Structure of a Conserved Golgi Complex-targeting Signal in Coronavirus Envelope Proteins

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Background: Coronavirus envelope (CoV E) proteins have a predicted β-coil–β motif reported to target the Golgi complex.
Results: This conserved domain forms β-structure on its own but is α-helical in the context of full-length SARS-CoV E protein.
Conclusion: This domain is potentially involved in large conformational transitions.
Significance: This is the first structural data of the extramembrane domain of any coronavirus E protein.

Coronavirus envelope (CoV E) proteins are ∼100-residue polypeptides with at least one channel-forming α-helical transmembrane (TM) domain. The extramembrane C-terminal tail contains a completely conserved proline, at the center of a predicted β-coil–β motif. This hydrophobic motif has been reported to constitute a Golgi-targeting signal or a second TM domain. However, no structural data for this or other extramembrane domains in CoV E proteins is available. Herein, we show that the E protein in the severe acute respiratory syndrome virus has only one TM domain in micelles, whereas the predicted β-coil–β motif forms a short membrane-bound α-helix connected by a disordered loop to the TM domain. However, complementary results suggest that this motif is potentially poised for conformational change or in dynamic exchange with other conformations.

Coronaviruses (CoV)³; order Nidovirales, family Coronaviridae, subfamily Coronavirinae) are enveloped viruses organized into three groups (1, 2): group 1 (α-coronaviruses), group 2 (β-coronaviruses) and group 3 (γ-coronaviruses). Coronaviruses have been known to cause common cold symptoms in humans and a variety of lethal diseases in birds and mammals (4). However, in 2003, the virus responsible for the severe acute respiratory syndrome (SARS-CoV) (5) produced a near pandemic with 8,273 cases and 775 deaths (6). In 2012, a novel β-coronavirus (HCoV-EMC) (7–9) was discovered that has already led to many fatalities (10–12).

The main coronavirus structural proteins are S (spike), E (envelope), M (membrane), and N (nucleocapsid), whereas the E protein and other CoV E proteins have channel activity in synthetic membranes (29–31). This channel activity is mediated by formation of pentameric oligomers (32–34) and is only very mildly selective for cations (35). The only available structural data for CoV E proteins, obtained using synthetic TM peptides, is derived from the channel-forming TM domain in SARS-CoV E (32, 34, 36). No structural data are available for the predicted N- or C-terminal extramembrane domains despite the latter being critical for viral assembly (37, 38), although some results have been obtained using shorter synthetic peptides encom-
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passing this domain (39). The C-terminal domain of E proteins contains a totally conserved proline residue, which in β- and γ-coronaviruses is at the center of a predicted β-coil-β motif (Fig. 1A), reminiscent of viral internal fusion peptides (40–42). In SARS-CoV E, this motif was found to be responsible for redirecting a plasma membrane protein to the Golgi region. Conversely, mutations designed to increase its propensity disrupted localization to membranes (43).

The C-terminal tail of E proteins is also important for its interaction with the C-terminal domain of M protein (18, 44, 45) at the cytoplasmic side of the endoplasmic reticulum-Golgi intermediate compartment, the budding compartment of the host cell. These interactions are the major drivers for envelope formation (46). The C-terminal tail of SARS-CoV E protein also interacts with the cellular protein PALS1 (47), which is thus depleted from the tight junctions in epithelial cells. Finally, SARS-CoV E interacts with the seven-domain (48) SARS non-structural protein 3 (Nsp3) (49).

Thus, it is crucial to determine the structure of the C-terminal predicted extramembrane domain of E proteins, especially in the context of a large construct that includes the TM domain. Until now, these structural studies have been hampered by problems in expression, purification, and stabilization of E proteins. We report herein the first detailed structure of a truncated form of the SARS-CoV E monomer that includes both its TM domain and its predicted β-coil-β motif at the C-terminal tail.

MATERIALS AND METHODS

SARS CoV E Protein Constructs—Full-length SARS CoV E and a truncated version were used in this work. Full-length SARS-CoV E protein (EFL) was cloned into pTBMalE plasmid downstream of the MBP fusion tag and tobacco etch virus cleavage sequence. The plasmid was transformed into Escherichia coli strain BL21(DE3) codon plus for protein expression. The truncated form, ETR, was cloned into pNIC28-Bsa4 with an N-terminal His6 tag followed by a tobacco etch virus cleavage sequence. The plasmid was transformed into E. coli strain BL21(DE3) Rosetta T1R for protein expression. In both constructs, all three native cysteines (Cys-40, Cys-43, and Cys-44) were mutated into alanines. In addition, two EFL mutants previously described by Cohen et al. (43) were prepared by site-directed mutagenesis: (i) P54A (EFP54A) and (ii) V56A/Y57A/V58A/Y59A (EFP54A).

