Supporting Information

Critical Effect of Detergent:Protein Ratio on the Formation of
Hepatitis C Virus p7 Channel

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Supplementary Methods

Protein expression and purification
Sample preparation for SEC analysis
NMR sample preparation
NMR spectroscopy

Figure S1-S4. 2D TROSY-HSQC spectra of mixed p7 sample (spin-labeled at various positions) in detergent micelles, before and after reducing the nitroxide with ascorbic acid.

Figure S5. Comparison between experimental PREs in DPC micelles and the relevant PRE distances derived from the hexamer structure.

Figure S6-S7. 2D TROSY-HSQC spectra of mixed p7 sample (spin-labeled at various positions) in bicelles, before and after reducing the nitroxide with ascorbic acid.

Figure S8. 2D TROSY-HSQC spectra of a regular p7 sample (without cysteines), treated with MTSL labeling and cleaning protocols, before and after reducing the nitroxide with ascorbic acid.
Supplementary Methods

Protein expression and purification

The Hepatitis C Virus p7 protein (EUH1480 strain, genotype 5a), designated p7(5a), was expressed and purified as previously described\textsuperscript{1-3}. Briefly, p7(5a) was expressed in \textit{E. coli} strain BL21(DE3) as a C-terminal fusion to the His\textsubscript{9}-trpLE sequence in the pMM LR6 vector. Protein expression was induced with 150 \(\mu\)M isopropyl-\(\beta\)-thiogalactopyranoside (IPTG) when cell culture OD\textsubscript{600} reached 0.7. Cells were harvested after overnight expression at 25°C and lysed by sonication in \textit{lysis buffer} (50 mM Tris, 200 mM NaCl, pH 8.0). The trpLE-p7(5a) fusion protein was extracted from inclusion bodies with \textit{extraction buffer} (6 M guanidine HCl, 50 mM Tris (pH 8.0), 200 mM NaCl, 1% (vol/vol) Triton X-100) and bound to HisPur Ni-NTA resin (Life technology). After sequential washing with 8M urea solution (20 mM Tris, pH 7.5) and water, the fusion protein was eluted with with 90% (vol/vol) formic acid. p7(5a) was then cleaved from the fusion protein by adding cyanogen bromide (0.2 g/ml; Sigma) to the formic acid solution for 1 hour under nitrogen gas. Pure p7(5a) was then separated from the cleaved mixture by reverse-phase HPLC on a Zorbax SB-C18 semi-preparative column (Agilent) using a linear acetonitrile gradient (40-60%) in the presence of 0.1% trifluoroacetic acid. The HPLC purified p7(5a) samples were then lyophilized and validated by SDS-PAGE. For protein deuteration and isotope labeling, cells were grown in M9 minimal medium with D\textsubscript{2}O and appropriate isotopes.

Sample preparation for SEC analysis

Pure lyophilized p7(5a) (1.2 mg) was dissolved in 6 M guanidine with various amounts of dodecylphosphocholine (DPC) (Anatrace) (final volume 0.5 ml), resulting in p7(5a) concentration of 0.35 mM and DPC concentration of 64, 197, or 654. For the detergent/peptide ratio of 10,000, 0.1 mM p7(5a) and 1 M DPC were used. The mixture was dialyzed against 25 mM MES buffer (pH 6.5) twice to remove the denaturant. DPC concentration after the dialysis was determined by \textsuperscript{1}H NMR to be 44.5, 155, 418, or 623 mM, respectively. For the sample with 623 mM DPC, additional 377 mM DPC was added to reach 1 M DPC. Hence, the DPC:p7 ratios of the four samples used for size-exclusion chromatography (SEC) analysis were 127, 443, 1194, and 10000. These samples were subject to fast protein liquid chromatography (FPLC) in a superdex 200 increase 10/300 GL column (GE Healthcare) in running buffer containing 3 mM DPC, 100 mM NaCl, and 25 mM MES (pH 6.5). Protein elution was monitored by UV absorption at 280 nm.
NMR sample preparation

Purified p7(5a), lyophilized, was reconstituted either in DPC micelles or in DMPC-DH₆PC bicelles using previously published protocols. For DPC reconstitution, 2 mg of p7(5a) was mixed with 20 mg of DPC in 6 M guanidine (the molar ratio between DPC and protein is around 180). The mixture was dialyzed against NMR buffer (25 mM MES, pH 6.5) twice, followed by size-exclusion chromatography. Hexamer containing fractions, the same pattern as shown in Fig. 1a, were collected, and concentrated to around 300 µl. For bicelle reconstitution, 2 mg of p7 was dissolved with 12 mg of 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) (Avanti Polar Lipids) and 16 mg of 1,2-Dihexanoyl-sn-Glycero-3-Phosphocholine (DH₆PC) in 6M guanidine. The mixture was dialyzed against NMR buffer for 3 hours, followed by an additional round of dialysis of 3 hours. During the second round of dialysis, 3 mg of DH₆PC was added to the sample after every hour to ensure bicelle q < 1. After dialysis, sample was concentrated to around 300 µl. The q was validated by 1D ¹H NMR and adjusted accordingly to 0.5-0.6 by addition of DH₆PC. General steps of bicelle sample preparation for NMR studies are described in Fu et al.⁴

