Supporting Information

Challenging the Limit: NMR Assignment of a 31 kDa Helical Membrane Protein

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Table of Content

Experimental details

Overexpression and purification of ^{2}H, ^{13}C, ^{15}N-labeled C-terminal domain of Stt3p

Recombinant uniformly triple labeled (^{2}H, ^{13}C, ^{15}N-labeled) C-terminal domain of Stt3p was expressed in *E. coli* BL21 (DE3) codon plus cells using pET-28c vector that contained an IPTG-inducible gene for the protein containing an N-terminal hexa-histidine tag. The transformed cells were grown in 3 mL of LB medium at 37 ℃ for 3 h, then transferred to 12.5 mL of unlabeled minimal M9/H_2O medium, and grown until an OD_{600} of ~ 0.5. The cells were then separated from the medium by centrifugation at 3,000 rpm for 15 minutes and transferred to 50 mL of M9/D_2O culture containing 0.12% (m/v) of $^{15}NH_4Cl$ as the sole nitrogen source and 0.4% (m/v) of ^{13}C-labeled glucose as the sole carbon source. At $OD_{600} \approx 0.5$, the culture was diluted to 300 mL with M9/D_2O. The expression of the His-tagged protein was induced at OD_{600} ~ 0.4 with 0.5 mM IPTG, and the culture was allowed to grow for an additional 11-12 h at 30 ℃ (final OD_{600} ~ 2.0). The cells were harvested by centrifugation. The cell pellet was passed through 4 cycles of freeze-thaw using liquid nitrogen and ice respectively and resuspended in B-PER solution (Pierce). The cells were then lysed by sonication (10 × 15 s) and the supernatant was removed after centrifugation at 10,000 × g for 30 min. The pellet was resuspended once with 10% B-PER solution, sonicated and centrifuged again as above. The inclusion bodies were stored at -20 ℃ until needed.

The purification of the triple labeled recombinant protein was carried out as reported recently.[1]

NMR samples and data collection

NMR samples contain 25 mM sodium phosphate buffer (pH 6.5), 1% (v/v) glycerol, 100 mM sodium dodecyl-d25 sulfate (SDS, Sigma-Aldrich), 1 mM EDTA, 10% D_2O. Protein concentration

was ~ 0.6 mM. Higher concentration of protein led to deterioration of spectral quality, presumably due to protein aggregation.

TROSY-based HNCA, HNCO, HN(CO)CA, HN(CA)CO, HNCACB, and CT- HN(CO)CACB were collected on a Bruker Avance 600-MHz spectrometer fitted with a triple resonance 1H/13C/15N TCI cryoprobe equipped with z-axis pulsed field gradients in the Department of Chemistry and Biochemistry, Auburn University. Moreover, TROSY-based HN(CA)CB and HN(CA)CO were also collected on a Varian Inova 900-MHz NMR spectrometer equipped with a triple resonance cold probe at the Southeast Collaboratory for High-Field Biomolecular NMR, a research resource at the University of Georgia.

NMR data processing and analysis

All NMR spectra were processed and analyzed using NMRPipe[2] and NMRView software.[3] Nearly complete NMR assignment of ^{1}H, ^{13}C, and ^{15}N backbone resonances (255 out of 274 residues) was achieved for the C-terminal domain of Stt3p with the exception of 19 residues including 11 N-terminal residues located near the hexa-histidine tag (M1-H9 and S19-H20) and eight other residues (L561, K562, I584, N585, I591, P559, P661 and S702). Residue P661 cannot be assigned because the residue 662 is also a proline. We observed that the N-terminal residues around the hexa-histidine tag are very weak and broad similar to what has been seen for other proteins (BMRB accession# 5833).[4] The unassigned residues could not be detected probably due to local exchange-induced line-broadening.

References

(1) Huang, C.; Mohanty, S.; Banerjee, M. *Biochemistry*. **2010**, *49*, 1115-1126.

(2) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G., Pfeifer, J.; Bax, A. *J. Biomol. NMR*. **1995**, *6*, 277-293.

(3) Johnson, B. A.; Blevins J. *J. Biomol. NMR*. **1994**, *4*, 603-614.

