Protein-Protein Interactions in an Alphavirus Membrane

RICHARD P. ANTHONY AND DENNIS T. BROWN*

*Cell Research Institute and Department of Microbiology, University of
Texas at Austin, Austin, Texas 78713-7640*

Received 15 March 1990/Accepted 20 November 1990

Using homobifunctional chemical cross-linkers with various span distances, we have determined the near-neighbor associations and planar organization of the E1 and E2 envelope glycoproteins which compose the icosahedral surface of Sindbis virus. We have found that E1-E2 heterodimers, which form the virus protomeric units, exist in two conformationally distinct forms, reflecting their nonequivalent positions in the icosahedron. Three of these heterodimers form the trimeric morphologic units (capsomeres) which are held together by central E1-E1 interactions. In addition, we present data which suggest that E2-E2 interactions organize the capsomeres into pentameric and hexameric geometric units and that E1-E1 interactions between capsomeres maintain the icosahedral lattice in mature virions.

Sindbis virus, the prototype alphavirus, comprises an icosahedral nucleocapsid surrounded by a host-derived lipid membrane containing 240 copies of each of the two viral glycoproteins, E1 and E2, arranged into a $T=4$ icosahedral lattice (1, 5, 13, 17). The virus has an average diameter of approximately 70 nm when viewed by cryoelectron microscopy (13).

The protomeric unit of the Sindbis virus membrane-based icosahedron is thought to be a heterodimer of E1 (63 kDa) and E2 (59 kDa) which is maintained by disulfide bridges and remains stable even after solubilization of the membrane with detergent (20, 35, 41, 42). Cross-linking experiments reported by Rice and Strauss (29) indicate that the protomers of Sindbis virus aggregate to form a capsomere containing a total of 6 proteins $[(E1-E2)_3]$. A thorough understanding of virus structure is possible only through X-ray crystallography. While many nonenveloped viruses have been crystallized, yielding three-dimensional structures at atomic resolution (2, 20, 26), satisfactory crystals of intact enveloped virions have not been obtained. High-resolution, three-dimensional analysis of the influenza virus membrane protein, HA, has been done after enzymatic cleavage from the membrane surface (39); however, the nature of the intertrimer relationships in the virion itself, if any, is not known. Indeed, the organization of the protein lattice in the bilayer of any enveloped virus has not been determined. In the absence of X-ray diffraction data, other methods to define the topological interactions of the envelope proteins responsible for virus assembly, stability, and subsequent disassembly during infection of a host cell must be found.

Chemical cross-linkers have been used in our laboratory (4) and in other laboratories to determine near-neighbor associations and spatial arrangement between proteins in complex biological aggregates such as erythrocyte proteins, the major histocompatibility complex proteins, adenovirus, Sendai virus, human immunodeficiency virus, murine and feline leukemia viruses, influenza virus, parvovirus, Semliki Forest virus, vesicular stomatitis virus, and ribosomes (6-9, 12, 15, 21, 23, 25, 31, 32, 36, 38, 40). Recently, Watts and Coombs (37) have used cross-linkers to determine the two-dimensional structure of the bacteriophage T4 baseplate, a structure containing 17 different proteins. Indeed, the only

information available on the spatial arrangement of protein components in several of the examples cited above has been obtained through the use of chemical cross-linkers.

The rationale behind the use of chemical cross-linkers for structural studies is based on their specificity and their well-defined reactivity. If two proteins can be locked together by a linkage that is shorter than 2 nm and that linkage is nonrandom and repeatable, then the near-neighbor contacts detected in the complex are structurally significant. In a geometrically ordered array with a defined number of components, the subunit organization can be characterized with a minimal amount of information on substructure contacts (3, 14, 24).

The research reported in this manuscript used chemical cross-linkers, among other techniques, to identify the planar and spatial organization of E1 and E2 on the surface of Sindbis virus. This study also characterizes the rearrangement of this organization induced by detergent treatments and exposure of the virus to acidic pH conditions.

MATERIALS AND METHODS

Cells, virus, and medium. Baby hamster kidney cells, strain 21 (BHK-21), were grown as monolayers in minimal essential medium supplemented with 10% (vol/vol) fetal calf serum, 5% tryptone phosphate broth, 1 mM glutamine, and 1% gentamicin and were grown at 37°C.

The growth of nonradiolabeled and [^{35}S]methionine-radio-labeled Sindbis virus has been described elsewhere (15). For the growth of [^{35}S]methionine-labeled virus, cells infected at a multiplicity of infection of 25 PFU per cell were labeled with 40 μCi of [^{35}S]Translabel (Du Pont, NEN Research Products, Boston, Mass.) per ml postinfection. Virus was harvested for purification 24 h postinfection.

Purification of virus. Medium from flasks (25 by 75 cm) of Sindbis virus-infected cells was pooled, and the cell debris was removed by centrifugation at 5,000 rpm for 15 min in a Sorvall RC-5B superspeed centrifuge. Virus was purified and concentrated from medium by gradient centrifugation twice through a linear 15 to 35% potassium tartrate gradient (in phosphate-buffered saline lacking calcium [PBS-D]) with a Sorvall 627 rotor for 12 h per run at 24,000 rpm. The purified virus bands were harvested and placed in a 12,000-molecular-weight cutoff dialysis tube, sealed, and dialyzed against HN buffer (10 mM HEPES [N -2-hydroxyethylpiperazine-

* Corresponding author.

N'-2-ethanesulfonic acid], 100 mM NaCl [pH 7.4]) at 4°C overnight. Aliquots of the dialyzed virus were prepared, assayed for protein concentration by the Bio-Rad method, trichloroacetic acid precipitated, counted to determine radiolabel incorporation, and then stored at 4°C for up to 7 days before use.

