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Structural model of the SARS coronavirus E channel in LMPG micelles

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A R T I C L E   I N F O

Keywords:
Envelope protein
Solution NMR
Transmembrane α-helices
Micelles
Oligomerization

A B S T R A C T

Coronaviruses (CoV) cause common colds in humans, but are also responsible for the recent Severe Acute, and Middle East, respiratory syndromes (SARS and MERS, respectively). A promising approach for prevention are live attenuated vaccines (LAVs), some of which target the envelope (E) protein, which is a small membrane protein that forms ion channels. Unfortunately, detailed structural information is still limited for SARS-CoV E, and non-existent for other CoV E proteins. Herein, we report a structural model of a SARS-CoV E construct in LMPG micelles with, for the first time, unequivocal intermolecular NOEs. The model corresponding to the detergent-embedded region is consistent with previously obtained orientational restraints obtained in lipid bilayers and in vivo escape mutants. The C-terminal domain is mostly α-helical, and extramembrane intermolecular NOEs suggest interactions that may affect the TM channel conformation.

1. Introduction

Coronaviruses (CoV) typically affect the respiratory tract and gut of mammals and birds. Approximately 30% of common colds are caused by two human coronaviruses - OC43 and 229E. Of particular interest are the viruses responsible for the severe acute respiratory syndrome (SARS), which produced a near pandemic in 2003 [1], and the recent Middle East respiratory syndrome coronavirus (MERS-CoV) [2].

No effective licensed treatments exist against coronavirus infections [3–5], but live attenuated vaccines (LAVs) [6–10] and fusion inhibitors [11] are promising strategies. One CoV component critical for pathogenesis is the envelope (E) protein, as reported in several coronaviruses, e.g., MERS and SARS-CoVs [12–14]. The CoV envelope (E) proteins are short polypeptides (76–109 amino acids) with a single α-helical transmembrane (TM) domain [15–21] that form homopentameric ion channels (IC) with poor ion selectivity [22,23]. CoV E proteins are mostly found in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) [24–29]. In animal models, deletion of SARS-CoV E protein reduced pathogenicity and mortality [30], whereas cellular models displayed up- and down-regulation of stress response and inflammation host genes, respectively [31]. The importance of E protein in pathogenesis has led to the development of LAVs based on deletion of E protein in SARS- and MERS-CoVs, although this led to compensatory mechanisms that recover virulence [32,33].

Specific critical features in the SARS-CoV E protein sequence have been identified that determine virulence, e.g., at the C-terminal tail [34] or in the TM domain [30], and precise structural characterization of these regions could help in the design of E protein-based CoV LAVs. However, detailed structural knowledge is still very limited in the case of SARS-CoV E, and non-existent for other CoV E proteins.

A pentameric model for SARS-CoV E was initially proposed by the authors after an in silico conformational search [15] of TM domain oligomers. In that report, two pentameric models (termed ‘A’ and ‘B’) were selected. In model A, V25 adopts a more lumenal position, whereas in model B, the position of this residue is clearly interhelical (Fig. 1). The pentameric organization of SARS-CoV E has been confirmed experimentally in various detergents: PFO, DPC or C-14 betaine [17,18], not only for synthetic TM (ETM), but also for an 8–65 (ET865) construct and for full length E protein (ET). To confirm experimentally the orientation of the α-helices in the pentameric model, site specific infrared dichroism (SSID) measurements [35] were obtained in hydrated lipid bilayers, with 13C = 18O isotopically labeled synthetic ETM. However, the orientation of the α-
helices turned out to be strongly dependent on the presence of 2 flanking lysine residues at each end of the peptides [16]: with flanking lysine residues, the orientation was a hybrid between models A and B (residues 17–24 were oriented consistent with model B, but from residue 24 onwards, orientation was as expected for model A), consistent with a 'bend of the α-helices around residues 25–27' [16]. Without terminal lysines, however, the orientation of the central five labeled consecutive residues, L21 to V25, was entirely consistent with model A [17].

