-borne encephalitis virus or its isolated envelope glycoprotein (E) in the form of rosettes yields an Mr 9000 fragment that is resistant to further digestion and carries polyclonal and monoclonal antibody-defined antigenic determinants. In a denaturation/renaturation experiment it was demonstrated that the antigenic reactivity of this domain, which was lost upon reduction and carboxymethylation, could be regained if the reducing agent was dialysed out before carboxymethylation. By the use of [35S]cysteine-labelled E protein and amino acid analysis it was confirmed that the reacquisition of antigenic reactivity in the renaturation experiment was associated with the reformation of disulphide bridges, which apparently confer structural stability to this part of the molecule. By the experiments performed we have identified an independently folding antigenically active domain of the E protein that is stabilized by disulphide bridges and has a strong tendency for renaturation.

Tick-borne encephalitis (TBE) virus is a human-pathogenic member of the family Flaviviridae (Westway et al., 1985). As is characteristic of flaviviruses in general, TBE virus is a small enveloped virus with an isometric nucleocapsid that contains a single molecule of plus-stranded RNA. The mature virion contains three structural proteins, which are termed E, C and M and have mol. wt. of 55000, 15000 and 7500, respectively (Heinz & Kunz, 1981). Both E and M are associated with the lipid envelope, whereas C forms the nucleocapsid. By the use of defined viral subunits it has been shown for TBE virus (Heinz et al., 1981) and also for other flaviviruses (Della-Porta & Westaway, 1977; Kitano et al., 1974; Trent, 1977) that the E protein represents the viral haemagglutinin, which induces neutralizing antibodies and mediates protection. In the course of investigating the antigenic structure of the TBE virus E protein we have identified eight monoclonal antibody-defined epitopes, which differ with respect to their functional activities (neutralization, protection, haemagglutination inhibition), serological specificities and topological relationship (Heinz et al., 1983a). Seven of the eight epitopes cluster to form two major antigenic domains (A and B) with different structural properties. The antigenic reactivities of the epitopes of domain A are sensitive to denaturing agents, whereas those of domain B are resistant even to boiling in SDS. The antigenic reactivity of domain B is, however, lost upon reduction and carboxymethylation (Heinz et al., 1983b). By Triton X-100 solubilization and centrifugation into detergent-free density gradients the native E protein is obtained in the form of rosettes. These rosettes contain both E and M, have a sedimentation coefficient of about 15S and a diameter of approximately 17 nm in electron micrographs of negatively stained material (Heinz & Kunz, 1980; Heinz et al., 1981). Proteolytic digestion of these complexes by any of several enzymes yields an Mr 9000 fragment, which is resistant to further proteolysis, suggesting the presence of a stabilized core structure. This fragment carries

† Present address: Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School, UMDNJ, Piscataway, New Jersey 08854, U.S.A.

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all the epitopes of domain B, reacts with polyclonal immune sera and induces haemagglutination inhibiting and neutralizing antibodies (Heinz et al., 1984). In the present study our goal was to characterize the structural properties underlying the resistance to denaturation and proteolysis of the $M_r$ 9000 fragment and its antigenic determinants.

TBE virus (strain Neudörl) was grown in primary chick embryo cells, concentrated by ultracentrifugation, and purified by rate-zonal followed by equilibrium sucrose density gradient centrifugation as described previously (Heinz & Kunz, 1981). For the preparation of $[^{35}S]$cysteine-labelled virus the growth medium was removed at 4 h post-infection and replaced by the same medium containing only 1/100 the normal cysteine concentration and 1 mCi per 30 ml of L-$[^{35}S]$cysteine (Amersham SJ.232). Concentration and purification of virus was performed as described above.

For the denaturation/renaturation experiment the viral glycoprotein was isolated by a two-step HPLC procedure essentially as described by Winkler et al. (1985). In brief, after precipitation with TCA of a purified virus suspension the proteins were solubilized in an SDS-containing phosphate buffer. This sample was either directly subjected to HPLC or it was reduced and carboxymethylated (see below) before chromatography. In the first purification step the E protein was separated from the other proteins on a TSK-G 3000 SW gel permeation column (600 × 7.5 mm internal diameter, Toyosoda) using a 50 mM-phosphate buffer pH 6-5, containing 0-1% SDS, for elution. Salts and SDS were then removed from the E protein peak by reversed phase chromatography on a C$_3$ support (Beckman RPSC, 5 µm, wide pore, 4-6 × 75 mm). Gradient elution was performed using 0-01 M-trifluoroacetic acid (TFA; Pierce Chemical, Rockford, Ill., U.S.A.) versus 0-01 M-TFA in a mixture of 50% acetonitrile and 50% 2-propanol at a flow rate of 2 ml/min. The linear increase of organic solvent was 6%/min. The purified protein was recovered in salt-free form by lyophilization.