(4) Ponchon, L.; Dumas, C.; Fesquet, D.; Padilla, A. *J. Biomol. NMR*., **2004**, *28*, 299-300

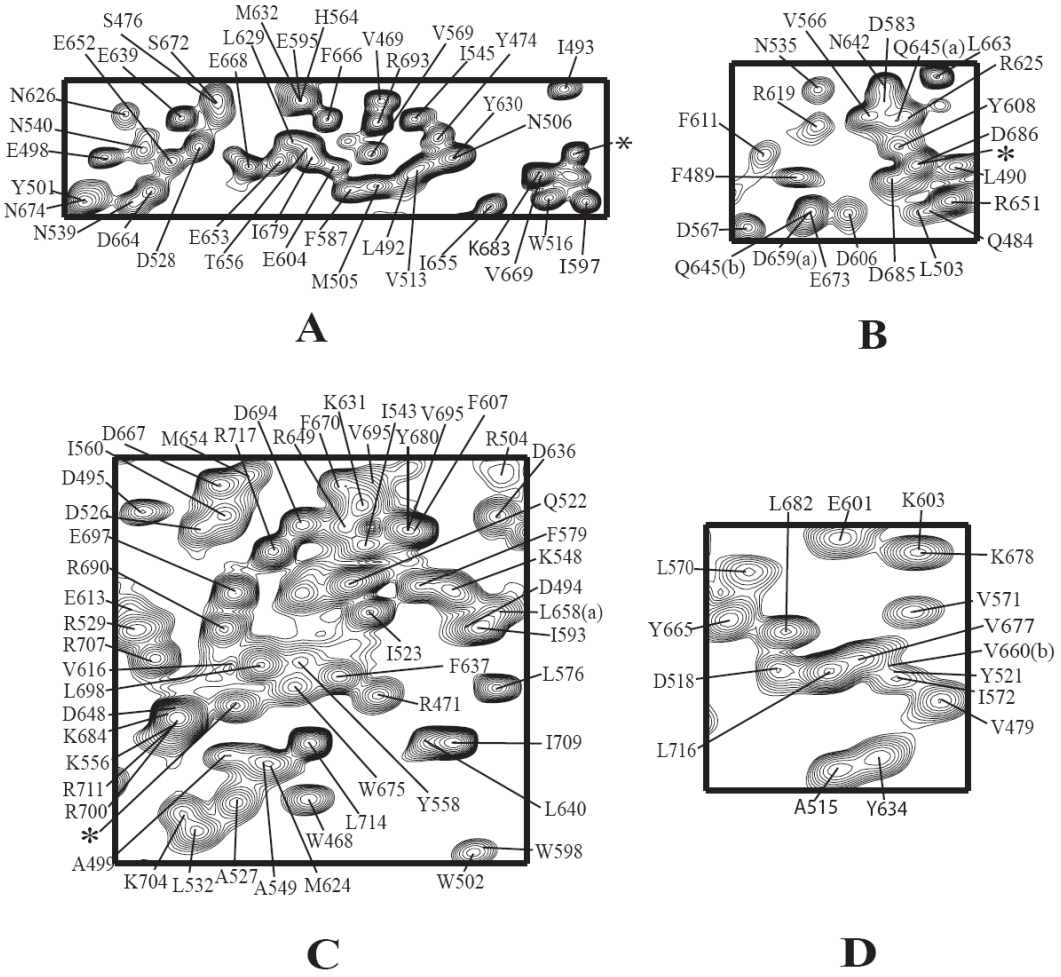

Figure S1. Four regions, labeled as A, B, C and D, of the ^{15}N-^{1}HN TROSY-HSQC spectrum (Figure 1 in the main text) of U-{^{15}N, ^{13}C, ^{2}H}-labeled C-terminal domain of Stt3p are expanded and peaks are labeled with residue numbers.

