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

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Supporting Information

ABSTRACT: The p7 protein encoded by the hepatitis C virus forms a cation-selective viroporin in the membrane. One of the most intriguing findings about the p7 viroporin is its unique hexameric structure in dodecylphosphocholine (DPC) micelles determined by nuclear magnetic resonance (NMR), but the hexameric structure was recently challenged by another NMR study of p7, also in DPC detergent, which claimed that the p7 in this detergent is monomeric. Here, we show that p7 oligomerization is highly sensitive to the detergent:protein ratio used in protein reconstitution and that the 40-fold difference in this ratio between the two studies was the cause of their different conclusions. In addition, we have performed extensive measurements of interchain paramagnetic relaxation enhancements (PREs) for p7 hexamers reconstituted in DPC micelles and in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine/1,2-dihexanoyl-*sn*-glycero-3-phosphocholine bicelles. In both cases, interchain PREs are overall consistent with the hexameric structure determined in micelles. Our data validate the overall architecture of the p7 hexamer while highlighting the importance of the detergent:protein ratio in membrane protein sample preparation.

Viroporin protein p7 encoded by the hepatitis C virus (HCV) genome is a 63-residue protein that oligomerizes in the membrane to form cation-selective channels,[1,2] with higher selectivity for Ca^{2+} than for K^+/Na^+.[3,4] The channel activity of p7 is important for the assembly and release of infectious viruses,[5,6] and thus, finding small molecules to block p7 is an alternative route of developing therapeutics for treating HCV infection.[1,3,4,7]

As in the cases of influenza M2 channels, structural characterization of p7 was impeded by various technical problems; the hydrophobic and dynamic nature of the protein made crystallization difficult, and the low molecular mass still poses a serious hurdle for cryo-electron microscopy (cryo-EM). In a previous study to enable solution nuclear magnetic resonance (NMR) measurements, we developed a sample of p7 from genotype 5a (EUH1480 strain), designated p7(5a), reconstituted in dodecylphosphocholine (DPC) micelles.[8] Negative-stain electron microscopy (nsEM) analysis of that sample generated two-dimensional (2D) reference-free class averages that are indicative of hexamers and that resemble the nsEM images of p7(2a) (JFH-1 strain) in the DH_7PC detergent.[9] Further NMR analysis of both intra- and interchain nuclear Overhauser effects (NOEs) led to a detailed model of the p7(5a) hexameric complex.[8,10]

The NMR structure shows that although p7 is only a 7 kDa protein, it forms a rather sophisticated hexameric, funnel-like architecture with six intertwined chains.[8] Each chain contains three helical segments, designated H1−H3. The H1 and H2 helices form the narrow and wide regions of the funnel-shaped cavity, respectively, and the H3 helices wrap the channel periphery by interacting with H2 of the $i + 2$ protomer and H1 of the $i + 3$ protomer. The high degree of interchain interactions allows a small peptide to form a 42 kDa channel complex.

The intriguing hexameric structure of p7 was, however, challenged recently by studies by Oestringer et al., which argued that the p7 reconstituted in the DPC detergent was monomeric.[11,12] The authors of the studies also suggested that the hexamer structure of p7 was an artifact generated using interchain structural restraints that did not exist. Because the oligomeric architecture of p7 represents only the second type of viroporin structures after that of the influenza M2 channels, the newly raised controversy was a major setback for the field of viroporin.

In this study, we performed experiments to understand why the DPC-reconstituted samples used by Oestringer et al. and us resulted in completely different observations. We found that the detergent:protein ratio used during p7 reconstitution has a strong influence on the oligomeric assembly of p7(5a), i.e., the hexamer dissociates as the DPC:protein ratio increases, and thus, the very different DPC:protein ratio used by the two studies was the cause of the major discrepancy. In addition, we have performed extensive interchain paramagnetic relaxation enhancement (PRE) analyses of p7(5a) in DPC micelles and in DMPC/DHPC bicelles that closely mimic a lipid bilayer. In both cases, the data are consistent with the published hexameric structure of p7(5a).

We prepared p7(5a) samples (UniProt entry 039928) with different amounts of DPC to examine the effect of detergent:protein molar ratios on p7 oligomerization. The

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p7(5a) protein was purified and reconstituted in DPC as described previously,[8,13] except size-exclusion chromatography (SEC) was not applied. The samples with various DPC:p7 ratios were analyzed by SEC using the *superdex* 200 *increase* 10/30 column. Although the same elution buffer containing 3 mM DPC was used, samples reconstituted originally with different DPC:p7 ratios showed very different elution profiles. When the DPC:p7 molar ratio was 127:1, p7 eluted at ∼14 mL as a single species (Figure 1a), indicative of a homogeneous

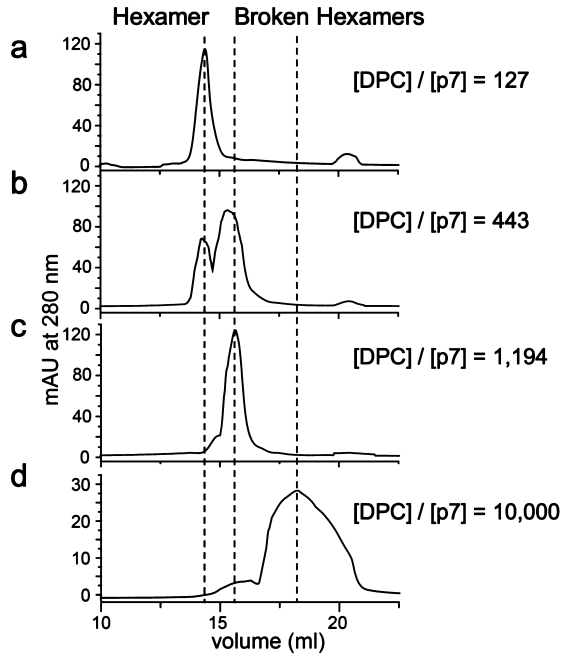

Figure 1. Size-exclusion chromatography (SEC) profiles of p7(5a) reconstituted in different amounts of DPC. SEC elution profiles from the *superdex* 200 *increase* 10/300 GL column of p7(5a) reconstituted initially at DPC:protein ratios of (a) 127, (b) 443, (c) 1194, and (d) 10000. [p7] = 0.35 mM in panels a−c and 0.1 mM in panel d. DPC concentrations of reconstituted samples after dialysis were determined by ^{1}H NMR.

hexameric complex observed previously by nsEM. When the DPC:p7 ratio was increased to 443:1, p7 eluted as two species, a minor peak at ∼14 mL and the major broader peak at ∼16 mL (Figure 1b), indicating that the hexameric complex has largely dissociated to lower, less homogeneous oligomers. When the DPC:p7 ratio was further increased to 1194:1, the p7 elution peak at ∼14 mL moved almost entirely to ∼16 mL (Figure 1c), indicating that the hexamer complex has completely dissociated to lower oligomers. Finally, at a DPC:p7 ratio of 10000:1, the elution peak shifted further to the right while becoming extremely broad, indicative of even more heterogeneous species of which the dominant species is probably monomeric.