Protein Expression and Purification—Non-labeled E protein was produced by growing the culture in Terrific broth medium at 37 °C until the culture density reached an A600 of 2. Protein expression was induced by adding 0.5 mM isopropyl 1-thio-β-d-galactopyranoside at 18 °C. When the culture density reached an A600 of 0.7, the medium was exchanged to M9 minimal medium at 25% of the initial volume to achieve a high density culture, as described previously (50). The M9 medium was appropriately supplemented with 15N-NH4Cl and [13C]glucose (Cambridge Isotope Laboratories) to produce 15N-labeled and 13C-labeled protein. Cultures were further grown for 1 h before inducing protein expression with 0.5 mM isopropyl 1-thio-β-d-galactopyranoside at 18 °C. After 6 h, cells were harvested as described above and stored at −80 °C.

Frozen cell pellets were resuspended in lysis buffer (20 mM Tris, pH 8.0, 300 mM NaCl, 5 mM imidazole, 2 mM β-mercaptoethanol, and 10% glycerol) supplemented with 1 mM PMSF and 1.5% Triton X-100. The cells were completely lysed by sonication and microfluidization. Insoluble particles were removed by centrifugation at 40,000 × g, and the supernatant was applied onto a pre-equilibrated nickel-nitrilotriacetic acid resin (Bio-Rad). The resin was washed with 20 mM Tris, pH 8.0, 300 mM NaCl, 20 mM imidazole, 2 mM β-mercaptoethanol, and 10% glycerol. Bound peptide was eluted in 20 mM Tris, pH 8.0, 300 mM NaCl, 250 mM imidazole, 2 mM β-mercaptoethanol, 10% glycerol, and 5 mM myristyl sulfobetaine (C14SB). ETR Protein was directly TCA-precipitated and lyophilized, whereas EFP54A was subjected to tobacco etch virus protease cleavage for 3 h at 30 °C prior to precipitation and lyophilization. Further purification was achieved by using reversed-phase HPLC on a Phenomenex Jupiter C4 semi-preparative column (250 × 10 mm, 300-Å pore size, 5-µm particle size). Lyophilized peptide was dissolved in 1% TFA in acetonitrile and separated under an isopropyl alcohol-acetonitrile linear gradient (4:1 (v/v) with 0.1% TFA). The identity and purity of peptide fractions were confirmed by SDS-PAGE and MALDI-TOF MS.

Gel Electrophoresis—Standard SDS-PAGE was performed in 13.5% Tris-glycine gel with TGS running buffer and stained with Coomassie Blue G-250. SDS-NuPAGE was performed in 4–12% NuPAGE® BisTris gel (Invitrogen) with NuPAGE® MES SDS running buffer and stained with SimplyBlueTM SafeStain (Invitrogen) according to the manufacturer’s protocol.

To perform electrophoresis in the presence of perfluorooctanoic acid (PFO) detergent, we modified Invitrogen’s SDS-NuPAGE protocol by replacing SDS with PFO. Lyophilized peptide was dissolved in sample buffer containing 4% PFO and heated at 65 °C for 5 min prior to loading. The gel was run at 80 V for 2–3 h with MES running buffer containing 0.5% PFO.

Blue native PAGE was performed as described previously (51). Lyophilized peptide was solubilized (0.1 mM) in sample buffer containing 25 mM SDS and either 25, 50, or 100 mM DPC. Aquaporin Z in 20 mM SDS (heated at 65 °C for 10 min) was included as an additional molecular weight marker.

Fourier Transform Infrared Spectroscopy—Sample preparation, data collection, and H/D exchange were performed essentially as described (32) on a Nicolet Nexus spectrometer (Madison, WI). The peptides were incorporated in multilamellar liposomes by dissolving a dry mixture of 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPC; Avanti Polar Lipids) and lyophilized peptide in HFIP at a 50:1 molar ratio. Fourier self-deconvolution was performed for some spectra using the following parameters: full width at half height, 20 cm−1; narrowing factor, k = 1.5) (52).

Analytical Ultracentrifugation—Sedimentation equilibrium experiments were performed using a Beckman XL-I analytical ultracentrifuge at 20 °C (53) and monitored by measuring the
absorbance at 280 nm. Lyophilized E_{TR} peptides were dissolved at A_{280} of 0.3, 0.5, and 0.8 (12-mm path length cell) in 20 mM sodium phosphate, pH 5.5; 50 mM NaCl; and for detergent either 5 mM C14SB, 100 mM DPC, or 12.5, 25, or 50 mM SDS. To match the density of the SDS-DPC mixture, D_{2}O was added at 61.6, 65, and 72.4%, respectively, to each SDS concentration. The samples were centrifuged in six-channel charcoal-filled Epon centerpieces using quartz windows. A radial distribution profile was acquired after sufficient time to reach equilibrium, as tested by HeteroAnalysis. The data were processed and fitted to several monomer/n-mer models in SEDFIT and SEDPHAT (54).