Acid or DTT treatment of virus. Virus purified as described above was placed in 12,000-molecular-weight cutoff dialysis tubing and dialyzed for 12 h at 4°C in HN buffer that had been adjusted to pH 5.3 for acid treatment or in HN buffer (pH 7.4) containing 5 mM dithiothreitol (DTT). After initial treatment, the acid-treated samples were shifted back to pH 7.4, and the thiol-reducing agent was washed out of DTT-treated virus by dialysis in fresh HN buffer.

Cross-linking of viral envelope proteins. The likelihood that the one cross-linking event required to bond two proteins together will occur is dependent upon the following factors. (i) Two free reactive groups must be facing each other within the span distance of the cross-linker used. (ii) The conformation of the reactive sites on the proteins must allow for the interaction with the reactive group on the cross-linker. (iii) A protein's reactive site cannot be occupied by a cross-linker which has its opposite reactive group hydrolyzed before the cross bridge formation is made. (iv) Sufficient cross-linker must be available to ensure cross bridge formation before total inactivation of the cross-linker by the competing hydrolysis reaction. (v) Intra-protein cross bridges should not form by using the only available interprotein reactive sites. (vi) Cross-linker concentration must be limited so that the inter- and intraprotein cross bridges within the protein suspension do not form an insoluble aggregate that is unresolvable. (vii) Reactive sites must be accessible to the cross-linker. These factors help to empirically define the reaction conditions that will produce the highest number of resolvable multimers. All of these variables result in cross-linking of only a fraction of the available reactive sites while increasing the reaction specificity. For further discussion of the mechanism of cross-linker reactions, see the reviews by Peters and Richards (24) and Gaffney (14).

Purified virus particles were treated with various concentrations (ranging from 500 mM to 100 nM by twofold dilutions) of homobifunctional chemical cross-linking reagents, all obtained from Pierce Chemical Company (Rockford, Ill.). These agents were selected for their different span lengths, solubilities in water, abilities to cleave, and reactive groups. The reagents used in these studies were disulfosuccinimidyl tartrate (SDST; span, 0.64 nm; cleaved by periodate), dimethyl 3,3'-dithiobispropionimidate (DTBP; span, 1.19 nm; cleaved by DTT), and ethylene glycolbis(sulfosuccinimidylsuccinate) (SEGS; span, 1.61 nm; cleaved by hydroxylamine at pH 8.2) (14, 24).

The extent of cross bridge formation at neutral pH (range, 6.0 to 8.0) and acidic pH (range, 4.8 to 5.5) increased with time. Little additional reaction occurred after 30 min; therefore, all cross-linking was performed at room temperature for 30 min. The reactions were quenched with equal volumes of 200 mM glycine, which completely blocked further cross-linking. The virions used in the study were kept suspended in HN buffer in 20- μ l aliquots with a total protein concentration not exceeding 1 μ g/ml. All cross-linkers were dissolved in HN buffer to make fresh stock solutions immediately before use. Stock solutions were mixed with the virus suspensions to give final working concentrations in 40- μ l volumes.

Our previous studies of nucleocapsid structure using chemical cross-linkers have shown that with each cross-linker, a specific set of reaction conditions (protein and

chemical cross-linker concentration and pH) is needed in order to detect specific domains of protein-protein association (4). In each of the experiments described below, when comparisons between cross-linkers or cross-linking conditions are made, the virus stocks used are from the same preparation and protein concentrations are identical. Differences in cross-linker characteristics and the tight packing order of the envelope proteins led us to alter some of the reaction conditions in order to expose cross-linking sites that may not normally be available.

Cross-linking under acidic conditions was performed by not adjusting the pH of the buffered stock solution, in which case the cross-linker reduced the pH as the concentration increased (100 mM, pH 4.8; 50 mM, pH 5.0; 25 mM, pH 5.3; 12.5 mM, pH 5.5). It was necessary to increase the concentration of the cross-linker in order to compensate for the rapid loss of reactivity in the more acidic pH range. The reactions were quenched with equal volumes of 200 mM glycine for 15 min and then solubilized with sample buffer (2% sodium dodecyl sulfate [SDS]–5% 2-mercaptoethanol–10% glycerol–1% bromophenol blue in 0.24 M Tris-HCl, pH 6.8), except in the case of DTBP, which is cleavable with thiol agents. In this case the reaction mixtures were dissolved in sample buffer lacking 2-mercaptoethanol. Samples were then heated in 100°C for 5 min before analysis by SDS-polyacrylamide gel electrophoresis (PAGE) (4), except for DTBP complexes, which were heated to 60°C for 5 min to limit cross-linker cleavage.

In order to study virus disassociation, virions were treated with the detergent Triton X-100 (Pierce) at a concentration of 0.24, 0.74, or 1.5 mM for 15 min at 39°C prior to cross-linking. Cross-linking treatments of Triton X-100-solubilized virus were adjusted to make final concentrations of cross-linker identical to those in non-detergent-treated controls. Preparation of samples for SDS-PAGE was identical to that mentioned above.

First- and second-dimension SDS-PAGE. Identical 4 to 10% gradient SDS-PAGE (14 by 12 by 0.15 cm) gels were used to resolve oligomers stabilized by chemical cross-linking. The polyacrylamide gels were modifications of the gels described previously by Coombs and Brown (4). The stacking gels were made with 3% (wt/vol) acrylamide in Tris-HCl buffer (pH 6.8), and the linear 4 to 10% (wt/vol) acrylamide separating gels were made with Tris-HCl buffer (pH 8.3). SDS (2%) was used throughout the slab gels and in the running buffer (Tris-HCl [pH 8.3], 200 mM glycine). Samples were prepared for electrophoresis as described above. After electrophoresis, the gels were fixed with dimethyl sulfoxide and impregnated with 2,5-diphenyloxazole (PPO). The gels were then exposed on X-Omat AR X-ray film (Eastman Kodak, Rochester, N.Y.), and the films were scanned with a Hoefer densitometer to quantify cross-linked products.