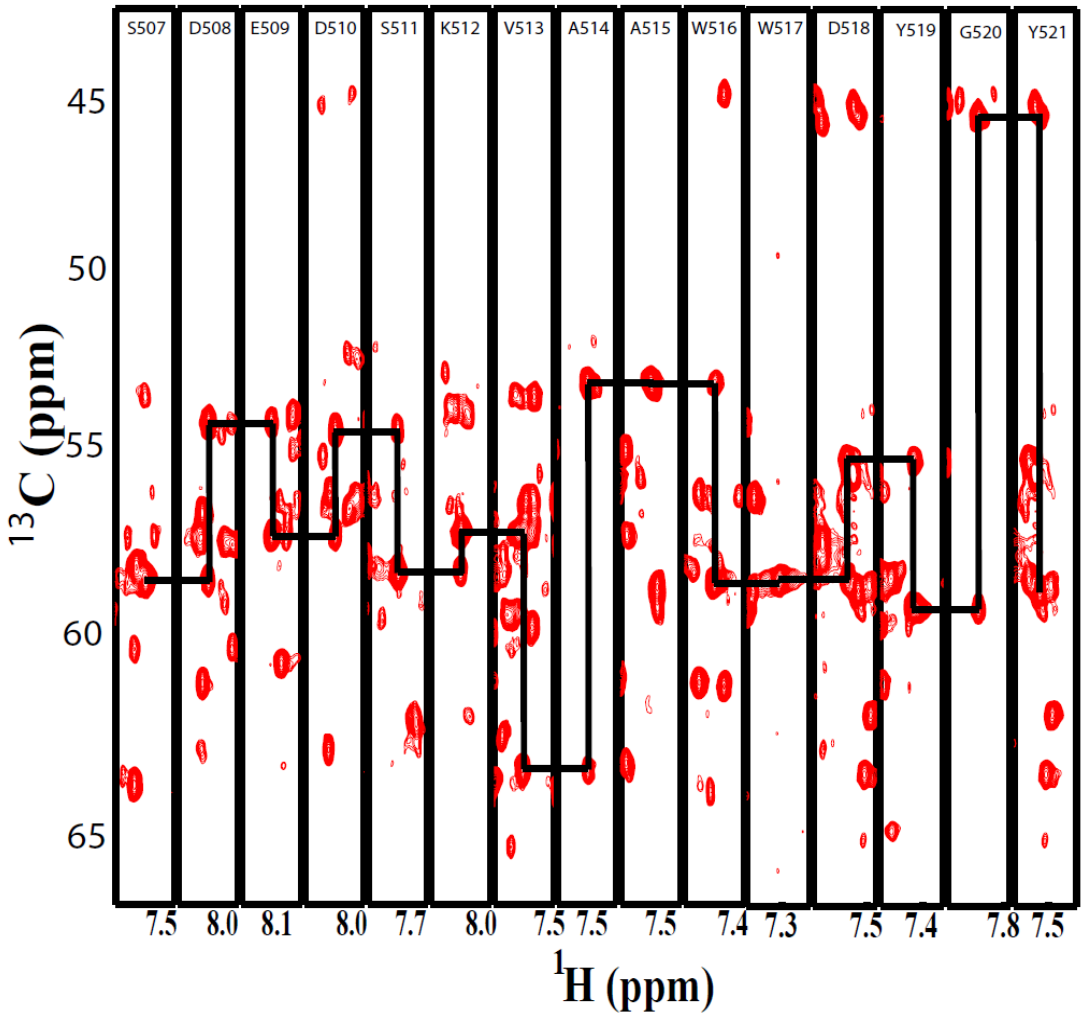

Figure S2. [^{1}H, ^{13}C]-strips for residue S^{507}-Y^{521} from 3D HNCA spectrum of U-{^{15}N, ^{13}C, ^{2}H}-labeled C-terminal domain of Stt3p showing sequential assignment. Residues are labeled and numbered on the top of each strip.