Circular Dichroism—CD data were acquired on a Chirascan CD spectrometer (Applied Photophysics) using a 0.2-mm quartz cuvette (Hellma). E_{TR} peptide samples were dissolved at pH 5.5; 50 mM NaCl; and for detergent either 5 mM C14SB, 100 mM DPC, or 12.5, 25, or 50 mM SDS. To match the density of the SDS-DPC mixture, D_{2}O was added at 61.6, 65, and 72.4%, respectively, to each SDS concentration. The samples were centrifuged in six-channel charcoal-filled Epon centerpieces using quartz windows. A radial distribution profile was acquired after sufficient time to reach equilibrium, as tested by HeteroAnalysis. The data were processed and fitted to several monomer/n-mer models in SEDFIT and SEDPHAT (54).

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NMR Sample Preparation—Approximately 1.2 mg of lyophilized E_{TR} protein was solubilized in 100 μl of methanol and dried under a dry stream of N_{2} gas, resulting in a thin protein film deposit. The tube was placed in a vacuum lyophilizer overnight to remove any residual methanol. The thin protein film was then solubilized with sample buffer containing 20 mM sodium phosphate, pH 5.5, 50 mM NaCl, and 50 mM SDS. The sample was vortexed and sonicated several times until a clear solution was obtained, indicating protein reconstitution into detergent micelles.

For paramagnetic relaxation enhancement (PRE) experiments, a single point mutation (S60C) was introduced into E_{TR} by site-directed mutagenesis using appropriate sets of primers. Expression and purification protocol of the E_{TR}-S60C mutant was the same as that of E_{TR} protein. For labeling, 0.3 mM 15N-labeled E_{TR}-S60C was dissolved in 20 mM sodium phosphate, 50 mM NaCl, 200 mM SDS, and 0.8 mM DTT at pH 5.5 and split into two equal portions for parallel labeling with (1-oxyl-2,2,5,5-tetramethyl-Δ^{3}-pyrroline-3-methyl) methanethiosulfonate (MTSSL) (Toronto Research Chemicals Inc.) and a diamagnetic analog of MTSSL: (1-acetyl-2,2,5,5-tetramethyl-Δ^{3}-pyrroline-3-methyl) methanethiosulfonate (dMTSSL; Toronto Research Chemicals Inc.). A 10-fold molar excess of both reagents was added from 75 mM stocks in methanol. The sample was vortexed for 30 min at high speed and incubated overnight at room temperature. A centrifugal filter unit (10,000 molecular weight cut-off; Millipore Corp.) was used to remove excess of both reagents. Labeled samples were washed four times by concentrating to 100 μl. After a fourth wash, the sample was concentrated to 180 μl for NMR measurements.

Partial alignment of the E_{TR} protein-micelle complexes relative to magnetic field was obtained by using stretched polyacrylamide hydrogels (58, 59). A 7% polyacrylamide gel was polymerized in a gel chamber of 5.4-mm inner diameter. After complete polymerization, gels were washed in H_{2}O overnight and then twice with sample buffer containing 20 mM sodium phosphate and 50 mM NaCl at pH 5.5. The gels were then completely dried at room temperature. The protein solution containing E_{TR}/SDS was soaked into the dried gels over 2 days to ensure complete rehydration. The hydrated 7% gel was then compressed into a 4.2-mm inner diameter open-ended tube using the gel press assembly (New Era Enterprise, Inc.).

NMR Spectroscopy—NMR experiments were performed at 308 K using an Avance-II 700 NMR spectrometer with cryogenic probe. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was used as the internal reference for 1H nuclei. The chemical shifts of 1^{3}C and 15N nuclei were calculated from the 1H chemical shifts. The NMR data were processed using TopSpin version 3.1 and analyzed using CARA. Sequence-specific assignment of backbone 1H, 15N, 13C, and 125C was achieved by using two-dimensional 1H-15N TROSY-HSQC, three-dimensional HNCO, HN(CA)CO, HNCA, HN(CO)CA, and HN(CO)CNA experiments on a 15N/13C-labeled E_{TR} protein. Side-chain resonances were assigned using three-dimensional 15N-resolved NOESY-HSQC (80-, 100-, and 150-ms mixing time), (H)CCH-TOCSY, and 13C-resolved NOESY-HSQC (120-ms mixing time). To identify membrane-embedded residues, the NMR sample was lyophilized overnight and reconstituted in 99% D_{2}O. Immediately after reconstitution, two-dimensional 1H-15N TROSY-HSQC was collected. For paramagnetic probe measurements, 15N-HSQC spectra were recorded in the presence and absence of 1 mM dry 5-doxyl stearic acid (5-DSA) or 16-doxyl stearic acid (16-DSA). Axially symmetric alignment tensor coefficients (axiality and rhombicity) were calculated using MODULE (60). The PRE effect was measured using 15N HSQC spectra of the S60C mutant before spin labeling and after MTSSL and dMTSSL labeling. The titration experiments with HMA, Nsp3a, or SH(45–65) were performed with 15N-labeled E_{TR} in a 1:4 molar ratio of SDS/DPC micelles at 318 K. Chemical shift perturbation (CSP) values were calculated using the following formula.