Western immunoblot assays. The cross-linking protocols were carried out on purified nonradiolabeled Sindbis virus and run with 4 to 10% SDS-PAGE gels as described above. The gels were then equilibrated for 60 min in 25 mM Tris–192 mM glycine (pH 8.3) and then electrophoretically transferred to a GeneScreen membrane (Du Pont, NEN). The membranes were then blocked for 1 h with 10% nonfat dry milk–1% bovine serum albumin in PBS. The membranes were then incubated in blocking buffer containing 0.3% Tween 20 and the appropriate antisera (polyclonal antisera to whole virus or a polyclonal monospecific antiserum to either E1 or E2). After being washed five times, the membranes were labeled for 1 h with ¹²⁵I-protein A (10 μ Ci/ μ g;

Du Pont, NEN). The membranes were then washed five times with PBS, dried, and autoradiographed.

Trypsin and V8 protease treatment of virus. Removal of E2 from intact virus was performed by protocols described by Edwards et al. (11). Aliquots of purified virus were treated with acid and then subjected to proteolytic digestion by trypsin treatment ($3\times$ crystallized trypsin treated with L-[tosylamido-2-phenyl]ethyl chloromethyl ketone; Worthington Diagnostics; used at a $25\text{-}\mu\text{g/ml}$ final concentration in PBS-D) for 3 h at 37°C . The trypsin reaction was then quenched with soybean trypsin inhibitor (final concentration, $100\text{ }\mu\text{g/ml}$; Worthington) for 15 min, and the virus was then cross-linked with DTBP.

RESULTS

Characterization of an E1-E2 heterodimer. Analysis of the proteins of purified Sindbis virus by SDS-PAGE frequently detects protein bands with molecular weights higher than expected for virus protein monomers (30). We have also identified a heterodimer (designated hd1; see figure 1), which is most likely equivalent to that described by Wahlberg and Garoff (35) and Ziemiecki and Garoff (41), who report the isolation of an E1-E2 heterodimer from Semliki Forest Virus and Sindbis virus after solubilization with detergent. The fact that oligomers are stable even when subjected to the denaturing conditions of SDS-PAGE suggests that they may represent important substructures on the surface of intact virions. We found the stability of the heterodimer to be sensitive to repeated heating (100°C) and to DTT or 2-mercaptoethanol. This indicates that the heterodimer may be stabilized by disulfide bridges in the intact virus. Similar interactions sensitive to heat and thiol reagents are found between F proteins in Sendai virus (31).

DTBP cross-linking of Sindbis virus. Since DTBP (span distance, 1.19 nm) is cleavable with thiol reducing agents, DTT and 2-mercaptoethanol were not used in the first-dimension gels or in sample buffers when analysis of cross-linked products was conducted. In the absence of reducing agents, monomeric E1 and E2 migrate to approximately the same position in SDS-PAGE, and the mobility of nonreduced oligomers formed from these monomers may also be altered.

Under neutral conditions, at all cross-linker concentrations tested, the multimers detected by DTBP (Fig. 1B) consisted of the E1-E2 heterodimer hd1 and a second heterodimer of E1 and E2 migrating at 115 kDa and designated hd2. We could also detect some oligomers which migrated at approximately 200 kDa that contain E1 and E2 and that probably represent dimers of the heterodimers. The lack of reducing agents in the analysis of DTBP-cross-linked oligomers could mean that the heterodimers and other trace oligomers detected under neutral cross-linking conditions are due to already present cysteine disulfide bridges rather than to cross-linking by DTBP. If this is true, then under neutral conditions this result would indicate that lysine residues are not available on the virus surface or are not within the span distance of DTBP. If DTBP is able to detect near-neighbor contacts, then it can cross-link only those within the heterodimers, as indicated by the lack of larger multimers.

To further define protein interactions in the Sindbis virus membrane, we investigated the stability of the oligomers by exposing the virus to progressively increasing concentrations of Triton X-100 before cross-linking. Triton X-100 was added to samples of virus at concentrations that are pre-

FIG. 1. Intact and Triton X-100-solubilized Sindbis virus cross-linked with DTBP. (A) ^{35}S -labeled Sindbis virus was cross-linked under acidic conditions with decreasing concentrations of DTBP (lanes: 1, 200 mM, pH 4.8; 2, 100 mM, pH 5; 3, 50 mM, pH 5.2; 4, 25 mM, pH 5.6) in HN buffer or with identical DTBP concentrations in HN buffer containing virus previously exposed to different concentrations of Triton X-100 (lanes: 5 to 8, 0.24 mM; 9 to 12, 0.78 mM; 13 to 16, 1.5 mM). Samples were then dissolved in SDS sample buffer lacking 2-mercaptoethanol, heated to 60°C for 5 min, and then analyzed on an SDS-PAGE gel (4 to 10% acrylamide). (B) ^{35}S -labeled Sindbis virus was cross-linked under neutral conditions (pH 7.2) with decreasing concentrations of DTBP (lanes: 1, 12.5 mM; 2, 6.25 mM; 3, 3.12 mM; 4, 1.6 mM) in HN buffer or identical DTBP concentrations in HN buffer containing virus previously exposed to different concentrations of Triton X-100 (lanes: 5 to 8, 0.24 mM; 9 to 12, 0.78 mM; 13 to 16, 1.5 mM). Samples were then dissolved in SDS sample buffer lacking 2-mercaptoethanol, heated to 60°C for 5 min, and then analyzed on an SDS-PAGE gel (4 to 10% acrylamide).

dicted to induce various states of virus disassociation: (i) intact virions with incorporated detergent (0.24 mM), (ii) intact viral membranes free of nucleocapsids (0.78 mM), and (iii) monomeric and dimeric envelope protein-lipid-detergent complexes (1.5 mM). These disassociation states described by Helenius and Soderlund (18) were verified by sedimentation analysis (data not shown).