For inter-chain PRE measurements, p7(5a) mutants containing a single cysteine (at designated site) and C-terminal His₆-tag were purified as described above except for the addition of 10 mM DTT in the relevant purification buffers. Equal amounts of (¹⁵N, 80% ²H)-labeled p7(5a) (also with C-terminal His₆-tag) and the single-cysteine mutant were mixed at 1:1 molar ratio after HPLC purification when the samples were still soluble in organic solvent. Mixed samples were lyophilized and then reconstituted in DPC micelles or DMPC-DH₆PC bicelles as described above except for the addition of 20 mM DTT in the reconstitution buffers. DTT was removed by passing the sample through PD-10 column in 3 mM DPC and 25 mM phosphate buffer (pH 7.5) for DPC reconstitution, or in 6 mM DH₆PC and 25 mM phosphate buffer (pH 7.5) for bicelle reconstitution. The spin label, 1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrrolone-3-methyl methanethiosulfonate (MTSL), was added to the protein sample at 10x the protein concentration to react with the free thiols of cysteines. The reaction mixture was incubated overnight in the dark. To remove free MTSL, the mixture was loaded to Ni-NTA resin in a gravity-flow column and washed with 20 column volumes of 3 mM DPC buffer for micelle reconstitution or 6 mM DH₆PC buffer for bicelle reconstitution. The mixed samples were then eluted with 500 mM imidazole in buffer containing either 6 mM DPC or 12 mM DH₆PC for micelle or bicelle sample, respectively. The mixed samples were dialyzed against the NMR buffer (25 mM MES, pH 6.5) to remove the imidazole. For the p7-DPC samples, the final protein concentration is estimated, based on the initial 2 mg peptide used, to be ~ 1 mM, and the DPC concentration was determined.
by 1D $^1$H NMR to be 75.3 mM (or a DPC:p7 ratio of ~75). For the p7-bicelle samples, the final protein concentration was ~1 mM, and the $q$ of the bicelle sample was examined by 1D $^1$H NMR and adjusted to 0.5-0.6 by adding DH$_6$PC.

To examine whether the above MTSL labeling protocol can thoroughly remove free MTSL, we prepared a non-mixed, uniformly ($^{15}$N, 80% $^2$H)-labeled sample of p7(5a) without any cysteines in DPC micelles ([p7] = 0.4 mM; [DPC] = 35.4 mM), and treated the sample with exactly the same MTSL labeling and cleaning procedures as above. This sample did not show any noticeable PREs (Fig. S8), indicating that the PREs observed for the Cys mutants could not have been generated by residual unconjugated MTSLs in micelles.

**NMR spectroscopy**

All NMR experiments were conducted at 30°C on a 600 MHz Bruker spectrometer equipped with cryogenic probe. NMR spectra were processed using NMRpipe$^5$ and analyzed using Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).

PRE measurements were performed using the $^1$H-$^{15}$N TROSY-HSQC experiment. PREs were calculated as the ratio of peak intensities before and after the reduction of MTSL with 20 mM ascorbic acid (prepared as a 500 mM stock, pH 6.5).

**References:**

Figure S1. 2D TROSY-HSQC spectra of mixed p7 sample (spin-labeled at Gly1) in micelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The protein was reconstituted in DPC. The spectra were recorded at 600 MHz $^1$H frequency.
Figure S2. 2D TROSY-HSQC spectra of mixed p7 sample (spin-labeled at His31) in micelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The protein was reconstituted in DPC. The spectra were recorded at 600 MHz $^1$H frequency.
Figure S3. 2D TROSY-HSQC spectra of mixed p7 sample (spin-labeled at Ser44) in micelles before (left) and after (right) reducing the nitrooxide with ascorbic acid. The protein was reconstituted in DPC. The spectra were recorded at 600 MHz $^1$H frequency.
Figure S4. 2D TROSY-HSQC spectra of mixed p7 sample (spin-labeled at Arg57) in micelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The protein was reconstituted in DPC. The spectra were recorded at 600 MHz $^1$H frequency.
Figure S5. Experimental PREs in DPC micelles (left) and the relevant PRE distances derived from the hexamer structure (right). The dominant PRE distances are distances from the $C_\beta$ of the MTSL-carrying residue to the amide protons of residues of the relevant neighboring chain, averaged over all neighboring chain pairs in the hexamer structure (PDB ID: 2M6X).
Figure S6. 2D TROSY-HSQC of mixed p7 sample (spin-labeled at His31) in bicelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The protein was reconstituted in DMPC-DH_{6}PC bicelle with \( q = 0.55 \). The spectra were recorded at 600 MHz \( ^1\text{H} \) frequency.
Figure S7. 2D TROSY-HSQC of mixed p7 sample (spin-labeled at Arg57) in bicelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The protein was reconstituted in DMPC-DH₂PC bicelle with \( q = 0.55 \). The spectra were recorded at 600 MHz \(^1\)H frequency.
Figure S8. 2D TROSY-HSQC spectra of non-mixed p7 sample (without any cysteines) in DPC micelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The DPC-reconstituted ($^{15}$N, 80% $^2$H)-labeled p7(5a) was treated with exactly the same MTSL labeling and cleaning procedures used for other mixed samples in Figs. S1-S4. The spectra were recorded at 600 MHz $^1$H frequency.