\[
\text{CSP} = \sqrt{\Delta \delta^{2} + (0.23 \times \Delta N)^{2}}
\]

Structure Calculation—NOE distance restraints were obtained from 15N NOESY-HSQC (mixing time 80, 100, and 150 ms) and 13C NOESY-HSQC (mixing time 120 ms) spectra, respectively. Backbone dihedral angle restraints (ϕ and ψ) were derived from 13C, 13Cα, 13Cβ, 1Hα, and 1Hβ chemical shift values using TALOS+ (61). The short range and medium range NOE connectivities were used to establish the sequence-specific 1H NMR assignment and to identify elements of the regular secondary structure. Hydrogen bonds were derived from the H/D exchange experiment and NOE connectivity.

Distance restraints were obtained from the measured PRE effect using the procedures described previously (62–64). The intensities of cross-peaks in the MTSSL (Ip) and dMTSSL (Id) were calculated in CARA. The correlation time was set to 10 ns. The ratios of intensities (Ip/Id) were normalized against a set of the eight highest Ip/Id ratios, which were assumed to belong to peaks unaffected by PRE. For peaks with ratios below 0.15, no lower distance restraints were used, whereas upper restraints were set to 15 Å. For peaks with ratios above 0.9, only upper restraints of 25 Å were utilized. For peaks with ratios between
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0.15 and 0.9, upper and lower distance restraints were generated using ±3 Å margins.

Structure calculations were performed using CYANA version 3.0 (65, 66) and visualized using PyMOL (Schrödinger LLC, New York). CNS 1.3 (67, 68) was used to refine the structure using the standard simulated annealing protocol. All of the restraints used in the calculations to obtain a total of 15 structures and all of the structure statistics are summarized in Table 1.

Single Channel Activity Measurement—Ion channel activity of ETR was measured by using Nanion Port-a-Patch®. Briefly, giant unilamellar vesicles of 1,2-diphytanoyl-sn-glycero-3-phosphocholine containing 10% cholesterol were prepared in 1 M sorbitol using Nanion Vesicle Prep Pro. The vesicles were subsequently deposited onto 6–8 megaohm NPC®-1 chips (Nanion). Conductance was measured under symmetrical buffer conditions (10 mM HEPES, 500 mM NaCl, pH 5.5).

Surface Plasmon Resonance—The nsp3a sequence was subcloned from pcDNA3(+) into pET28b upstream of a C-terminal His₆ tag for expression in E. coli. The protein was expressed and purified as described previously by Serrano et al. (69). A negative control, consisting of C-terminal peptide from the small hydrophobic (SH) protein of human respiratory syncytial virus (RSV SH(45–65)), was synthesized by standard solid phase and purified by reverse-phase HPLC. Surface plasmon resonance measurements were performed on a Biacore 3000 system (GE Healthcare) using 1 mM phosphate buffer at pH 6.5, 100 mM NaCl, 3 mM EDTA, 0.05% n-octyl-β-D-glucopyranoside, and 0.27% C14-betaine at 25 °C. Eₜᵣ was immobilized to 15,000 RU onto a research grade CM5 sensor chip (GE Healthcare) using standard amine-coupling chemistry. Briefly, a buffer-equilibrated carboxymethyl dextran surface was activated with a 10-min injection of a 1:1 mixture of 0.05 M N-hydroxysuccinimide and 0.2 M N-ethyl-N-[3-(diethylamino)propyl]carbodiimide. Eₜᵣ Peptides dissolved in 10 mM sodium acetate, 15 mM DPC (pH 5.0) were passed over the activated surface to achieve the desired response level. Another 10-min injection of 1 M ethanolamine-HCl (pH 8.5) was used to deactivate the surface and remove any non-covalently bound protein. Kinetic measurements of immobilized Eₜᵣ association with Nsp3a and SH(45–65) (49 mM to 25 μM in 10 2-fold serial dilutions) were performed with a 1-min association phase and 5-min dissociation phase at a 30 μl/min flow rate. Each concentration was tested in duplicate. No regeneration was necessary because all complexes dissociated within the monitored time. Sensorsgrams were double-referenced (70) and globally fit to a steady-state model to obtain affinity values.