These initial results suggested that the conformation of the E\textsubscript{TM} pentamer may be very sensitive of the presence of extra residues and probably also, extramembrane domains. An NMR study was performed on a synthetic E\textsubscript{TM} (residues 8–38) in DPC detergent micelles, where E\textsubscript{TM} was selectively labeled [20]. E\textsubscript{TM} was \textsuperscript{15}N-labeled at A22, V24, V25, and \textsuperscript{13}C, \textsuperscript{15}N-labeled at L18, L19 and L21. Intermonomeric NOEs were assigned indirectly, i.e., when cross-peaks could not be explained by intramonomer interactions. Of these, derived from difference 2D homonuclear $^1$H, $^1$H\text{aromatic} band-selected NOESY, only four NOEs were labeled 'strong', and involved the $^1$H\text{phenyl} ring of Phe23, to $^1$H\text{δ\epsilon}$ of either Leu18 (two NOEs) or Leu21 (two NOEs). These

![Fig. 1. Comparison of orientation of residue V25 in SARS-CoV E\textsubscript{TM} pentameric models. Orientation of computational models A (orange) and B (cyan) [15], where the side chain of V25 (F26 is only used to guide the eye) is indicated. The 'A-like' model obtained by NMR [20] is shown in red. In model B, the position of V25 is clearly interhelical.](image)

![Fig. 2. Hydrogen-deuterium exchange protected region and secondary structure of E\textsubscript{TM} monomer in LMPG. (A) $^1$H-$^1$N-TROSY-HSQC spectra in H\textsubscript{2}O (left) and 99% D\textsubscript{2}O (right), with cross-peaks labeled by one-letter code and residue number; (B) Secondary structure prediction obtained using TALOS+ [43], comparing E\textsubscript{TM} in LMPG, SDS, and SDS/DPC [19]. (Layout note: 1 column).](image)
intermolecular NOEs were insufficient to distinguish between models A and B, and the monomer structure was fit to a model A template.

More recently, recombinant SARS-CoV escape mutants were recovered after introducing a V25F channel-inactivating mutation in the E protein, [36], that led to attenuation in a mouse model [30]. Revertant mutants regained fitness and pathogenicity whereas mutated E protein regained channel activity [30]. Surprisingly, escape mutations in E protein clustered along the helix interface opposite to residue V25, consistent with an interhelical orientation of this residue, as found in model B (Fig. 1, cyan).

In the present paper, we report a more accurate model of the SARS-CoV E protein pentamer, in LMPG micelles. The construct we have used prolongs the TM domain with another 27 residues in the C-terminal domain (residues 8–65). Following established protocols [37], two types of monomers were mixed, bearing different isotopical labels, that allowed unambiguous identification of ten intermonomeric NOEs. In a nutshell, the results are consistent with a TM model that appears to be a hybrid between models A and B: while overall being closer to model A, residue V25 has a clear ‘model B-like’ interhelical orientation, consistent with the revertant mutants that appeared in vivo.

2. Materials and methods

2.1. Protein expression and purification

The expression and purification methods for the truncated SARS-CoV E construct corresponding to residues 8–65 (ETR) have been described previously [19]. This construct does not have cysteines, as these are not required for oligomerization [18,19,28,38]. In the present work, M9 media was supplemented with an appropriate combination of 15NH4Cl, 13C-glucose, 2H-glucose, and 2H2O (Cambridge Isotope Laboratories) to produce 15N-, 13C-, 15N/13C- and 15N/2H-labeled ETR samples. For preparation of fully deuterated 15N/2H-labeled samples, freshly transformed E. coli cells were doubly-selected in LB agar plates and media prepared with 30% and 60% 2H2O, successively, and later grown in M9 media prepared with 99.9% 2H2O [39,40].

2.2. Gel electrophoresis

Blue-native PAGE (BN-PAGE) was performed as described previously [41]. Lyophilized ETR protein was solubilized (0.1 mM) in sample buffer containing LMPG (lyso-myristoyl phosphatidylglycerol, Anatrace) at the indicated concentrations.

2.3. Residue rotational pitch calculations

For α-helical bundle models, the rotational pitch angle of a residue, ω, defined arbitrarily as 0° or 180° when transition dipole moment, helix director, and the z-axis all reside in a single plane, was calculated as described elsewhere [42]. The final result is the average of the ω values calculated in each monomer. For a canonical α-helix, it is expected that Δω between two consecutive residues is ~100°.