Virus treated with 0.24 mM Triton X-100 and then cross-linked at neutral pH with DTBP does not stabilize any additional multimers at the cross-linker concentrations tested (Fig. 1B). Increasing the detergent concentration to 0.74 mM, which produces intact envelopes containing Triton X-100 that are free of nucleocapsids, produces hd1 and hd2 and many distinct higher-molecular-weight multimers (Fig.

1B). A second-dimensional analysis of the multimers detected after 0.74 mM detergent treatment reveals that all of the multimers greater than the heterodimers contain only E2 (see Fig. 3). The lack of E1 in these complexes indicates a change in conformation in either E1 or E2 which masks intraheterodimer cross-linking sites. Increasing the detergent concentration to 1.5 mM causes a loss of E2 multimers.

Experiments using low-pH treatment of influenza virus and Sindbis virus have shown that conformational change which reveals fusion activity in the envelope proteins occurs in these proteins (6, 10, 11). With Sindbis virus, acid treatment alone appears to change conformation of the spike complex without dramatically changing virus morphology at the electron microscope level (11, 22). This result implies that protein-protein contacts important for maintaining the icosahedral lattice are not affected by this treatment. To examine whether protein interactions in other regions of the spike complex are responsible for maintaining structure, virus was cross-linked at acidic pH. The chemical cross-linkers have a pH range of 7 to 9 for optimum reactive stability and a half-life of several hours at pH 7.5. At pH 5.3, stability decreases rapidly, with the half-life of the cross-linkers being reduced to a few minutes. To compensate for the reduction of active cross-linker, higher concentrations were used under acidic pH conditions (see Materials and Methods).

Under acidic pH conditions (pH 4 to 5.5), DTBP detected distinct protein oligomers that migrated at approximately 120 and 180 kDa, as determined by comparison to nonreduced markers (Fig. 1A). When these oligomers were analyzed on a second-dimension gel in the presence of reducing agent to cleave the DTBP cross bridges, the 120- and 180-kDa bands, as well as smaller amounts of other oligomers, were found to contain only E1 (Fig. 2B). This analysis identified the 120-kDa oligomer as a dimer of monomeric E1 and the 180-kDa oligomer as a trimer of E1. Under acidic cross-linking conditions, DTBP was not able to detect E2-containing oligomers at any concentration. This indicates that lysine residues exposed on E2 must be more than 1.19 nm apart between E2s and between E1s and E2s.

The addition of Triton X-100 (Fig. 1A) to virus before DTBP cross-linking under acid pH conditions reveals that the trimeric aggregate of E1 is very sensitive to changes in membrane integrity. As the DTBP-susceptible contact points in the E1 trimers are lost, E1 dimers are still detected, implying differences in contact points between E1 monomers within or between specific geometric units (i.e., trimeric capsomeres versus pentamer-hexamer junctions [see Fig. 7]). The E1 dimers require higher concentrations of detergent to alter their contact points, but as E1 dimer contact points are lost, multimers of heterodimers are detected, as shown by the two-dimensional gel of detergent-treated samples in Fig. 2A.

These data show that E1-E1 interactions detectable after exposure to acid are masked in the virus surface at neutral pH. In addition, E2-E2 contacts cross-linkable by DTBP after Triton X-100 solubilization under neutral conditions are either in an undetectable arrangement or do not exist until alterations of E1 trimers occur. The E2-E2 multimers detected in nucleocapsid-free envelopes produced by moderate concentrations of detergent (Fig. 1B and 3) may be due to random collision of E2s that are no longer in the constraints of an icosahedral lattice.

Cross-linking Sindbis virus after proteolytic removal of E2. The removal of the E2 protein ectodomain from the surface of the virus is possible through protease digestion of virus

FIG. 2. Two-dimensional analysis of oligomers detected by DTBP under acidic pH conditions from both intact and Triton X-100-solubilized virus. (A) Triton X-100-treated virus cross-linked at pH 5.3 (sample used in Fig. 1A, lane 12) was run on an SDS-PAGE gel (5 to 10% acrylamide) and then soaked for 2 h in SDS-PAGE running buffer containing 5% 2-mercaptoethanol in order to cleave the DTBP cross-links. The first dimension was then placed on top of a second-dimension SDS-PAGE gel (10% acrylamide). hd indicates the heterodimer from cleavage products of an oligomer larger than monomeric E1 and E2. (B) Virus cross-linked at pH 5.3 (sample used in Fig. 1A, lane 2) was run on an SDS-PAGE gel (5 to 10% acrylamide) and then soaked for 2 h in SDS-PAGE running buffer containing 5% 2-mercaptoethanol in order to cleave the DTBP cross-links. The first dimension was then placed on top of a second-dimension SDS-PAGE gel (10% acrylamide). Arrowheads indicate cleavage products from the 120-kDa [(E1)₂] and 180-kDa [(E1)₃] oligomers. C, Capsid protein.

exposed to a pH of 5.3. This treatment does not alter the virus particle morphology, as shown by electron microscopy (1a, 22), and the intact membrane contains undigested E1 and the carboxy transmembranal tail of E2 (11, 22, 27). The retention of morphology after removal of E2 would imply that E1-E1 contacts are still intact in the virus lacking E2 ectodomain. Cross-linking virus that does not contain E2 does not affect the detection of dimers and trimers of E1 with DTBP (Fig. 4). This indicates that near-neighbor contact between E1s that generate E1 multimers is independent of E2 interactions within the icosahedral lattice.