In the study by Oestringer et al.,[12] a DPC:p7 ratio of 10000 [5 μM p7(5a) and 50 mM DPC] was used in the same reconstitution protocol as the one used in this study. For this sample, the SEC elution volume from the *superdex* 200 *increase* 10/30 column was 18.7 mL,[12] consistent with our result in Figure 1d. The SEC data collectively show that oligomerization of p7(5a) is highly sensitive to the DPC:protein ratio, and the ∼40-fold higher DPC:protein ratio used by Oestringer et al. compared to that in our previous structural study explains why they observed only p7 monomers in their SEC analysis.[12]

We next performed a qualitative evaluation of the p7 hexamer structures in DPC micelles and in the more membrane-like DMPC/DHPC bicelles by measuring interchain PREs for the two reconstitution systems. In the earlier study, we developed an NMR-feasible sample of p7(5a) in DMPC/DHPC bicelles with q = 0.5, showing that p7(5a) also forms a hexamer in bicelles.[14] The study presented here takes a step further to qualitatively address the oligomeric arrangement in bicelles relative to the hexameric structure in micelles.

For measuring interchain PREs, the NMR-visible, (^{15}N, 80% ^{2}H)-labeled chain was mixed with the NMR-invisible, spin-labeled chain at a 1:1 ratio, such that the detected PREs are exclusively intermolecular. Each spin-labeled chain contains a single mutation to cysteine for labeling with MTSL (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methylmethanethiosulfonate). Interchain PREs were examined for four different single-site MTSL labels at residue positions 1, 31, 44, and 57. The (^{15}N, 80% ^{2}H)- and spin-labeled chains were mixed in an organic solvent, followed by reconstitution in DPC and MTSL labeling (Supplementary Methods, NMR sample preparation). The DPC:protein ratio for the PRE samples was ∼75. Residue-specific PREs were measured as the ratio of NMR peak intensities in the ^{1}H−^{15}N TROSY-HSQC spectra (Figures S1−S4) before (I) and after (I_0) the samples had been reduced with ascorbate.

The strongest interchain PREs generated by the four spin-labels range from 0.3 to 0.7, expected of 1:1 mixture of ^{15}N- and spin-labeled chains. The spin-label at the N-terminal residue (Gly1) generated the strongest PRE, consistent with the funnel-like structure in which the N-termini of the protomers at the funnel tip are very close, N-termini of the neighboring chains being within 14.8 Å of each other (Figure 2a). C-Terminal residues 59−63 also show obvious PRE (∼0.6) because they are relatively close to the N-termini of neighboring chains. The spin-label at His31 induced strong PREs (0.5−0.6) near the C-terminal end (residues 36−42) of the H2 helix (Figure 2b), also consistent with the interaction between the H2 segments from the neighboring chains shown by the hexamer structure. Ser44 is one of the most peripheral residues on the wider opening of the funnel, located in the flexible loop connecting the H2 and H3 helices. The spin-label at Ser44 generated modest but obvious interchain PRE (∼0.7) for residues 41−45 (Figure 2c). Finally, the spin-label at Arg57 induced substantial interchain PRE (∼0.6) for residues 17 and 18, in good agreement with the proximity of Arg57 to the short joint (His17 and Gly18) between H1 and H2 (Figure 2d). Overall, the interchain PREs of p7(5a) in DPC are in agreement with the previously reported hexamer structure derived from interchain NOE data[8] (see PRE distances in Figure S5).

We then performed similar interchain PRE measurements for p7(5a) reconstituted in DMPC/DHPC bicelles with q = 0.55 (Figures S6 and S7). In this case, mixed isotope and spin-labeled chains were first reconstituted in bicelles, followed by labeling with MTSL and Ni-NTA affinity chromatography for removing excessive MTSLs bound to the bicelles. The spin-label at His31 induced strong interchain PREs very similar to those observed for the micelle sample, although the PRE is on average stronger and a wider range of residues (36−47) are affected (Figure 3a). The slightly stronger interchain PREs were probably due to more intimate contacts between the H2

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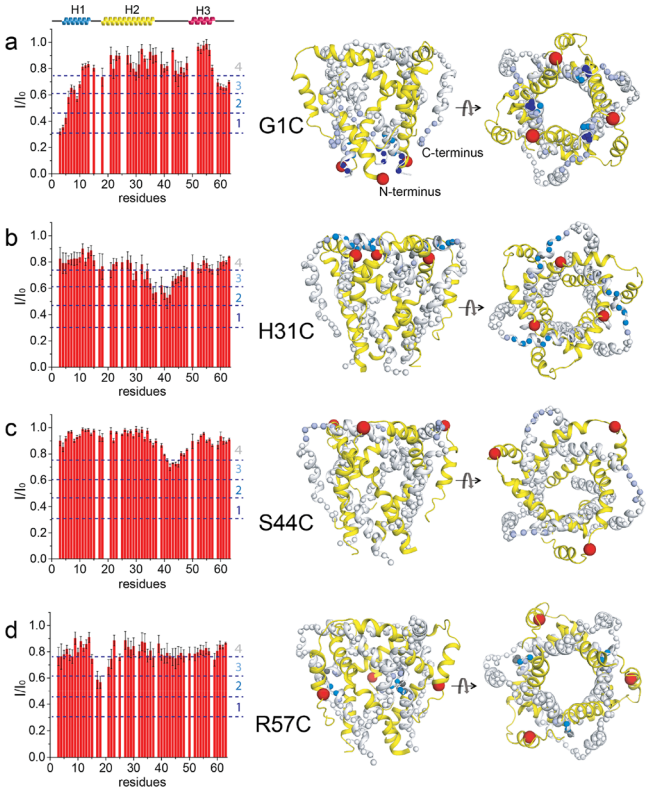

Figure 2. Interchain PREs of p7(5a) samples in DPC micelles. The samples consist of a 1:1 ratio of (^{15}N, ~80% ^{2}H)-labeled protein and unlabeled protein with MTSL labeled at residue position (a) 1, (b) 31, (c) 44, and (d) 57, reconstituted in DPC micelles. The left column shows residue-specific PREs, defined as the ratio of the intensity before (I) and after (I_0) reduction with ascorbate. Regions between the dashed lines define different PRE ranges. The right columns show structural views (Protein Data Bank entry 2M6X) of backbone amide protons (spheres) that show strong PRE. Ribbon representations of the ^{15}N- and MTSL-labeled strands are colored gray and yellow, respectively (note the mixture is purely random). The MTSL position is indicated by the red sphere. The amide protons are colored according to the PRE ranges defined on the left.