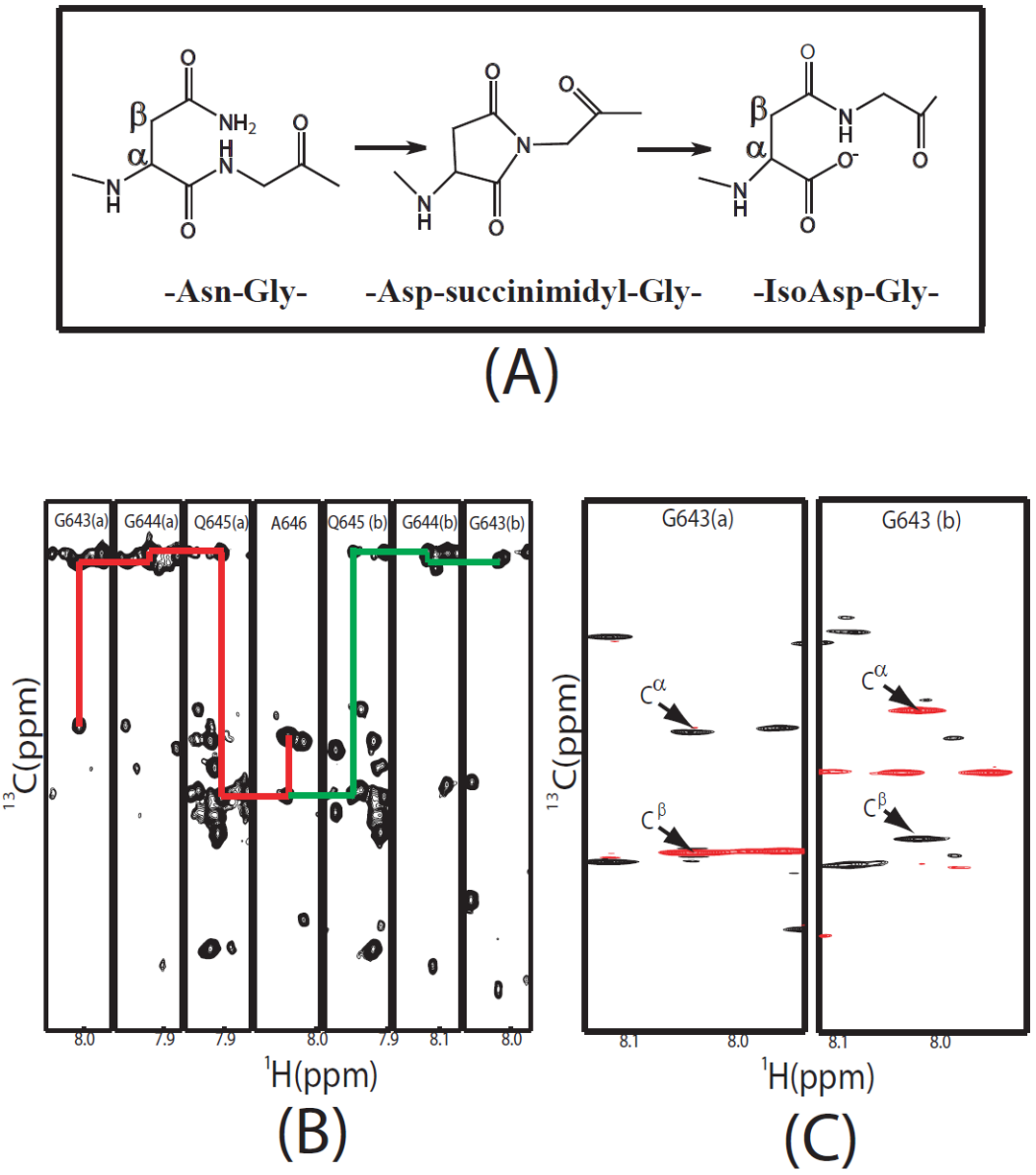

Figure S3. (A) The deamidation of the N^{642} side chain resulting in the isoasparaginyl linkage between the N^{642}-G^{643} pair. (B) Two sets of HNCA spectrum strip plots of assignments for residues G^{643}-Q^{645} (shown as red and green line connectivity respectively). These two sets of assignment correspond to the residues following the –Asn-Gly- (residues connected by red lines) and –IsoAsp-Gly- linkages (residues connected by green lines) respectively. (C) CT-HN(CO)CACB strip plot of the two assigned G^{643} residue. Negative peaks are shown in red while positive peaks are shown in black. Note in the left strip, the negative sign of C^{α} and positive sign of C^{β} for residue N^{642} are unambiguously indicative of –IsoAsp-Gly- linkage between the N^{642}-G^{643} pair; while the right strip demonstrate a normal –Asn-Gly- linkage (positive sign for C^{α} and negative sign for C^{β}).