SEGS cross-linking. Figure 5A shows the oligomers detected by reacting Sindbis virus with selected concentrations of SEGS (span distance, 1.6 nm) under neutral pH reaction conditions. The greatest number of resolvable oligomers was produced by treatments with 114 μ M SEGS (Fig. 5A, lane 3). At the optimal concentration, oligomers of increasing

FIG. 3. Two-dimensional analysis of oligomers detected by DTBP under neutral pH conditions after Triton X-100 solubilization. Triton X-100-treated virus cross-linked at neutral pH (sample used in Fig. 1B, lane 10) was run on an SDS-PAGE gel (5 to 10% acrylamide) and then soaked for 2 h in SDS-PAGE running buffer containing 5% 2-mercaptoethanol in order to cleave the DTBP cross-links. The first dimension was then placed on top of a second-dimension SDS-PAGE gel (10% acrylamide). Cleavage products from oligomers larger than monomeric E1, monomeric E2, and heterodimers contain only E2. C, Capsid protein.

molecular mass were formed in successively decreasing amounts, with the largest being an oligomer migrating at approximately 580 kDa. No higher-molecular-mass oligomers could be detected by densitometer tracing of extended gel exposure, except for those proteins exceeding 2×10^6 Da, which remain in the stacking gel. Distinct bands of E2 multimers with masses of up to 1.0×10^6 Da have been resolved in this gel system (see the E2 multimers detected by DTBP in Fig. 3).

A two-dimensional analysis (Fig. 5B) of the products of

FIG. 4. Trypsin-treated and untreated virus cross-linked with DTBP. 35 S-labeled Sindbis virus was left untreated (pH 7.2) (A), acid treated (pH 5.3) (B), or acid treated (pH 5.3) for 15 min, returned to pH 7.4, and then digested with 100 μ g of trypsin per ml to remove E2 (C). The samples were then cross-linked with DTBP (lanes: 1, 100 mM; 2, 50 mM; 3, 25 mM). Numbers between panels B and C represent molecular masses in kilodaltons.

FIG. 5. Intact and Triton X-100-solubilized Sindbis virus cross-linked with SEGS. (A) Aliquots of 35 S-labeled Sindbis virus were cross-linked with 1.14 mM (lanes 2, 5, 8, and 11), 114 μ M (lanes 3, 6, 9, and 12), or 11.4 μ M (lanes 4, 7, 10, and 13) SEGS in HN buffer. The virus was either intact (group a) or gently solubilized with Triton X-100 at a final concentration of 0.24 mM (group b), 0.78 mM (group c), or 1.5 mM (group d) before cross-linking. The control lanes contain un-cross-linked Sindbis virus (lane 1) or SAAR86-S12 (lane 14; a Sindbis virus mutant defective in its ability to cleave PE2 to E2). The labels 2mer through 5mer indicate multiples of the 115-kDa heterodimer (hd2) stabilized by the SEGS. (B) SEGS-cross-linked (114 μ M, neutral conditions), 35 S-labeled Sindbis virus was run on an SDS-PAGE gel (4 to 10% acrylamide) in the first dimension and then soaked for 2 h in HN buffer (pH 8.2) containing 1 M hydroxylamine and 2% 2-mercaptoethanol. After the gel slice was equilibrated in electrophoresis stacking buffer for 1 h, the sample was placed across the top of an SDS-PAGE gel (10% acrylamide) and imbedded in SDS-3% polyacrylamide. All higher-molecular-weight multimers contain both E1 and E2. In both panels, C indicates the capsid protein.

the 114 μ M SEGS-cross-linked virus was performed to determine the protein species present in the oligomers. All higher-molecular-weight species resolved in the first dimension were found by densitometer analysis to contain equal molar amounts of E1 and E2. This indicates that SEGS detects aggregates of E1 and E2, with the smallest being hd2 and the rest being oligomers of this heterodimer corresponding to di-, tri-, tetra-, and pentamers. Since the heterodimer is not found to form a distinct band exceeding approximately 580 kDa in molecular mass (a pentamer), it may be concluded that SEGS can stabilize hd2 associations in oligomers which form pentamers but does not cross-link oligomer

associations within hexamers. SEGs may therefore be capable of detecting only one conformational form of heterodimer that makes up the pentameric geometric units on the virus surface.

Exposure of the virus to detergent (Fig. 5A) or acidic conditions (data not shown) prior to cross-linking did not produce significant differences in the types of multimers formed (all of which contained E1 and E2) but did alter the amounts of resolvable heterodimer multimers. Acid exposure before cross-linking eliminated the ability to detect multimers larger than hd1. When detergent is added before cross-linking under neutral conditions, the amount of hd2 progressively decreases as the detergent concentration is increased. The amount of hd1 increases as intra- and intermolecular contact points necessary for cross-linking the heterodimers into hd2 conformation are no longer available. These data indicate that E2s at the spike surface are within 1.6 nm of one another and are not interspersed with E1s in the pentamer rings. The loss of detectable hd2 caused by acid and detergent pretreatment, along with simultaneous decreases in amounts of higher-molecular-weight multimers, may be due to the acid-induced conformational change in the heterodimer, which alters associations important for maintaining intrapentamer (and most likely intrahexamer) surface interactions.