RESULTS AND DISCUSSION

Expression and Purification of SARS-CoV E Protein—Initially, we successfully expressed and purified full-length SARS-CoV E protein (Eₚ, Fig. 1B) by using either a β-barrel (71) or MBP as fusion tags. However, the yield of pure protein was low due to the presence of truncations (not shown). Nevertheless, the pure sample obtained (Fig. 1, C and D) was sufficient for backbone assignment in SDS micelles, although not to produce a three-dimensional model. Therefore, a series of hexahistidine-tagged SARS-CoV E constructs were screened to obtain an expressing and well behaved sample. The best construct, encompassing residues 8–65, was successfully expressed in E. coli and purified by affinity chromatography in milligram amounts without any enzymatic cleavage steps. This truncated construct (ETᵣ; see Fig. 1B) has an N-terminal His tag and a 16-residue linker that connects it to residues 8–65. The purified peptide appeared in MALDI-TOF MS as a single-charged peak at 8,997 Da and a double-charged peak at 4,512 Da, consistent with the calculated molecular mass of Eₜᵣ, 8,995 Da, and a small proportion of larger (dimer to pentamer) oligomers (Fig. 1E). After HPLC, the Eₜᵣ monomer (9 kDa) showed anomalous migration in standard SDS-PAGE (Fig. 1F), as reported previously for the full-length protein (31).

Identification of the Membrane-embedded Region of Eₜᵣ by NMR—Screening of reconstitution conditions identified SDS as the best environment to achieve good peak dispersion in both dimensions (Fig. 2A). Comparison of 1H,15N TROSY-HSQC spectra in water and in 99% D₂O (Fig. 2 A and B) identified a stretch of 21 residues, from Leu-18 to Leu-39, protected from H/D exchange. The same residues were protected from H/D exchange in Eₚ (not shown), which indicates that both Eₜᵣ and Eₚ have only one TM domain.

The topology of Eₜᵣ was also delineated by introducing the paramagnetic probes 5-DSA and 16-DSA. Upon the addition of 5-DSA, a slight intensity reduction was observed around residues 11–20 and 40–55 (Fig. 2C), suggesting that these residues are located at or near the micelle surface. The addition of 16-DSA caused pronounced intensity reduction in the stretch of TM residues 19–40 and periodically in the stretch 48–61 (Fig. 2D). Together with the H/D exchange data (Fig. 2 A and B), these results again demonstrate the presence of a single TM domain and suggest that the stretch of residues 55–65 corresponds to a domain bound to the surface of the micelle. Finally, a 1H,15N steady-state heteronuclear NOE experiment (Fig. 2E) showed that the protein forms a well folded structure in both dimensions (Fig. 3). Comparison of 1H-15N TROSY-HSQC spectra in water and in 99% D₂O (Fig. 2, A and B) identified a stretch of 21 residues, from Leu-18 to Leu-39, protected from H/D exchange. The same residues were protected from H/D exchange in Eₚ (not shown), which indicates that both Eₜᵣ and Eₚ have only one TM domain.

NMR Structure Determination of Eₜᵣ in SDS Micelles; Structure Calculation—The restraints shown in Table 1 were used in a calculation to obtain a total of 15 structures, with a root mean square deviation of 0.27 ± 0.11 and 0.70 ± 0.13 Å for backbone and all heavy atoms, respectively (Fig. 3A). A longer α-helix (residues 15–45) encompasses the TM domain, which is connected to another shorter C-terminal α-helix (residues 55–65) by a flexible (see Fig. 2E) linker domain (residues 46–54), forming an L-shape. The short extramembrane helix may be partially bound to the micelle surface, as suggested by the pattern of intensity attenuation of paramagnetic reagents (see Fig. 2 A, B and C). The most affected residues, which would face the micelle surface, are Val-52, Thr-55, Tyr-59, and Lys-63 (Fig. 3B).

The TM α-helix has a slight bend at residues 26–30, consistent with previous results obtained for the synthetic TM
FIGURE 1. Sequences, expression, and purification of SARS-CoV E<sub>TR</sub>. A, alignment of representative sequences of E proteins in α-, β-, and γ-coronaviruses. The cysteine residues are underlined, the conserved proline is highlighted (gray), and the four residues mutated to alanine in the E<sub>ALA</sub> mutant (see "Materials and Methods") are shown in red. For these four proteins, the prediction of secondary structure is shown below in a color code, with the TM domain indicated as a black line; B, proteins used in the present work: a His-tagged construct (E<sub>TR</sub>) encompassing residues 8–65 (boldface type, underlined), and full-length SARS-CoV E<sub>FL</sub>. In E<sub>FL</sub>, the fragment SNA results from the cleavage of the tag. In both proteins, the native cysteines were mutated to alanine (C40A, C43A, and C44A; see asterisks); C and D, MALDI-TOF MS spectra (C) and standard SDS-PAGE (D) of pure E<sub>FL</sub> with the species labeled; E and F, same as for purified E<sub>TR</sub>; the identities of various single- and double-charged species are indicated. The calculated mass of E<sub>TR</sub> is 8,995 Da.
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A