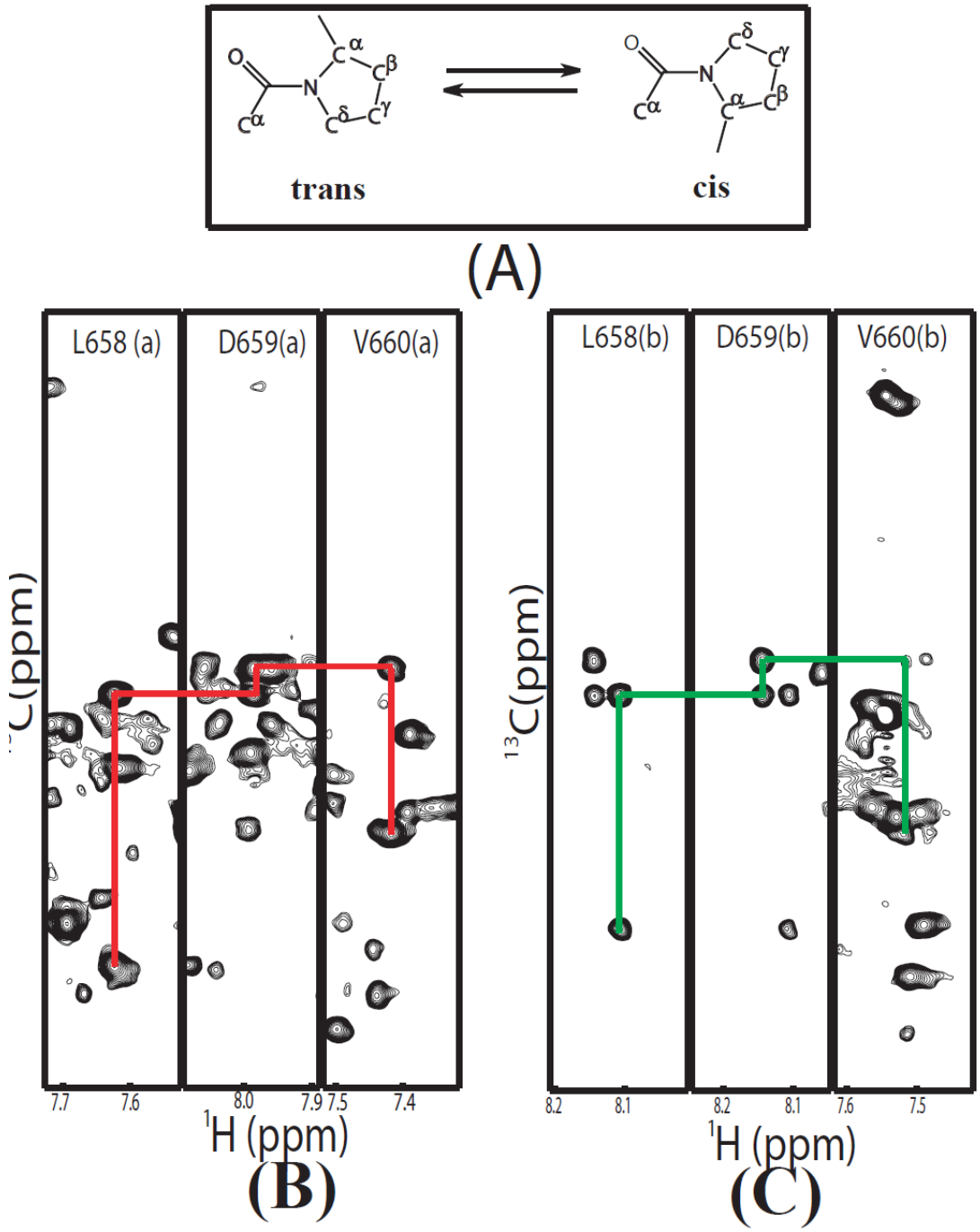

Figure S4. Identification of proline trans/cis isomerizational linkage in the C-terminal domain of Stt3p. (A) proline trans/cis isomerization. (B) and (C) are two sets of HNCA spectrum strip plots of assignments for residues L^{658}-V^{660}, indicating peptide bond V^{660}-P^{661} adopts both trans and cis isomers.