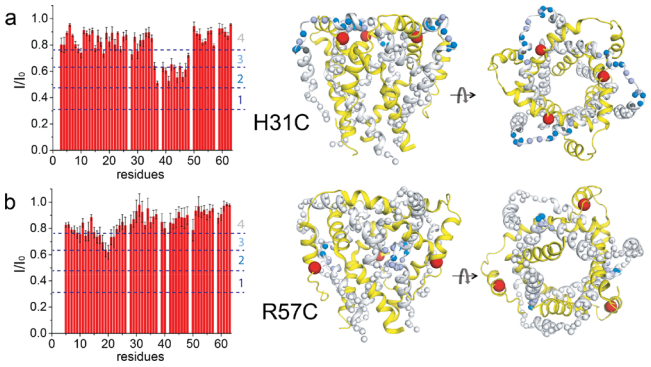

Figure 3. Interchain PREs of p7(5a) samples in bicelles. The samples consist of a 1:1 ratio of (^{15}N, ~80% ^{2}H)-labeled protein and unlabeled protein with MTSL labeled at residue positions (a) 31 and (b) 57, reconstituted in DMPC/DHPC bicelles with q = 0.55. Presentations in the left and right panels are the same as in Figure 2. The MTSL position is indicated by the red sphere. The amide protons are colored according to the PRE ranges defined on the left.

helical segments of the neighboring chains in the more membrane-like bicelles. The spin-label at Arg57 also induced interchain PREs (~0.6) similar to that detected in the micelle sample; e.g., residues around Gly18 were most affected (Figure 3b). In addition to the joint between H1 and H2, however, H1 in bicelles shows substantially stronger PREs than in micelles (Figure 3b), possibly due to the more dynamic H1 helices in bicelles shown previously by NMR measurements of the apparent rotational correlation times.[14] The interchain PRE patterns generated by the two spin-labels in bicelles are overall in agreement with the PREs induced by the same spin-labels in micelles, indicating that the hexameric conformations in bicelles and micelles are similar.

Our SEC analysis of p7(5a) reconstituted at different detergent concentrations shows that the detergent:protein ratio has a profound influence on the oligomerization of p7(5a). Previous studies have already shown that, for certain small oligomeric membrane proteins, a high detergent:protein ratio can cause membrane protein denaturation or misfolding.[15−17] More importantly, our SEC data address the major discrepancy between what appeared to be extremely similar NMR studies of HCV p7 by OuYang et al.[8] and Oestringer et al.[12] The two studies used the same p7 construct [p7(5a)] and the same detergent (DPC) and yielded very similar 2D NMR spectra. However, the SEC result from Oestringer et al. is clearly indicative of monomeric p7 in micelles, in sharp contrast to the hexameric species and interchain NOEs observed by OuYang et al. The results in Figure 1 show that the very different detergent:protein ratios used during protein reconstitution were the cause of the discrepancy, providing an unambiguous resolution to the ongoing controversy about the HCV viroporin structure.

The strong dependence on the detergent:protein ratio, however, suggests the fragility of the p7 oligomers in detergent micelles. Indeed, the interchain NOEs of p7(5a) in DPC micelles appeared to be weaker than those of transmembrane helix oligomers in bicelles,[18−20] and hence, the intriguing architecture of the p7 oligomer revealed in DPC micelles has been challenged. Our site-specific PRE measurements of p7(5a) in DPC micelles and in DMPC/DHPC bicelles both show specific interchain PREs that are overall consistent with the published hexamer structure in micelles. Notably, the PREs generated by spin-labels at different positions along the 6-fold axis all can be explained by the unusual fold of the protein, and this level of agreement is not possible if the protein were monomers generating PREs via nonspecific aggregation.

Finally, an earlier study of p7(5a) in DMPC/DHPC bicelles (q = 0.6) has already shown that the protein forms hexamers in bicelles.[14] The lipid and water NOE data from the same study are also consistent with the hexameric NMR structure; i.e., the H3 helices that wrap the channel periphery exhibited strong lipid NOEs, whereas the pore-lining H2 helices did not. In the study presented here, our interchain PREs generated by spin-labels at His31 and Arg57 further suggest that the mode of hexameric assembly (or arrangements of the helical segments) in bicelles is similar to that in micelles. The interchain PRE data are nevertheless semiquantitative. Detailed structural differences between the two systems would require comprehensive interchain NOE analysis.

■ ASSOCIATED CONTENT

Supporting Information

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Detailed description of the method and Figures S1−S8 (PDF)

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Author Contributions

W.C., B.O., and J.J.C. conceived the study. W.C. prepared the samples and conducted experiments. W.C., B.O., and J.J.C. wrote the paper.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

DPC, dodecylphosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; SEC, size-exclusion chromatography; PRE, paramagnetic relaxation enhancement; MTSL, 1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methylmethanethiosulfonate.

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Supporting Information

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

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Supplementary Methods

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[1-3]. Briefly, p7(5a) was expressed in *E. coli* strain BL21(DE3) as a C-terminal fusion to the His_9-trpLE sequence in the pMM LR6 vector. Protein expression was induced with 150 μM isopropyl-β-thiogalactopyranoside (IPTG) when cell culture OD_{600} reached 0.7. Cells were harvested after overnight expression at 25˚C and lysed by sonication in *lysis buffer* (50 mM Tris, 200 mM NaCl, pH 8.0). The trpLE-p7(5a) fusion protein was extracted from inclusion bodies with *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_2O 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 ^{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-DH_6PC bicelles using previously published protocols[1,3]. 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 (DH_6PC) 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 DH_6PC 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 DH_6PC. 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 His_6-tag were purified as described above except for the addition of 10 mM DTT in the relevant purification buffers. Equal amounts of (^{15}N, 80% ^2H)-labeled p7(5a) (also with C-terminal His_6-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-DH_6PC 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 DH_6PC and 25 mM phosphate buffer (pH 7.5) for bicelle reconstitution. The spin label, 1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-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 DH_6PC buffer for bicelle reconstitution. The mixed samples were then eluted with 500 mM imidazole in buffer containing either 6 mM DPC or 12 mM DH_6PC 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_6PC.