B

C

D

E

F
TABLE 1

| Restraints and structure statistics for the selected 15 structures of ETR |
|----------------------------------|-------------------|-----------------|-----------------|
| NMR restraints                   | Total unambiguous distance restraints | 2,258           |                  |
|                                  | Intraresidual      | 1,334           |                  |
|                                  | Sequential (|i| − |j| = 1) | 230             |                  |
|                                  | Short-range (|i| − |j| = 1) | 1,564           |                  |
|                                  | Medium (2 ≤ |i| − |j| ≤ 4) | 321             |                  |
|                                  | Long range (|i| − |j| ≥ 5) | 11              |                  |
| Dihedral angle restraints        | 85                |                 |                  |
| Hydrogen bond restraints         | 20                |                 |                  |
| RDC restraints                   | 44                |                 |                  |
| PRE restraints                   | 38                |                 |                  |

Root mean square deviation from the experimental residual dipolar couplings (Hz)

| 1Dexpt | 0.71 ± 0.03 |

Root mean square deviation from the average atomic coordinates (residues 12–63, Å)

| Backbone atoms | 0.27 ± 0.11 |
| All heavy atoms | 0.70 ± 0.13 |

Ramachandran analysis (%)

| Residues in most favored regions | 87.5 |
| Residues in additional allowed regions | 12.5 |
| Residues in generously allowed regions | 0.0 |
| Residues in disallowed regions | 0.0 |

a Backbone hydrogen bonds of α-helix were applied to regions confirmed to be α-helical, according to the local NOE pattern and H²O chemical exchange experiments.

b Statistics were calculated and averaged over an ensemble of 15 structures with lowest target function according to CYANA.

domain in DPC micelles (34). A kink near this location is suggested by the short distance (2.0 ± 0.1 Å) between Thr-30 Hβ and the carbonyl oxygen at Phe-26, in the range of a hydrogen bond (Fig. 3C).

Effect of Truncation and Environment on ETR Secondary Structure—To assess the effect of the truncation and the presence of a His tag on the ETR secondary structure, ETR and EFL were compared. The possible effect of the reconstitution environment was also determined.

CD and IR Spectra of ETR and EFL in Detergent and Lipid Membranes—The CD spectra of ETR in DPC, SDS, and mixed (1:2 molar ratio) SDS/DPC micelles are almost superimposable (Fig. 4A) with minima at 209 and 222 nm. Also, these data are entirely consistent with the CD spectra of EFL, which was predominantly α-helical in both SDS and DPC micelles (71). When reconstituted in DMPC membranes, ETR and EFL produced an almost identical spectrum, with an amide I band centered at 1,655 cm⁻¹ and 1,652 cm⁻¹, respectively (Fig. 4B), characteristic of a predominantly α-helical conformation. Overall, these data show that both ETR and EFL (i) are predominantly α-helical and (ii) have a secondary structure that is not significantly affected by the reconstitution environment, supporting the relevance of the ETR structure (Fig. 3).

13Ca Chemical Shifts—13Ca chemical shifts are highly correlated with secondary structure (72, 73). Comparison of 13Ca chemical shifts of ETR and EFL in SDS (Fig. 4C) shows that ETR shifts (red) are almost identical to their counterpart residues (residues 8–65) in EFL (blue). In EFL, the shifts for the last 10 residues (residues 66–76) are predicted to correspond to random coil, which is consistent with results obtained previously with a synthetic peptide spanning residues 59–76 (39). This peptide produced a broad amide I band in the IR spectrum centered around 1,650 cm⁻¹ and immediately experienced complete H/D exchange.

When the 13C shifts for ETR were compared for SDS and an SDS/DPC mixture (1:4 molar ratio), only the N-terminal region (residues 7–15) showed minor differences. Finally, a comparison between EFL and ETR in an SDS/DPC mixture (1:4 molar ratio) also revealed an almost identical pattern in the C-terminal tail (not shown), although the spectral resolution for EFL was reduced in other regions. Overall, these results are consistent with those shown above (Fig. 4, A and B), indicating that the detergent used, the tag, and the truncation has a minimal on ETR secondary structure.

Ion Channel Activity of ETR and Its Inhibition by HMA—Purified EFL has channel activity (71), which is inhibited by the drug HMA (30). An I/V plot obtained in a symmetrical 0.5 M NaCl experiment for purified ETR in 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (Fig. 5A) was used to determine a conductance of 0.39 ± 0.02 nanosiemens. For comparison, synthetic full-length SARS-CoV E and ETM (residues 7–38) produced single channel conductances of 0.19 ± 0.06 picosiemens and 0.18 ± 0.12 nanosiemens in 1 M NaCl (35), although the values were higher in 1 M KCl, with 0.37 ± 0.16 and 0.31 ± 0.12 for full-length SARS-CoV E and EETM, respectively. The lower conductance observed in synthetic samples may be due to extraneous modifications or impurities resulting from exposure to harsh chemicals. Representative traces of ETR channel activity (Fig. 5B) and complete inhibition after the addition of 10 μM HMA (Fig. 5C) suggest that ETR is entirely functional.