2.4. NMR sample preparation

Lyophilized ETR protein (0.67 mM) was solubilized in 20 mM sodium phosphate pH 5.5, 50 mM NaCl, and 200 mM LMPG, i.e., a protein:detergent (P/D) molar ratio of 1:300. The same protein concentration and P/D ratio was used for the mixture of 15N-D and 13C-labeled samples. The solution was vortexed and sonicated several times until a clear solution was obtained, indicating protein reconstitution into detergent micelles.

2.5. NMR spectroscopy

NMR experiments were performed at 308 K using an Avance-II 700 NMR spectrometer with cryogenic probes. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as the internal reference for 1H
nuclei. The chemical shifts of $^{13}C$ and $^{15}N$ nuclei were calculated from the $^1H$ chemical shifts. The NMR data were processed using TopSpin 3.1 (www.bruker-biospin.com) and analyzed using CARA (www.nmr.ch). Sequence-specific assignment of backbone $^1H$, $^{15}N$, $^{13}C$ and $^{13}C^\alpha$ was achieved by using 2D $[^1H-^{15}N]$-TROSY-HSQC, 3D HNCA and HN(CO)CA experiments on a $^{15}N$/$^{13}C$-labeled ETR protein. Side-chain resonances were assigned using 3D $^{15}N$-resolved NOESY-HSQC (120 ms mixing time), (H)CCH-TOCSY and $^{13}C$-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, 2D $[1H-15N]$-TROSY was collected. The pentamer structure was calculated using HADDOCK 2.2 [46] according to standard protocols. Ten inter-monomeric NOE restraints (defined as above) were described as ambiguous and unambiguous 5.0 Å distance restraints. Two segments were described as fully flexible: residues 37–47 and 40–54. A C5 symmetry restraint between all 5 subunits and pairwise non-crystallographic symmetry restraints between neighbouring subunits were applied. Initial rigid-body docking yielded 1000 structures, out of which 200 top-scoring structures (i.e., based on HADDOCK target function score) were selected for refinement by semi-flexible simulated annealing. These were then clustered based on RMSD, and the top-scoring cluster was selected (all 16 structures within the said cluster were grouped to form an ensemble).

3. Results and discussion

3.1. Helical structure and TM domain of SARS-CoV E monomer (ETR) in LMPG micelles

Despite phospholipid isotropic bicelles may have been more membrane-like than detergent micelles, in our hands, phospholipid bicelles did not produce suitable spectra of ETR (not shown). Examples of significant differences observed in bicelles vs micelles have been reported, e.g., in the study of the integrin TM heterodimers [47–52] or in viral channels [53].

Nevertheless, we have shown previously that ETR is pentameric in various detergents [17,18], although none of them was suitable for NMR studies of ETR or EFL (not shown). ETR only produced reasonably good NMR spectra in DPC when SDS was also present [19], but since SDS disrupts ETR oligomerization, we searched for other micellar environments. Lipid-like LMPG was found to produce good NMR spectra for ETR, although not for EFL. Therefore, ETR in LMPG was used in subsequent experiments. The use of the ETR construct instead of the full-length E protein (E123) is justified since the $^{13}C_\alpha$ chemical shifts of ETR and E123 protein in SDS or SDS/DPC were almost identical for residues 8–65 [19]. In addition, the secondary structure, obtained by CD/FTIR [18], of ETR and E123 is similar and predominantly α-helical, whether in DPC, SDS, mixed (1:2 M ratio) SDS/DPC micelles or DMPC synthetic membranes [18,19].

Comparison of the HSQC spectrum of ETR/LMPG before and after exposure to D$_2$O (Fig. 2A) shows that only 20 residues are protected from hydrogen/deuterium (H/D) exchange. The protected residues correspond to the stretch L18-L37, unequivocally indicating the presence of a single TM domain in SARS-CoV E. This result is consistent with SEM images and the intrinsic fluorescence of C540 on the extracellular side of the protein, as well as with circular dichroism (CD) data, which show a high content of α-helical structure in the TM domain of SARS-CoV E.
with the stretch L18-L39 found to be protected in SDS micelles [19]. The chemical shift index (CSI)-based secondary structure of ETR (calculated by using TALOS+) obtained in LMPG (Fig. 2B), has significantly higher helicity in C-terminal residues 52–55, when compared with the data obtained SDS or with a mixture SDS/DPC [19].