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:

(1) OuYang, B.; Xie, S.; Berardi, M. J.; Zhao, X.; Dev, J.; Yu, W.; Sun, B.; Chou, J. J. *Nature* **2013**, *498*, 521.

(2) Dev, J.; Bruschweiler, S.; Ouyang, B.; Chou, J. J. *Journal of biomolecular NMR* **2015**, *61*, 369.

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(4) Fu, Q.; Piai, A.; Chen, W.; Xia, K.; Chou, J. J. *Nature protocols* **2019**.

(5) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. *Journal of biomolecular NMR* **1995**, *6*, 277.

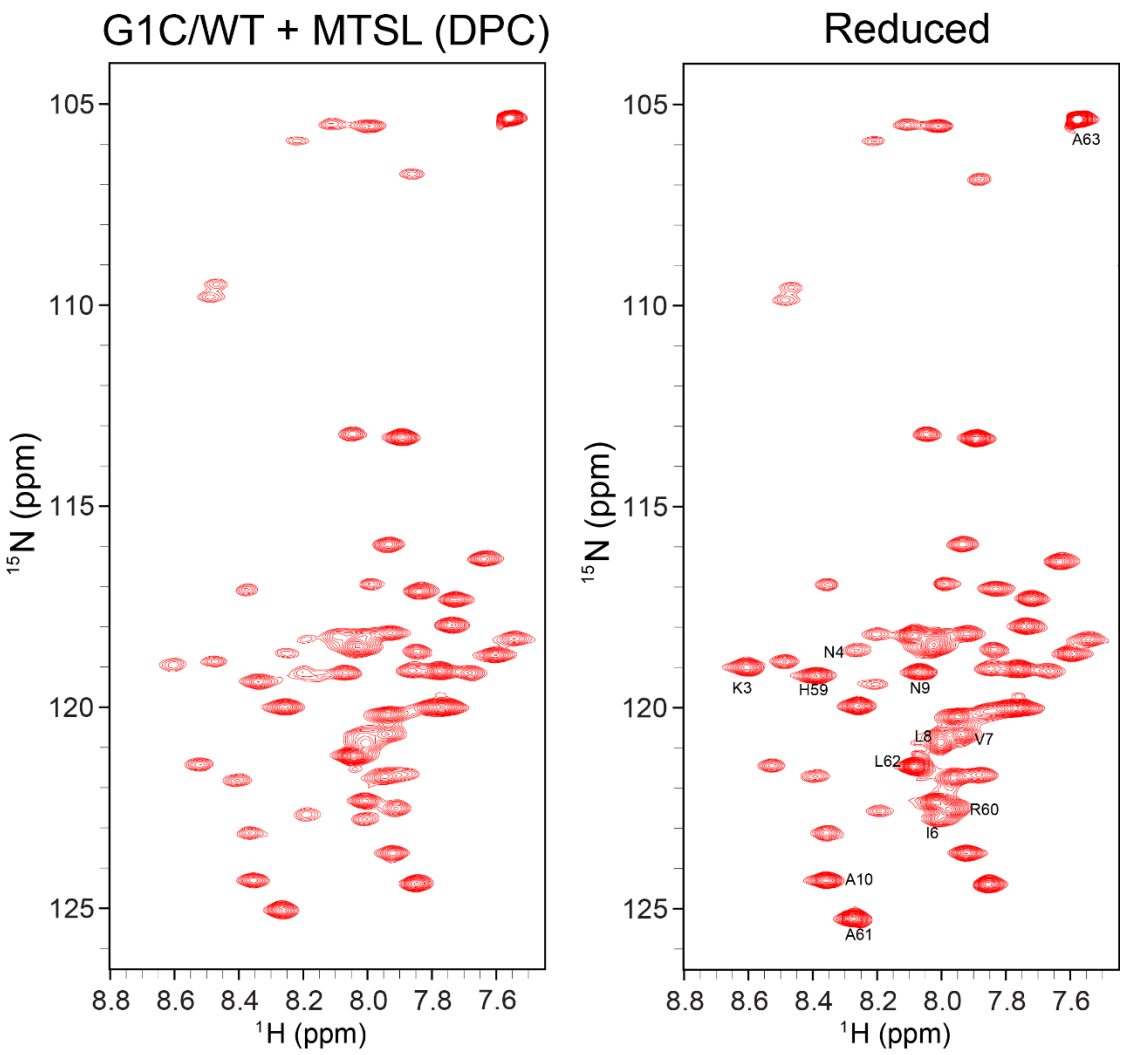

Figure S1. 2D TROSY-HSQC spectra of mixed p7 sample (spin-labeled at Gly1) in micelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The protein was reconstituted in DPC. The spectra were recorded at 600 MHz ^{1}H frequency.