To determine the binding site for HMA, we measured the differences in CSP values before and after the addition of the drug (Fig. 5, D and E). In SDS, the average CSP value was low, 0.006 ppm (Fig. 5F), even at an HMA/ETR 10:1 molar ratio, suggesting no significant binding. However, the same panel shows that the addition of HMA to ETR in mixed SDS/DPC (1:4 molar ratio) micelles produced an average CSP value of 0.013 ppm, even at an HMA/ETR 2:1 molar ratio.

Except for Asn-64 and Leu-65, the residues that showed significant CSP (CSP ≥ 0.025 ppm) clustered near the membrane interface regions of the TM domain (Fig. 5F, see arrows). Near the N-terminal side of the TM domain, the most affected were Glu-8, Gly-10, Thr-11, Val-14, Asn-15, and Ser-16, the latter two consistent with observations made on the TM channel in DPC (34). At the C-terminal end of the TM, Leu-37 was the most affected, suggesting that the interaction of HMA at He of Arg-38 reported previously (34) may have been an artifact due to the use of a TM peptide.

Oligomeric State of ETR

Gel Electrophoresis—The localized changes in chemical shifts observed after HMA addition to ETR in mixed DPC/SDS micelles (Fig. 5E), but not in SDS micelles (Fig. 5D), suggest that binding sites for HMA may have been induced after DPC addi-

FIGURE 2. Topology and secondary structure of ETR. 1H, 15N TROSY-HMQC spectra of 0.2 mM ETR in 50 mM SDS in H₂O (A) and in 99% D₂O (B). The cross-peaks are labeled by one-letter code and residue number; C and D, peak intensity reduction upon the addition of 5-DSA (C) and 16-DSA (D), calculated as the ratio of peak intensity before and after the addition of the paramagnetic reagents; E, 1H, 15N steady-state heteronuclear NOE experiment; F, sequential and medium-ranged NOE connectivity between residues, displayed as bands under the respective residues.
tion to SDS, possibly through E$_{TR}$ increased oligomerization and population of the pentameric form. Thus, we examined the oligomeric state of E$_{TR}$ using gel electrophoresis performed in the presence of SDS or an SDS/DPC mixture. In contrast with the results obtained in normal SDS-PAGE (Fig. 1), we used NuPAGE, where E$_{TR}$ migrated with its expected monomer molecular weight, as a single band at 9 kDa (Fig. 6A). No oligomerization is consistent with the lack of interaction between HMA and E$_{TR}$ observed in SDS micelles.

To test the effect of DPC, in a blue native polyacrylamide gel, a constant SDS concentration (25 mM) was titrated with increasing DPC (Fig. 6B), from 1:1 to a 1:4 molar ratio. Bands
consistent with dimers and trimers were observed at 1:1 and 1:2 molar ratios, whereas tetramers and pentamers were observed at a 1:4 molar ratio. This increasing oligomerization is again consistent with the binding of HMA observed in the presence of DPC.

_Sedimentation Equilibrium, SDS/DPC Micelles_—For a sample corresponding to a 1:4 molar ratio of SDS/DPC, the equilibrium sedimentation data (Fig. 7A) could be fitted to several oligomeric models, from trimers to heptamers (Fig. 7B). Similar ambiguous results were obtained for a 1:2 molar ratio SDS/DPC mixture (Fig. 7C), suggesting that E

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**FIGURE 5. Channel activity of E

A, I/V plot for E

B, selected traces of 12 s each, recorded at various holding potentials of E

C, channel activity recorded at 60 mV holding potential and after the addition of 10 μM HMA (arrow) D, 1H-15N TROSY-HSQC spectra of 0.1 mM 15N-labeled E

E, same as for 0.2 mM 15N-labeled E

F, CSP of the backbone amide resonances of E

Note that the HMA/E

molar ratio was 10 in SDS and only 2 in SDS/DPC micelles. The arrows show residues with significant change in chemical shifts after the addition of HMA. The TM domain is indicated only to guide the eye.
Overall, the above results show that higher oligomeric states, including pentamers, are observed when increasing the DPC concentration, consistent with the larger shifts observed after adding HMA to mixed DPC/SDS micelles.

**E	extsubscript{TR} Forms Pentamers in C14 Betaine and PFO**—We have shown previously that SARS-CoV E TM domain and E	extsubscript{FL} form pentamers (36, 71). When E	extsubscript{TR} was solubilized in C14 betaine detergent (Fig. 7D), data could be optimally fitted to a monomer-pentamer model ($K_a = 10^{16} M^{-4}$) with a significantly lower $\chi^2$ than that obtained in SDS/DPC. Also, the mobility of E	extsubscript{TR} in PFO-NuPAGE (Fig. 7E) corresponds to a molecular mass of 45 kDa (i.e., a pentameric form). Thus, E-TM, E	extsubscript{TR}, and E	extsubscript{FL} show a similar oligomerization behavior, being able to form pentameric channels that are inhibited by HMA.