For reduction and carboxymethylation the TCA-precipitated proteins (200 to 400 µg) were dissolved in 500 µl 1 M-Tris- HCl buffer pH 8-1, containing 2% SDS and 100 mM-dithiothreitol (Serva). The solution was flushed with N$_2$ and heated in a boiling water bath for 5 min. Iodoacetic acid, sodium salt (Serva, Heidelberg, F.R.G.) was added to a final concentration of 300 mM and the sample was incubated in an N$_2$ atmosphere for 1 h at 37 °C. Subsequently the E protein was purified by gel permeation chromatography (TSK-G 3000 SW, see above). Carboxymethylation of free SH groups was performed in the same manner, except that dithiothreitol was omitted. For the renaturation experiment the sample was split after reduction. One half was immediately carboxymethylated, whereas the second part was dialysed against 1 M-Tris-HCl buffer pH 8-1, at 4 °C for 24 h. After addition of bovine serum albumin (2% w/v), carboxymethylation was performed as described.

The chemical nature of cysteine sulphur in the untreated, reduced, and renatured $[^{35}S]$cysteine-labelled glycoprotein E was examined by direct determination of the cystine/carboxymethylcysteine ratio after carboxymethylation. After isolation and purification of the glycoprotein by a two-step HPLC method (see above) the polypeptide was totally hydrolysed with 6 M-HCl at 110 °C for 26 h in evaporated, sealed glass vials. Unlabelled cystine (Sigma) and carboxymethylcysteine (Fluka, Buchs, Switzerland) were then added to allow optical detection at 214 nm. The amino acid mixture was applied to a C$_{18}$ column (Altex; Ultrasphere-ODS, 5 µm, 4-6 × 250 mm) and separation was achieved by isocratic elution using 0-01 M-TFA at a flow rate of 0-5 ml/min. Small fractions (250 µl) were collected; the location of radioactivity under the optically detected cystine or carboxymethylcysteine peaks indicated the presence of disulphide bridges or free SH groups, respectively.

Fig. 1 shows the comparative digestion of isolated glycoprotein rosettes and whole purified virions with α-chymotrypsin. In both samples a decreasing amount of E protein in the course of the digestion is accompanied by an increasing concentration of the $M_r$ 9000 fragment. This result proves that the protease resistance observed is not an artifact seen only with the isolated glycoprotein due to detergent-protein associations, but it also represents a property of the E protein in its most native conformation as an integral component of the viral membrane. The varying amounts of the C protein in Fig. 1 (b) are due to inconsistent precipitation of this protein by TCA.
Short communication

Fig. 1. Time course of proteolytic digestion of TBE virus E protein rosettes (Heinz et al., 1981) (a) and whole purified virus (b) (each at 40 μg/ml in 0.05 M-triethanolamine, 0.1 M-NaCl, pH 8.0) with α-chymotrypsin (1 unit per mg protein). Incubation was carried out at 37 °C and after (lanes 1) 0, (lanes 2) 1, (lanes 3) 2, (lanes 4) 4, (lanes 5) 8, (lanes 6) 24 and (lanes 7) 36 h samples containing approximately 1 μg of protein were precipitated with TCA, and run on 15% SDS gels according to Laemmli & Favre (1973). The separated proteins were blotted onto nitrocellulose sheets (Towbin et al., 1979) which were incubated first with mouse immune serum, then with rabbit anti-mouse Ig, and finally with peroxidase-conjugated rabbit anti-mouse Ig, each for 1 h at 37 °C. For visualization diaminobenzidine and H2O2 were used as a substrate. I, Immunoreactive fragments, M, 9000; E, C, M, virus proteins.

The availability of this distinct antigenic domain allowed its further characterization with respect to the presence of carbohydrate side chains as analysed by concanavalin A binding. As can be seen in the time course experiment shown in Fig. 2, only the whole undegraded E protein and a large intermediate cleavage product react with concanavalin A whereas the M, 9000 fragment completely lacks reactivity. This findings suggests that the carbohydrate-containing part of the E protein is not located on the protease-resistant fragment but is degraded to smaller peptides which cannot be analysed in SDS gels.