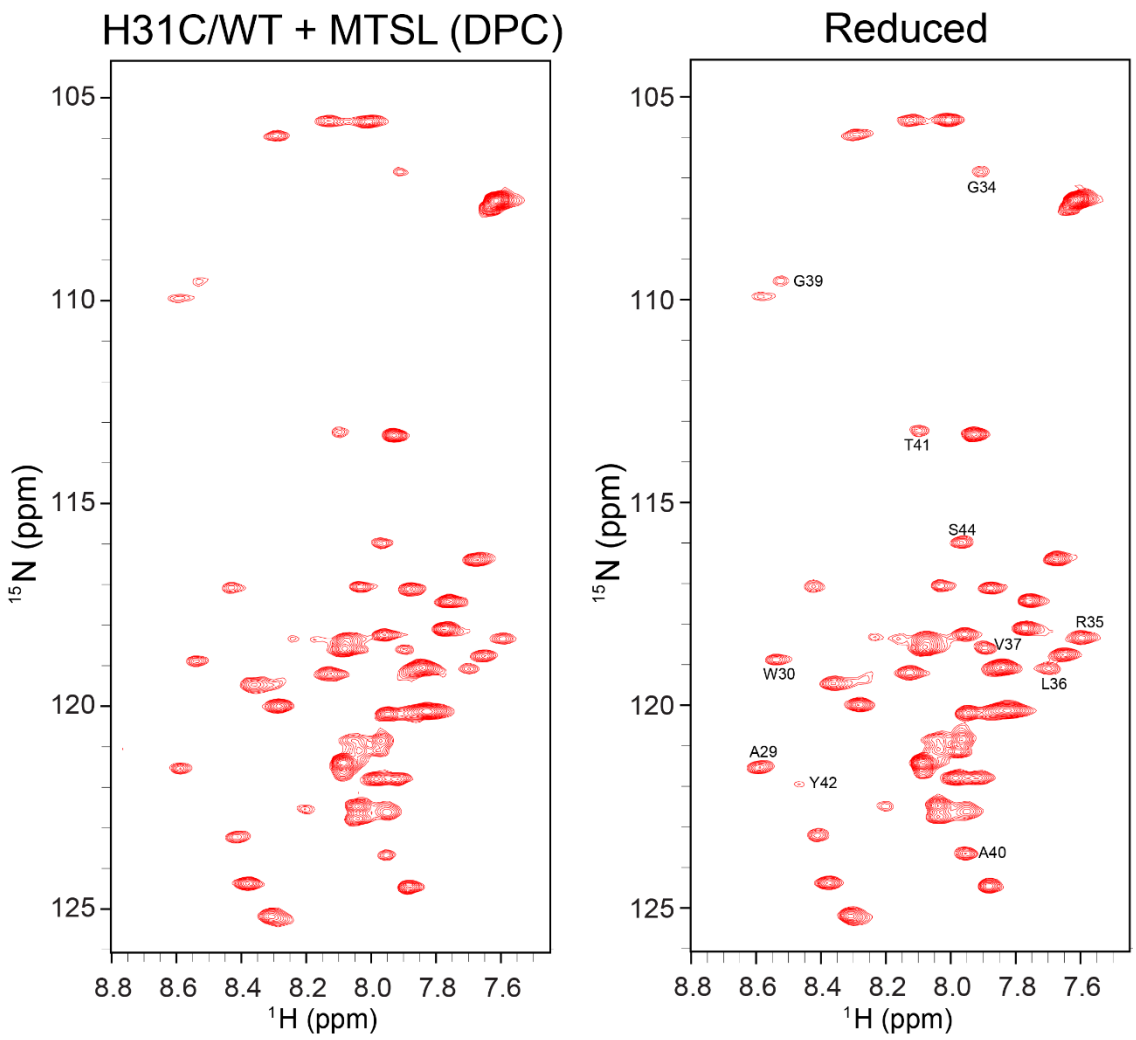

Figure S2. 2D TROSY-HSQC spectra of mixed p7 sample (spin-labeled at His31) in micelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The protein was reconstituted in DPC. The spectra were recorded at 600 MHz ^{1}H frequency.

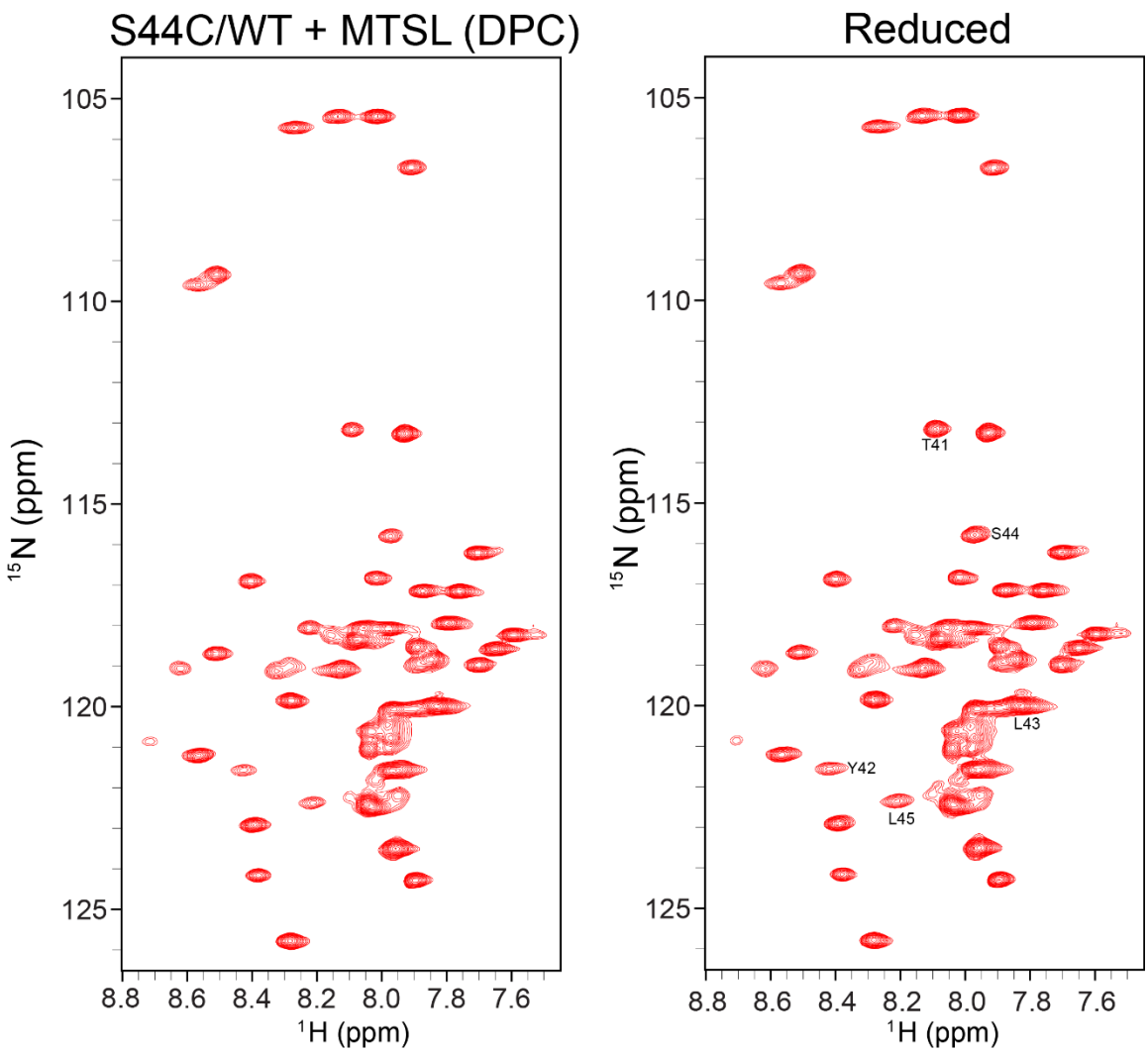

Figure S3. 2D TROSY-HSQC spectra of mixed p7 sample (spin-labeled at Ser44) in micelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The protein was reconstituted in DPC. The spectra were recorded at 600 MHz ^{1}H frequency.