Cross-linking of capsomeric units with SDST. Sindbis virus cross-linked with SDST (span distance, 0.6 nm) under acid (100, 50, or 25 mM) or neutral (12.5 or 6.25 mM) conditions stabilizes hd1 into a heterodimer resistant to heat, SDS, and 2-mercaptoethanol (Fig. 6A). The next higher-molecular-mass oligomers detected by SDST are small amounts of a 200-kDa oligomer and two major bands between approximately 300 and 350 kDa. Western blot assays were performed (Fig. 6B) on the SDST-cross-linked samples by using polyclonal monospecific antisera produced against E1 or E2. hd1, as well as the higher-molecular-mass oligomers, was found to contain both E1 and E2. These data identify the bands at 300 to 350 kDa as trimers of heterodimers which may represent the basic capsomere in the Sindbis virus icosahedron. When virus was treated with Triton X-100 (as described above) prior to cross-linking with SDST, the higher-molecular-weight oligomers were no longer detected (Fig. 6A). The trimeric heterodimers were easily disrupted by detergent treatment, losing their contact points at low Triton X-100 concentrations. This result suggests that intercapsomeric interactions detectable by this cross-linker within a mature virion are very sensitive to changes in membrane integrity.

DISCUSSION

Using homobifunctional chemical cross-linkers, we have found that the E1-E2 heterodimers (protomers) are organized into trimers which are held together by central E1-E1 associations with E2 members of the trimer located peripherally. This trimer represents the basic capsomere, with 80 capsomeres forming the 20 triangular faces of the icosahedron. Multiple associations of these trimers generate the 30 hexamers and 12 pentamers which characterize the T=4 icosahedral lattice (3) (Fig. 7).

This model is consistent with the computerized reconstructions of cryoelectron-microscopic images of the Sindbis virus surface produced by Vogel et al. (33) and by Fuller (13). Each of the spikes resolved in the three-dimensional reconstruction produced by these authors corresponds to

FIG. 6. Intact and Triton X-100-solubilized Sindbis virus cross-linked with SDST. (A) ^{35}S -labeled Sindbis virus cross-linked at pH 5.3 with SDST at a concentration of 100 mM (lanes 1, 6, 11, and 16), 50 mM (lanes 2, 7, 12, and 17), or 25 mM (lanes 3, 8, 13, and 18) or at neutral pH with a concentration of 12.5 mM (lanes 4, 9, 14, and 19) or 6.25 mM (lanes 5, 10, 15, and 20). Samples were either cross-linked in HN buffer or solubilized prior to cross-linking in HN buffer containing 0 mM (group a), 0.24 mM (group b), 0.78 mM (group c), or 1.5 mM (group d) Triton X-100. Aliquots were then dissolved in SDS sample buffer containing 2% 2-mercaptoethanol and boiled for 5 min. Three major species were detected: a 105-kDa (hd1) species and two bands migrating between 300 and 350 kDa which represent trimers of hd1 and hd2 (see below). (B) Western blot assay of SDST-cross-linked Sindbis virus. Unlabeled SDST-cross-linked virus was assayed by Western blotting (described in Materials and Methods) with polyclonal antiserum prepared against whole virus (group A), a polyclonal monospecific antiserum to E1 (group B), or a polyclonal monospecific antiserum to E2 (group C). In group A, all monomeric E1s, E2s, and cleavage products (arrowhead C) as well as larger oligomers are detected with whole virus antiserum. In group B, monomers of E1 and oligomers containing E1 are detected. In group C, monomeric E2 and oligomers containing E2 are detected. SDST concentrations were 6.25 mM (lanes 1), 12.5 mM (lanes 2), 25 mM (lanes 3), 50 mM (lanes 4), 100 mM (lanes 5), and 200 mM (lanes 6).

one of the trimeric aggregates of E1-E2 heterodimers shown in Fig. 7.

Von Bonsdorff and Harrison (34) have shown that a hexagonal array of protomers (containing no pentamers) is maintained in membranes derived from Sindbis virus in which the underlying nucleocapsid is removed. This result shows that the icosahedral lattice on the virus surface is not dependent on the presence of the nucleocapsid and supports the view that interactions among virus envelope proteins play a role in arranging the icosahedron. The near-neighbor associations described in these experiments probably represent protein-protein interactions essential for organizing and maintaining the virus membrane structure.

Our data further suggest that E1-E2 heterodimers (protomers) occupy two nonequivalent positions in the icosahedron. We have identified two conformations of the het-

FIG. 7. Sindbis virus envelope protein organization model. See Discussion for description of model. Pictured is one face of a $T=4$ icosahedron plus one complete fivefold and one complete sixfold symmetry axis. The Texas-shaped symbols represent E2, the bean-shaped symbols represent E1, the dumbbells represent SEGs, the hatched boxes represent SDST, and the stars represent DTBP.

erodimer, which we have designated hd1 and hd2, in intact virus. The differences in migration exhibited by heterodimers composed of proteins with identical sequences is attributed to the ability of the cross-linker to lock heterodimers into specific conformations which have different SDS-binding and hydrodynamic characteristics and Stokes radii (24). The different conformations detected, hd1 and hd2, may reflect the positions of the heterodimers in the icosahedral lattice (fivefold versus twofold axis interactions [Fig. 7]) which fulfill the predictions of quasiequivalence. The disappearance of hd2 and appearance of hd1 (Fig. 5) imply that hd1 represents the "relaxed" conformation that exists between E1 and E2 subunits when they are not in the topologic constraint of an icosahedron. This infers that hd2 is metastable, as indicated by a requirement for cross-linking in the intact virion in order to detect the dimer in the hd2 conformation.