**Interaction of E	extsubscript{TR} with Nsp3a**—The interaction of Nsp3 with SARS-CoV E protein, thought to be related to E ubiquitination, was previously mapped to an N-terminal acidic domain, Nsp3a (49). Although the site of interaction with E is not known, it is likely to involve the C-terminal tail because it is the largest extramembrane domain. Thus, to test that the conformation of

\[ \Delta G^{\circ}_{o} \approx -4 \text{ kcal mol}^{-1} \]
ETR is structurally equivalent to the corresponding sequence in EFL, we tested its ability to interact with Nsp3a (49) using surface plasmon resonance.

As a negative control, the mock peptide RSV SH(45–65) (see “Materials and Methods”) was used to bind to immobilized ETR in detergent (see “Materials and Methods”) (Fig. 8A). Binding and unbinding of RSV SH(45–65) was very slow (>1 min), and the data could not be fitted to any model, suggesting a nonspecific interaction.

In contrast, rapid and reversible interaction was observed using purified Nsp3a (Fig. 8B). These data could be fitted with a stoichiometry of 1:1, although the end point could only be estimated. From that model, affinity was determined as 1.6 mM, although we note that binding is already evident even at concentrations as small as 1–10 μM (see Fig. 8B, inset).

The residues involved in the interaction between Nsp3a and ETR were identified by the differences in CSP values before and after the addition of Nsp3a to ETR in SDS/DPC micelles (1:4 molar ratio). Large chemical shift changes at Leu-39, Val-49, and Leu-65 (Fig. 8C) indicated a potential binding site at the C-terminal tail, whereas the central TM region did not experience any change. Overall, these data suggest that ETR has a structure in SDS/DPC micelles that is similar to the native fold of EFL in biological membranes.
Structural Discordance at the C-terminal Tail—Having determined the suitability of the construct used by comparison with the full-length protein and shown that the nature of the detergent did not affect the secondary structure significantly, we investigated the structure of the C-terminal extramembrane domain of ETR and its apparent contrast with secondary structure predictions.

The secondary structure predictions clearly suggest the presence of a β-turn-β motif in the C-terminal region, not only in SARS-CoV E but also in other representative sequences of other coronaviruses (see Fig. 1A). Indeed, we have reported previously that the synthetic peptide E46–60, which encompasses this putative β-hairpin, folds as β-strands, is completely resistant to H/D exchange, and has a very high tendency to aggregate in solution (39). However, we have shown here that in the context of EFL or ETR, this domain does not adopt a β-structure. To test if at least some part of the population adopts this structure in lipid membranes, we mutated EFL residues 56–59 to increase the predicted helicity of this part of the molecule (43), from β-branched Val or bulky Tyr to small side chain Ala,
to obtain the construct E4ALA. Last, to test the effect of the conserved Pro-54 at the center of this putative motif, we generated the mutant E54A.

The IR spectrum of these mutants when reconstituted in DMPC membranes (Fig. 9A) shows a similar amide I band. However, for the mutant E4ALA, two shoulders are eliminated (see arrows). This indicates that these shoulders in EFL do not represent misfolded protein, but they may correspond to the bona fide β-structure conformation present in a small part of the population. However, the significance of these mutations is not completely clear because, although the mutations introduced in E4ALA prevented Golgi complex accumulation when the C terminus tail of SARS-E was coupled to VSV-G (43), a similar effect was also observed for the P54A mutant, which in our IR spectrum showed no obvious differences with respect to EFL.

As stated above, the synthetic peptide E46–60 (39), which encompasses the predicted β-hairpin in SARS-CoV E, was found to produce ~100% β-structure and was completely resistant to H/D exchange (Fig. 9C). Combined with the effect observed for the E4ALA mutant, we propose that this β structure may be in dynamic equilibrium with the much more abundant α-helical form (Fig. 9D). A delicate balance between these two forms may alter processes in the infected cell (e.g. membrane scission, binding to protein partners, or E protein localization).

Finally, the HMA titration results showed large CSP values for residues Val-49 and Leu-65, which are far apart in the sequence. Using the SymmDock server (75, 76), a reconstructed ETM pentameric model was obtained based on the published structure of the E-TM pentamer (34) and the current ETM structure. The model suggests that these two residues may be in fact spatially close (Fig. 10) and belong to different monomers, providing a rationale for the observation above.

CoV E proteins have been proposed to have at least two roles. One is related to their TM channel domain. This would be active in the secretory pathway, altering luminal environments and rearranging secretory organelles and leading to efficient trafficking of virions (38, 77). The other would be related to their extramembrane domains, particularly the C-terminal domain. This is involved in protein-protein interactions and targeting, among other roles.

E protein participates in M-M and E-M interactions (17, 44), which are interesting targets for drug discovery. Also, formation of viral particles appears to be facilitated by a broad range of E sequences (78), which suggests that a common topology is more important than sequence requirements. E proteins have been suggested to act as chaperones during packaging (79), but the precise mechanism by which this takes place is not known. In this context, the structure determined here sheds light on a critical domain present in most CoV E proteins.

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