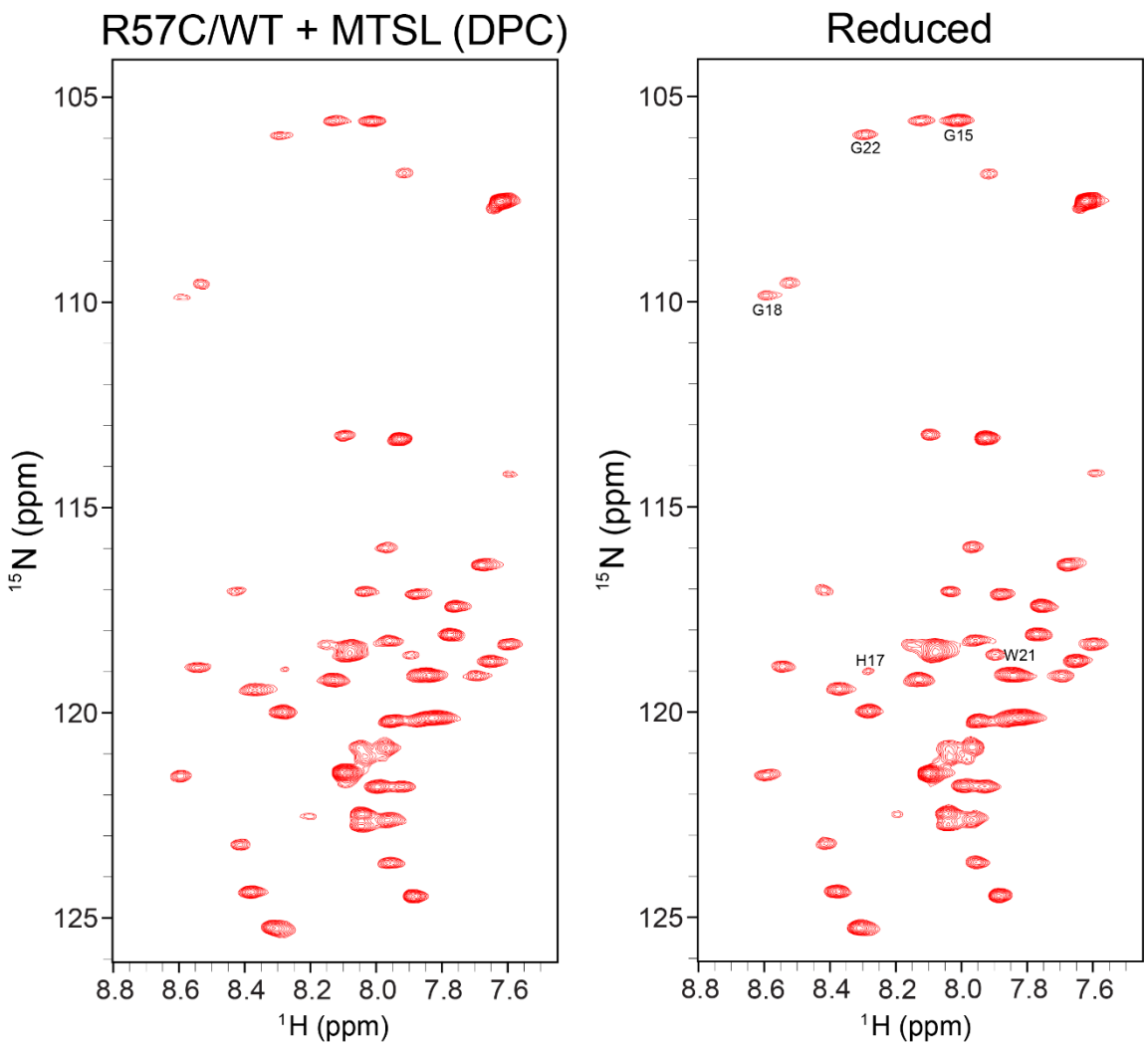

Figure S4. 2D TROSY-HSQC spectra of mixed p7 sample (spin-labeled at Arg57) in micelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The protein was reconstituted in DPC. The spectra were recorded at 600 MHz ^{1}H frequency.