Trimers (capsomeres) of the basic heterodimer (protomers) are recovered as two distinctly migrating species after cross-linking with SDST (Fig. 6). These differences in migration reflect the possibility that these trimers contain different numbers of the two conformationally different heterodimers (hd1 and hd2). Our model (Fig. 7) predicts that three of the trimers in each of the 20 faces would contain one copy of one of the heterodimers (involved in a pentamer) and two copies of the other heterodimer (involved in hexamers). The fourth trimer in the center of each face would contain only heterodimers involved in hexamers. Ideally, this would reveal a 3:1 ratio of vertex trimers to face-center trimers. Although accurate measures of such ratios require reagents which detect only hexamer and pentamer units, we do find

that the ratio of these two species detected by SDST corresponds to approximately 3:1 (Fig. 6).

The cross-linking of E1 homotrimers by DTBP (Fig. 1 and 2) suggests that a capsomere with the E1 component of the heterodimers facing the center and the E2 components facing outward is the correct orientation of the protomers. The ability to detect E1 homotrimers with DTBP after removing E2 (Fig. 4) supports the contention that E1-E1 interactions maintain the basic trimer. These data further suggest that E1-E1 associations maintain trimers and link trimers between hexamers and pentamers in the icosahedron (Fig. 7) and that these interactions are sufficient for maintenance of the icosahedral lattice. We cannot exclude the possibility that the transmembrane and cytoplasmic domain of E2 not removed by trypsin treatment participates in stabilizing the lattice. The absence of cross-linkable residues in this 40-amino-acid polypeptide of E2 precludes detection of any near-neighbor associations by the methods employed in this study (27, 28).

The inability of SEGs to cross-link hd1 or hd2 into hexamers (Fig. 5) indicates that the inter-E2 domains containing reactive lysine residues are available to SEGs only on members of the pentameric geometric units. Detergent treatments reveal that alternate lysine domains separate from the E1 trimer contact sites allow the cross-linking of E1 dimers within and between hexameric geometric units.

The identification of various morphological entities in the envelope of the Sindbis virus virion has important implications for the process of assembly and disassembly of the virus membrane. The basic disulfide-linked E1-E2 promoter has been detected in infected cells (35, 42), and the interaction of three of these protomers through disulfide-mediated E1-E1 associations formed in the rough endoplasmic reticulum could generate the basic trimeric capsomere which is to be delivered to the plasma membrane (19). At the plasma membrane, the presence of multiple copies of the trimer sets the stage for newly formed nucleocapsids to initiate envelopment (17, 19, 30). The interaction of capsid protein with the cytoplasmic tail of E2 would organize the trimer into a position in the membrane corresponding directly to that of the underlying trimer of capsid protein. Interaction of neighboring capsid protein with the tails of other trimeric aggregates of E1-E2 in the plasma membrane would bring the trimers into juxtaposition for E2-E2 interactions to generate the hexamers at the twofold edges of the icosahedron and pentamers at the vertices. Additional E1-E1 associations would lock the subunits into the characteristic $T=4$ icosahedral lattice (Fig. 7).

ACKNOWLEDGMENTS

We thank Ruben Mitchell and Jerry Fay for their help with the graphics and photo layout of this report.

This work was supported by grants AI 14710 and AI 19545 from the U.S. Public Health Service and by funds generally appropriated by the state of Texas.

REFERENCES

1. Adrian, M., J. Dubochet, J. Lepault, and A. W. McDowell. 1984. Cryo-electron microscopy of viruses. *Nature (London)* **308**:32-36.
- 1a. Anthony, R. P., and D. T. Brown. Unpublished data.
2. Arnold, E., and M. G. Rossmann. 1990. Analysis of the structure of a common cold virus, human rhinovirus 14, refined at a resolution of 3 Å. *J. Mol. Biol.* **211**:763-801.
3. Caspar, D. L. D., and A. Klug. 1962. Physical principles in the construction of regular viruses. *Cold Spring Harbor Symp. Quant. Biol.* **27**:1-24.