Although the antigenic reactivity of domain B and the M, 9000 fragment is resistant to SDS it is lost upon reduction and carboxymethylation (Heinz et al., 1983b). Reactivity is however retained in Western blots, which also involves reduction in addition to SDS treatment prior to electrophoresis. This suggests not only that disulphide bridges are involved in stabilizing the native structure of domain B but also that renaturation can probably occur during electrophoresis and/or blotting. To test this hypothesis the following renaturation experiment was carried out (Fig. 3). SDS-solublized E protein isolated by a two-step HPLC procedure (Winkler et al., 1985) (a) was either carboxymethylated without reduction (b), carboxymethylated in the presence of dithiothreitol (c), or treated with dithiothreitol, dialysed to remove the reducing agent and allow for renaturation and then carboxymethylated (d). Each of these preparations was dotted onto nitrocellulose and the antigenic reactivity of domain B was assessed by probing with monoclonal antibodies B1, B2, B3 and B4. As can be seen in Fig. 3(a)
Fig. 2. Time course of $\alpha$-chymotryptic digestion of the TBE virus E protein on whole purified virus. After SDS-PAGE and blotting onto nitrocellulose sheets (Towbin et al., 1979) the glycosylation of cleavage products was assessed by incubating the sheets with concanavalin A and then with horseradish peroxidase essentially as described by Hawkes (1982). For visualization diaminobenzidine and $H_2O_2$ were used as a substrate. Numbers on top of the figure indicate the time (h) of proteolysis.

and (b), antigenicity is retained after SDS treatment and carboxymethylation, whereas it is lost upon reduction and carboxymethylation. However immunological reactivity is regained if the reducing agent is removed by dialysis prior to carboxymethylation (Fig. 3d).

In order to demonstrate that reformation of disulphide bridges did indeed occur during renaturation, the same experiment was carried out using E protein [$^{35}$S]cysteine-labelled in vivo. Each of the four samples was hydrolysed in order to determine whether the [$^{35}$S]cysteine label was present in the form of cystine or carboxymethylcysteine (experimental details are described above). The result is shown in Fig. 4. In the SDS-treated sample (a) all the radioactivity is found under the cystine peak. The same distribution is also obtained after carboxymethylation (b) indicating that no free SH groups are accessible in the SDS-treated protein without reduction. After reduction and carboxymethylation (c) a large amount of radioactivity shifted to the carboxymethylcysteine peak. The residual cystine radioactivity indicates that either reduction
Fig. 3. Renaturation of antigenic domain B of the TBE virus E protein after reduction and SDS treatment. A dilution series containing equal amounts (5 μg, 0.5 μg, 0.05 μg) of four different preparations of the SDS gel permeation HPLC-purified SDS-treated E protein were dotted onto nitrocellulose sheets. (a) Only SDS-treated; (b) carboxymethylated; (c) reduced and carboxymethylated; (d) reduced, dialysed (renatured) and then carboxymethylated. The antigenic reactivity was assessed by probing the dots with monoclonal antibodies defining antigenic domain B (B1 to B4). The immunoassay was performed as described in the legend to Fig. 1.

Fig. 4. Analysis for the presence of cystines or carboxymethylcysteines in four different preparations of the SDS-treated [35S]cysteine-labelled E protein. The protein samples were purified by SDS gel permeation chromatography and subsequent reversed-phase HPLC (see text). After total hydrolysis in 6 M-HCl, non-radioactive cystine and carboxymethylcysteine (CMC) were added and applied to a C18 column. The separation of the two amino acids was followed by detection of the u.v. absorbance at 214 nm (———). The distribution of radioactivity (-----) was determined by taking samples every 0.5 min (250 μl). (a) SDS-treated only; (b) carboxymethylated; (c) reduced and carboxymethylated; (d) reduced, dialysed and then carboxymethylated.

or carboxymethylation was not complete under the conditions employed. Clearly, in the sample in which the protein was reduced and then allowed to renature before carboxymethylation, the radioactivity has again shifted back to the cystine peak (d). These results together with those of Fig. 3 demonstrate that the reacquisition of the antigenic reactivity of domain B is associated with the reformation of disulphide bridges.

These data suggest that the \( M_r \) 9000 proteolytic fragment contains an independently folding domain of the E protein which is stabilized by disulphide bridges and has a strong tendency to renature even after reduction and SDS treatment. This also implies that, despite their resistance to boiling in SDS and their detectability in Western blots after reduction, the monoclonal antibody-defined epitopes B1 to B4 represent highly conformation-dependent antigenic sites. Consequently, positive reactivity of monoclonal antibodies in Western blots...
should not generally be taken as evidence for the presence of conformation-independent or sequential antigenic determinants.

The location of disulphide bridges in the E protein of West Nile virus has recently been determined by Nowak & Wengler (1987) and a comparison of the recently published amino acid sequences of the E proteins of yellow fever virus (Rice et al., 1985), West Nile virus (Wengler et al., 1985), Murray Valley encephalitis virus (Dalgarno et al., 1986) and the Far Eastern subtype of TBE virus (Pletnev et al., 1986) reveals that the location of the cysteine residues is highly conserved among the E proteins of these flaviviruses. This high degree of conservation of cysteine is comparable to that found between the haemagglutinins of different influenza A virus subtypes (Webster et al., 1982) and is also characteristic for the otherwise highly polymorphic class I major histocompatibility complex antigens (Shiroishi et al., 1984) suggesting their critical role for determining the protein structure and consequently for the expression of biological functions.

**REFERENCES**


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