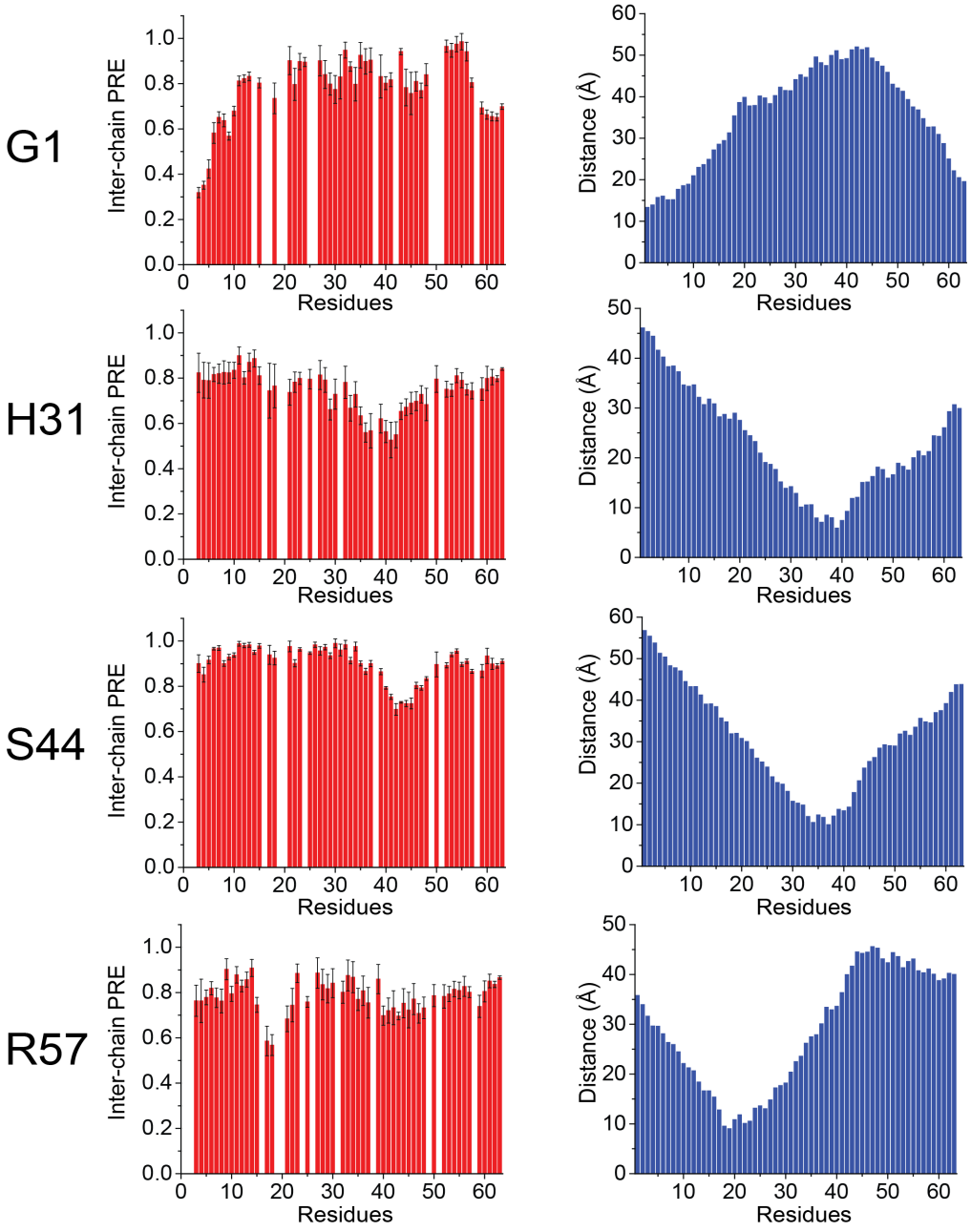

Figure S5. Experimental PREs in DPC micelles (left) and the relevant PRE distances derived from the hexamer structure (right). The dominant PRE distances are distances from the C_β of the MTSL-carrying residue to the amide protons of residues of the relevant neighboring chain, averaged over all neighboring chain pairs in the hexamer structure (PDB ID: 2M6X).

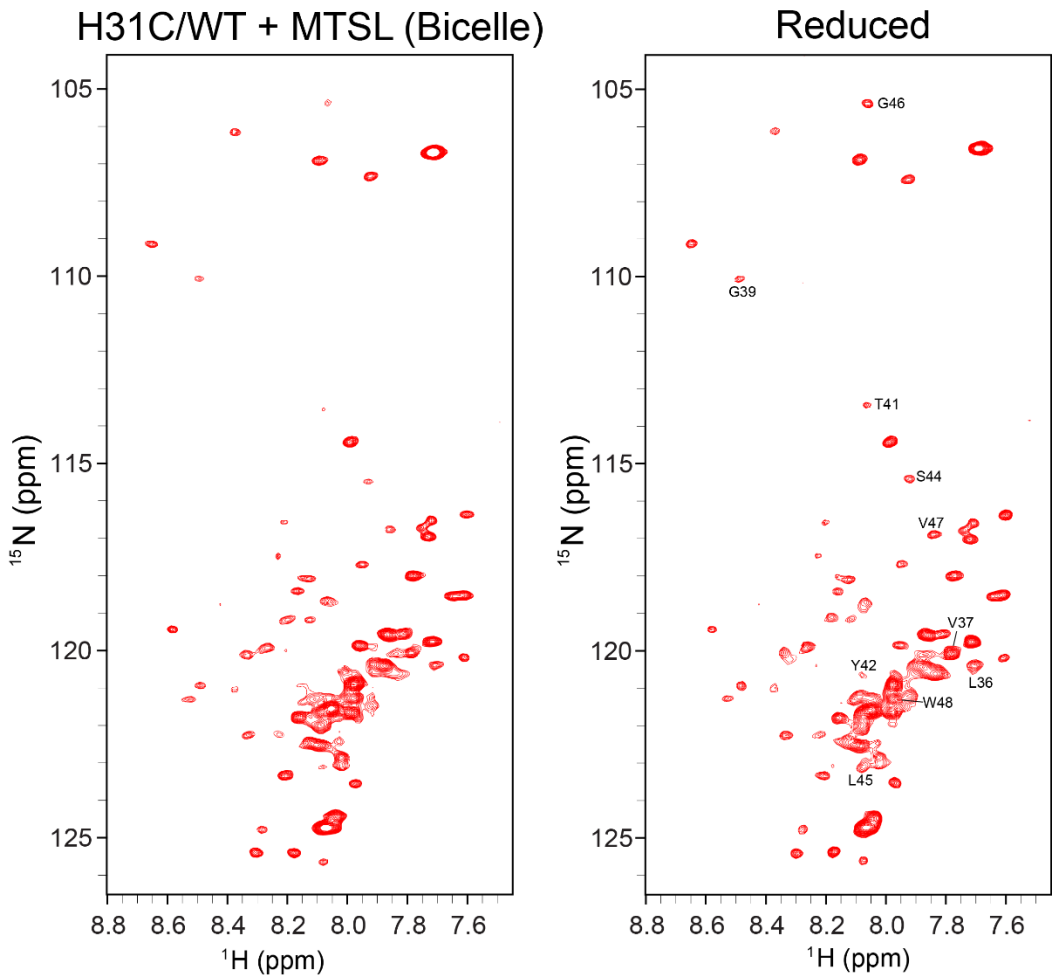

Figure S6. 2D TROSY-HSQC of mixed p7 sample (spin-labeled at His31) in bicelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The protein was reconstituted in DMPC-DH_6PC bicelle with q = 0.55. The spectra were recorded at 600 MHz ^{1}H frequency.