The structure of ETR was calculated from 10 ETR monomer structures (Fig. 3A) and the structure statistics are summarized in Supplementary Table S1. The ETR monomer in LMPG consists of three helical segments: the one encompassing the TM domain (H1, residues 12–37), a juxta-membrane middle helical segment (H2, residues 39–47), and a C-terminal helix (H3, residues 52–65) (Fig. 3B). In contrast, ETR in DPC/SDS [19] was formed by only two helical segments separated by a long flexible link (Fig. 3C). Compared to the results in SDS or SDS/DPC [19], in LMPG helix H3 is extended by 3 residues on its N-terminal side, whereas a new helical segment, H2, is formed.

### 3.2. Oligomeric state of SARS-CoV E in LMPG

To assess the oligomerization of ETR in LMPG micelles, its migration in a BN-PAGE gel was analyzed at various protein-to-detergent (P/D) ratios (Fig. 4). At the lowest P/D molar ratio (1:1000), ETR migrates as a ladder of increasingly larger oligomers where the fastest migrating band is assumed to correspond to monomers (lower star), ~8 kDa, whereas at a high P/D ratio (1:125), ETR migrates with an apparent molecular weight of ~150 kDa. These results are almost identical to those obtained previously for MERS-CoV E, for which a pentameric oligomer was determined using analytical ultracentrifugation in C-14 betaine. In that case, migration in BN-PAGE gels was also observed as a single ~150 kDa band in detergents DPC, DHPC and LMPG [21], and the ladder observed at higher detergent concentration conveniently provided an internal reference that served as an oligomeric size marker. Similar to ETR, by comparison with that ladder, we confidently assigned the single band observed for MERS-CoV E to pentameric oligomers. It should be noted that in BN-PAGE gels of membrane proteins, molecular weights can appear up to 80% higher due to a contribution of the dye [54]. We have shown this for tetrameric AQPZ, which migrated at ~170 kDa instead of the expected ~100 kDa, and with a viroporin, the SH protein pentamer [41], which migrated as ~66 kDa instead of ~40 kDa. In the case of envelope E proteins, the effect is even more pronounced. In both SARS-CoV ETR and MERS-CoV E, the pentameric form appears at ~150 kDa, therefore the monomer should appear at >30 kDa. This is consistent with its migration above the AQPZ monomer (~25 kDa). The ladder ends with a pentamer, which is the predominant band at high P/D ratios. The proportion of large oligomers naturally decrease at low P/D ratios, but a significant amount of pentamer species is still present even at the 1:1000 P/D ratio. The NMR data was collected at a P/D molar ratio of 1:300, which should mostly be formed by pentamers.
3.3. HMA binding to oligomeric E\textsubscript{TR}

In a previous paper, we showed that monomeric E\textsubscript{TR} in SDS micelles was not affected by addition of the drug HMA \cite{19}. However, after addition of DPC to SDS, to a SDS/DPC 1:4 M ratio, HMA induced clear chemical shift perturbations (CSPs), concomitant with E\textsubscript{TR} oligomerization. The oligomerization in DPC/SDS was not homogeneous, which precluded a more detailed study, whereas in LMPG a predominant oligomeric size is observed at a high protein-detergent ratio (Fig. 4). Therefore, in LMPG the changes observed after HMA addition should more reliably represent the binding of HMA to E\textsubscript{TR}. HMA-induced CSPs were detected herein after addition of 7.75 mM HMA to 0.25 mM E\textsubscript{TR} in 200 mM LMPG micelles (P/D molar ratio 1:800) (Fig. 5).

The average CSP value was 0.019 ppm, and several residues showed CSP > 1 S.D. from the average value, notably Thr-9, Leu-12, Ile-13, Ala-36 and Val-47. These results suggest the presence of two binding sites located at both ends of the TM domain. Given the long distance between Ala-36 and Val-47, the two HMA-interacting residues may be located in different monomers.

3.4. Pentameric model of E\textsubscript{TR}

A pentameric model was obtained by docking the monomeric form of E\textsubscript{TR} using HADDOCK 2.2 \cite{46}, which incorporated 10 inter-monomeric NOE restraints (Fig. 6A). We note that 2 inter-monomeric NOEs are located at the extramembrane C-terminal tail: L39 HN - Y57 HB and V47 HN - N64 HN. The same figure shows a representative example of NOE E\textsubscript{TR} inter-monomer connectivity (green lines). The remaining plots of inter-monomeric NOEs are shown in Fig. S1. Structure statistics are summarized in Supplementary Table S2.