4. Coombs, K., and D. T. Brown. 1987. Organization of the Sindbis virus nucleocapsid as revealed by bifunctional cross-linking agents. *J. Mol. Biol.* **195**:359–371.
5. Crowther, R. A., L. A. Amos, J. T. Finch, D. J. De Rosier, and A. Klug. 1970. Three dimensional reconstructions of spherical viruses by Fourier synthesis from electron micrographs. *Nature (London)* **226**:421–425.
6. Dimmock, N. J., H. S. Dolbear, and A. R. Guest. 1989. Chemical cross-linking of proteins of the influenza virion: 2. Acid-induced irreversible conformational changes in HA1 and HA2. *Arch. Virol.* **108**:183–190.
7. Dimmock, N. J., H. S. Dolbear, and A. R. Guest. 1989. Chemical cross-linking of proteins of the influenza virion: 1. Interrelationships. *Arch. Virol.* **108**:169–182.
8. Dubovi, E. J., and R. R. Wagner. 1977. Spatial relationships of the proteins of vesicular stomatitis virus: inductions of reversible oligomers by cleavable protein cross-linkers and oxidation. *J. Virol.* **22**:500–509.
9. Earl, P. L., R. W. Doms, and B. Moss. 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. *Proc. Natl. Acad. Sci. USA* **87**:648–652.
10. Edwards, J., and D. T. Brown. 1986. Sindbis virus-mediated cell fusion from without is a two-step event. *J. Gen. Virol.* **67**:377–380.
11. Edwards, J., E. Mann, and D. T. Brown. 1983. Conformational changes in Sindbis virus envelope proteins accompanying exposure to low pH. *J. Virol.* **45**:1090–1097.
12. Everitt, E., L. Lutter, and L. Philipson. 1975. Structural proteins of adenoviruses. *Virology* **67**:197–208.
13. Fuller, S. D. 1987. The T=4 envelope of Sindbis virus is organized by interactions with a complementary T=3 capsid. *Cell* **48**:923–934.
14. Gaffney, B. J. 1985. Chemical and biochemical crosslinking of membrane components. *Biochim. Biophys. Acta* **822**:289–317.
15. Garoff, H. 1974. Cross-linking of the spike glycoproteins in Semliki Forest virus with dimethylsuberimide. *Virology* **62**:385–392.
16. Garoff, H., K. Simmons, and O. Renkonen. 1974. Isolation and characterization of the membrane proteins of Semliki Forest virus. *Virology* **61**:493–504.
17. Harrison, S. C. 1986. Alphavirus structure, p. 21–34. *In* M. J. Schlesinger, and S. Schlesinger (ed.), *The Togaviridae and Flaviviridae*. Plenum Press, New York.
18. Helenius, A., and H. Soderlund. 1973. Stepwise dissociation of the Semliki Forest virus membrane with Triton X-100. *Biochim. Biophys. Acta* **307**:287–300.
19. Jones, K. J., R. K. Scupham, J. A. Pfeil, K. Wan, B. P. Sagik, and H. R. Bose. 1977. Interactions of Sindbis virus glycoproteins during morphogenesis. *J. Virol.* **21**:778–787.
20. Liljas, L., T. Unge, K. Friedborg, T. A. Jones, S. Lovgren, O. Skoglund, and B. Strandberg. 1982. Structure of satellite tobacco necrosis virus at 3.0 Å resolution. *J. Mol. Biol.* **159**:93–108.
21. Mudd, J. A., and R. E. Swanson. 1978. In situ crosslinking of vesicular stomatitis virus proteins with reversible agents. *Virology* **88**:263–280.
22. Omar, A., and H. Koblet. 1988. Semliki forest virus particles containing only the E1 envelope glycoprotein are infectious and can induce cell-cell fusion. *Virology* **166**:17–23.
23. Paradiso, P. R. 1983. Analysis of the protein-protein interactions in the parvovirus H-1 capsid. *J. Virol.* **46**:94–102.
24. Peters, K., and F. M. Richards. 1977. Chemical crosslinking reagents and problems in studies of membrane structure. *Annu. Rev. Biochem.* **46**:523–551.
25. Pinter, A., and E. Fleissner. 1979. Structural studies of retroviruses: characterization of oligomeric complexes of murine and feline leukemia virus envelope and core components formed upon cross-linking. *J. Virol.* **30**:157–165.
26. Rayment, I., T. S. Baker, D. L. D. Caspar, and W. T. Murakami. 1982. Polyoma virus capsid structure at 22.5 Å resolution. *Nature (London)* **295**:110–115.
27. Rice, C. M., J. R. Bell, and M. W. Hunkapiller. 1982. Isolation and characterization of the hydrophobic COOH-terminal domains of the Sindbis virion glycoproteins. *J. Mol. Biol.* **154**:355–378.
28. Rice, C. M., and J. H. Strauss. 1981. Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins. *Proc. Natl. Acad. Sci. USA* **78**:2062–2066.
29. Rice, C. M., and J. H. Strauss. 1982. Association of Sindbis virus glycoproteins and their precursors. *J. Mol. Biol.* **154**:325–348.
30. Schlesinger, M. J., and S. Schlesinger. 1986. Formation and assembly of alphavirus glycoproteins, p. 121–148. *In* M. J. Schlesinger and S. Schlesinger (ed.), *The Togaviridae and Flaviviridae*. Plenum Press, New York.
31. Sechoy, O., J. R. Philippot, and A. Bienvenue. 1987. F protein-F protein interaction within the Sendai virus identified by native bonding or chemical cross-linking. *J. Biol. Chem.* **262**:11519–11523.
32. Uchiumi, T., A. Wahba, and R. R. Traut. 1987. Topography and stoichiometry of acidic proteins in large ribosomal subunits from *Artemia salina* as determined by cross-linking. *Proc. Natl. Acad. Sci. USA* **84**:5580–5584.
33. Vogel, R. H., S. W. Provencher, C.-H. von Bonsdorff, M. Adrian, and J. Dubochet. 1986. Envelope structure of Semliki Forest virus reconstructed from cryo-electron micrographs. *Nature (London)* **320**:533–535.
34. von Bonsdorff, C.-H., and S. C. Harrison. 1975. Sindbis virus glycoproteins form a regular icosahedral surface lattice. *J. Virol.* **16**:141–145.
35. Wahlberg, J. M., W. A. M. Boere, and H. Garoff. 1989. The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. *J. Virol.* **63**:4991–4997.
36. Wang, K., and F. M. Richards. 1975. Reaction of dimethyl-3,3'-dithiobispropionimide with intact human erythrocytes. *J. Biol. Chem.* **250**:6622–6626.
37. Watts, N. R. M., and D. H. Coombs. 1990. Structure of the bacteriophage T4 baseplate as determined by chemical cross-linking. *J. Virol.* **64**:143–154.
38. Wiley, D. C., J. J. Skehel, and M. Waterfield. 1977. Evidence from studies with a cross-linking reagent that the haemagglutinin of influenza virus is a trimer. *Virology* **79**:446–448.
39. Wilson, I. A., J. J. Skehel, and D. C. Wiley. 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature (London)* **289**:366–373.
40. Zarling, D. A., A. Watson, and F. H. Bach. 1980. Mapping of lymphocyte surface polypeptide antigens by chemical cross-linking with BSOCOES. *J. Immunol.* **124**:913–920.
41. Ziemiecki, A., and H. Garoff. 1978. Subunit composition of the membrane glycoprotein complex of Semliki Forest virus. *J. Mol. Biol.* **122**:259–269.
42. Ziemiecki, A., H. Garoff, and K. Simons. 1980. Formation of the Semliki Forest virus membrane glycoprotein complexes in the infected cell. *J. Gen. Virol.* **50**:111–123.