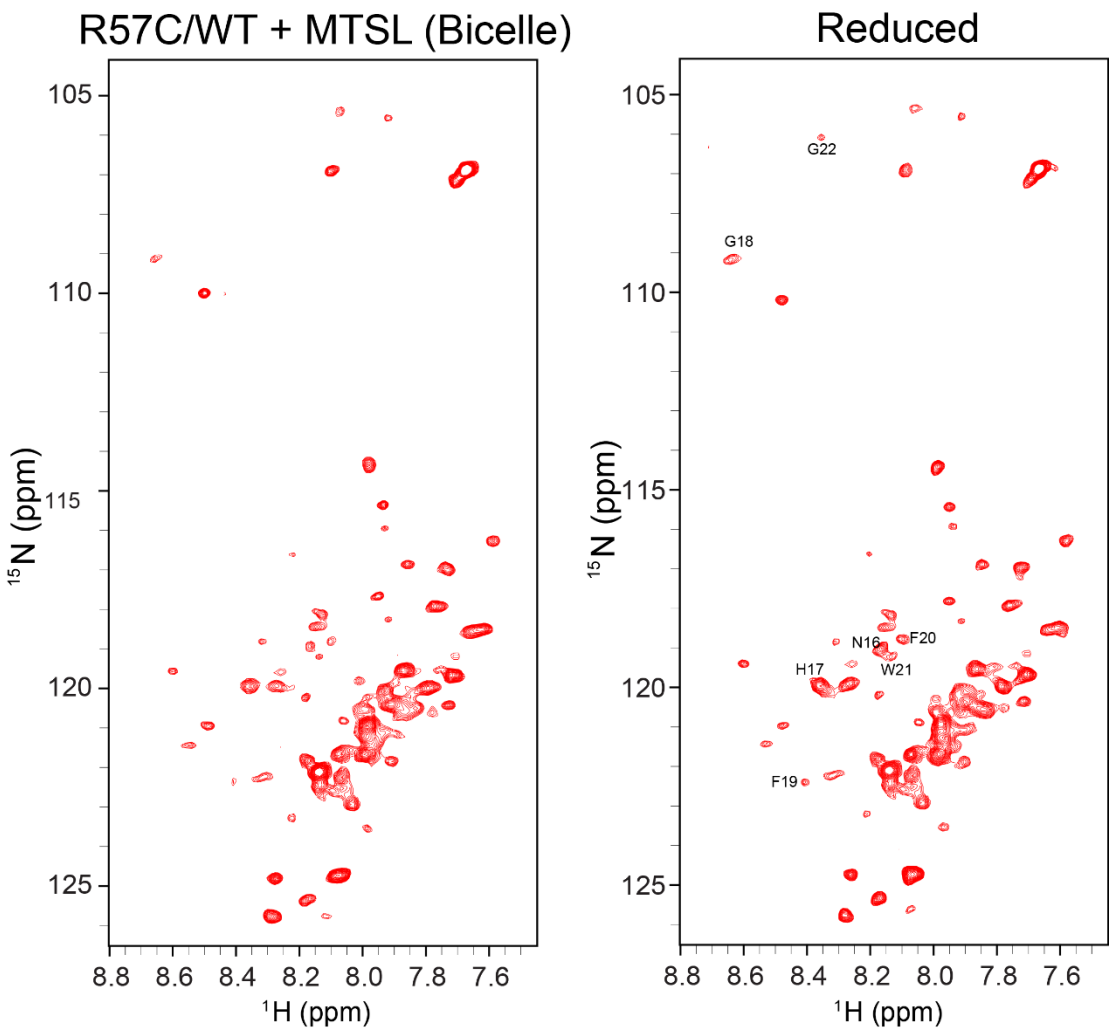

Figure S7. 2D TROSY-HSQC of mixed p7 sample (spin-labeled at Arg57) in bicelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The protein was reconstituted in DMPC-DH_6PC bicelle with q = 0.55. The spectra were recorded at 600 MHz ^{1}H frequency.

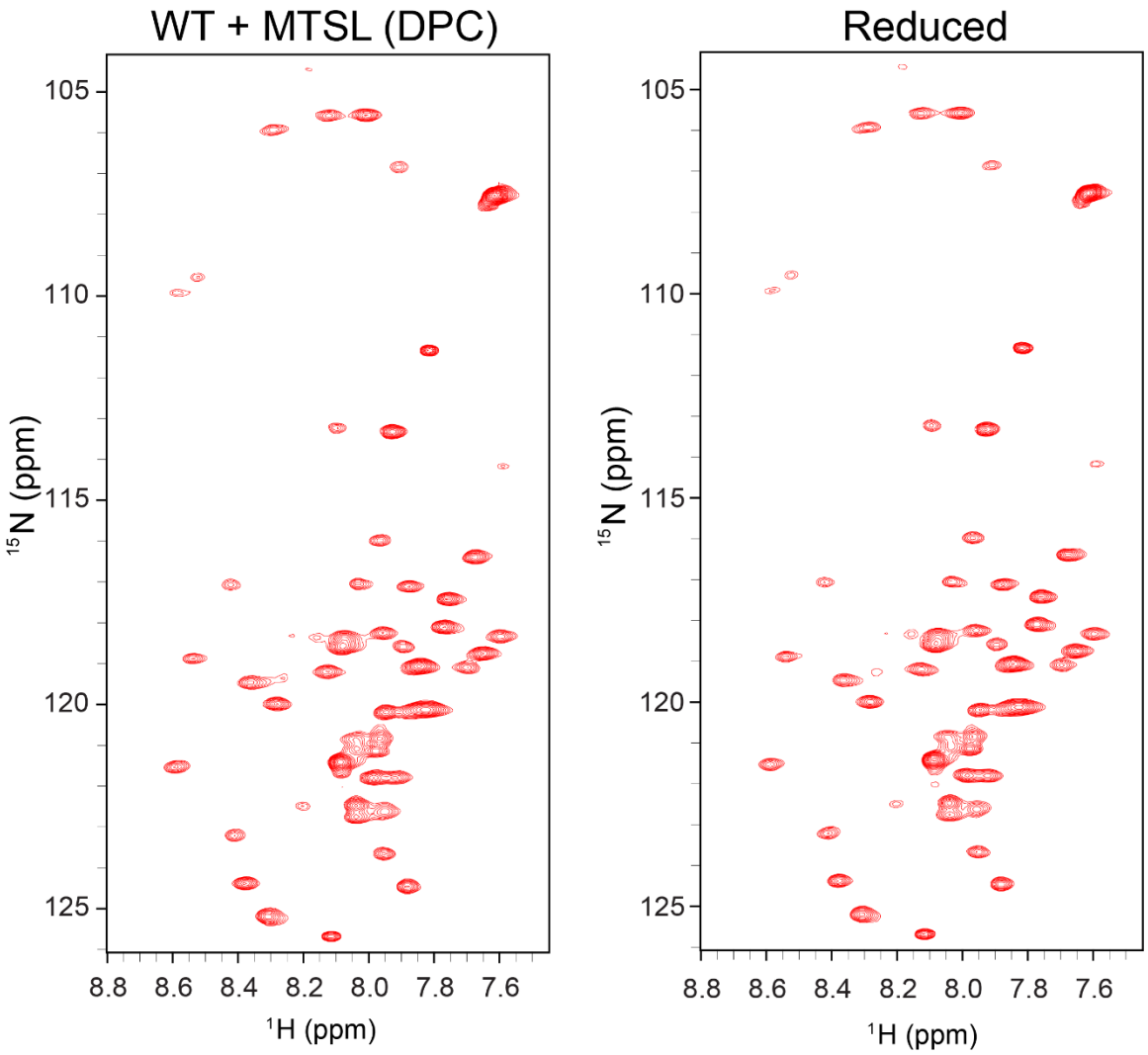

Figure S8. 2D TROSY-HSQC spectra of non-mixed p7 sample (without any cysteines) in DPC micelles before (left) and after (right) reducing the nitroxide with ascorbic acid. The DPC-reconstituted (^{15}N, 80% ^{2}H)-labeled p7(5a) was treated with exactly the same MTSL labeling and cleaning procedures used for other mixed samples in Figs. S1-S4. The spectra were recorded at 600 MHz ^{1}H frequency.