The E\textsubscript{TR} pentamer is a right handed \(\alpha\)-helical bundle where the C-terminal tails coil around each other (Fig. 7A) likely owing to the 2 inter-monomeric restraints between the two C-terminal helices. Each pentamer subunit (Fig. 7B) has better defined structure compared to the monomer alone (Fig. 3A). This is mainly due to decreased flexibility at the inter-helical segments, which were kept flexible during the docking, as the two C-terminal helices now adopt a relatively fixed conformation. This is also apparent from the RMSD values; the pentamer subunit RMSD values are significantly reduced as compared to the monomer (Fig. 7C).

Notably, in this pentameric model, the location of V25 is inter-helical (Fig. 8B–C), whereas in the previously proposed model it was closer to a lumenal orientation \cite{16}. The rotational pitch of the residues in the TM domain of this pentameric model were measured individually \cite{35} and compared to those from the computational models A and B \cite{15} (Fig. 8D). While values for residues 25–27 are closer to model B, the rest of the sequence is similar to model A, except at residue 28 which deviates from both models. For comparison, the rotational pitch close to model A for residues in ETM obtained previously by NMR in DPC micelles \cite{20} is also shown. The present model has been constructed independently from A and B model templates, and the result appears to be a hybrid between the two \cite{15}. This is not surprising since the in silico study assumed a certain rigidity in the TM \(\alpha\)-helices \cite{15}.

Most of the residues in the model we report have an orientation consistent with model A. This is not surprising, since model A had the lowest energy value for each individual E protein homologs \cite{15}. However, the model gets closer to model B in the turn that contains V25 (Fig. 8D). This enables V25 to adopt a more interhelical orientation consistent with the revertant mutants that appeared in vivo \cite{30}. Additionally, the helix kink region suggested by infrared dichroism data in lipid bilayers \cite{16} is also observed, which supports the validity of the membrane-mimic environment used herein.

Finally, in LMPG micelles, the C-terminal tail of SARS-CoV E protein is \(\alpha\)-helical, more so than observed in mixed DPC/SDS micelles \cite{19}, and the presence of extramembrane NOEs suggest interactions between the C-terminal domains that may affect the pentameric conformation.

Accession numbers

The atomic coordinates have been deposited in the Protein Data Bank.
Fig. 7. Structure of the $E_{18}$ pentamer. (A) Top view of the $E_{18}$ pentamer showing an ensemble of 16 structures obtained using HADDOCK and 10 inter-monomeric NOE restraints (see Materials and Methods); (B) Side view of one subunit of the pentamer showing the backbone as line representation; (C) RMSD values (per-residue) of the monomer ensemble (see Fig. 3A, black), structured helical segments of the monomer (see Fig. 3B, blue), and the pentamer ensemble (Fig. 7A, red). The average RMSD value of the monomer (dashed line) and ± 1 S.D. values (grey band) are indicated. (Layout note: 1.5 column).

Fig. 8. Orientation of the $E_{18}$ pentamer. (A) Top view and (B) side view of average structure of the $E_{18}$ pentamer bundle in cartoon representation. The N- and C-terminus of one monomeric unit is indicated; (C) top view of a monomer-monomer TM interaction, showing the distances between the side chain of V25 and those of residues appearing in SARS-CoV E V25F revertant mutants [30]; (D) differences in TM residue rotational orientation, $\omega$, between the experimental model proposed here (LMPG) versus computational models A and B [18] and that of $E_{18}$ obtained by NMR in DPC micelles [20]. The region with larger differences between the present model (LMPG) and model A (residues 25–28) is highlighted. (Layout note: 1.5 columns).
Bank (PDB ID: 5X29). Assigned chemical shifts have been deposited at the Biological Magnetic Resonance Bank (BMRB ID: 36049).

Transparency document

The http://dx.doi.org/10.1016/j.bbamem.2018.02.017 associated with this article can be found, in online version.

Acknowledgements

J.T. acknowledges the funding from Singapore MOE Tier 1 grant RG 51/13. The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2018.02.017.

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