NMR Solution Structure of an Invisible Protein State at the Edge between Folding and Aggregation into Amyloid Fibrils

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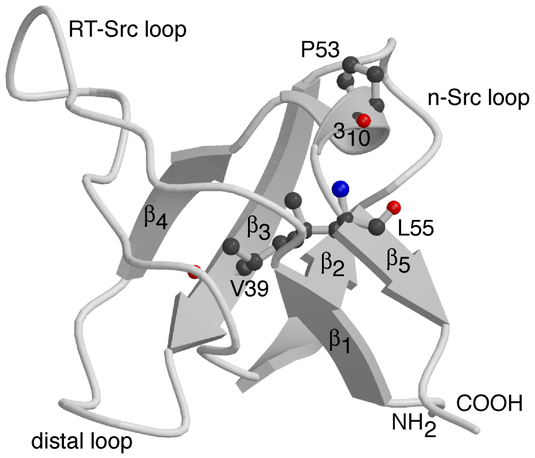
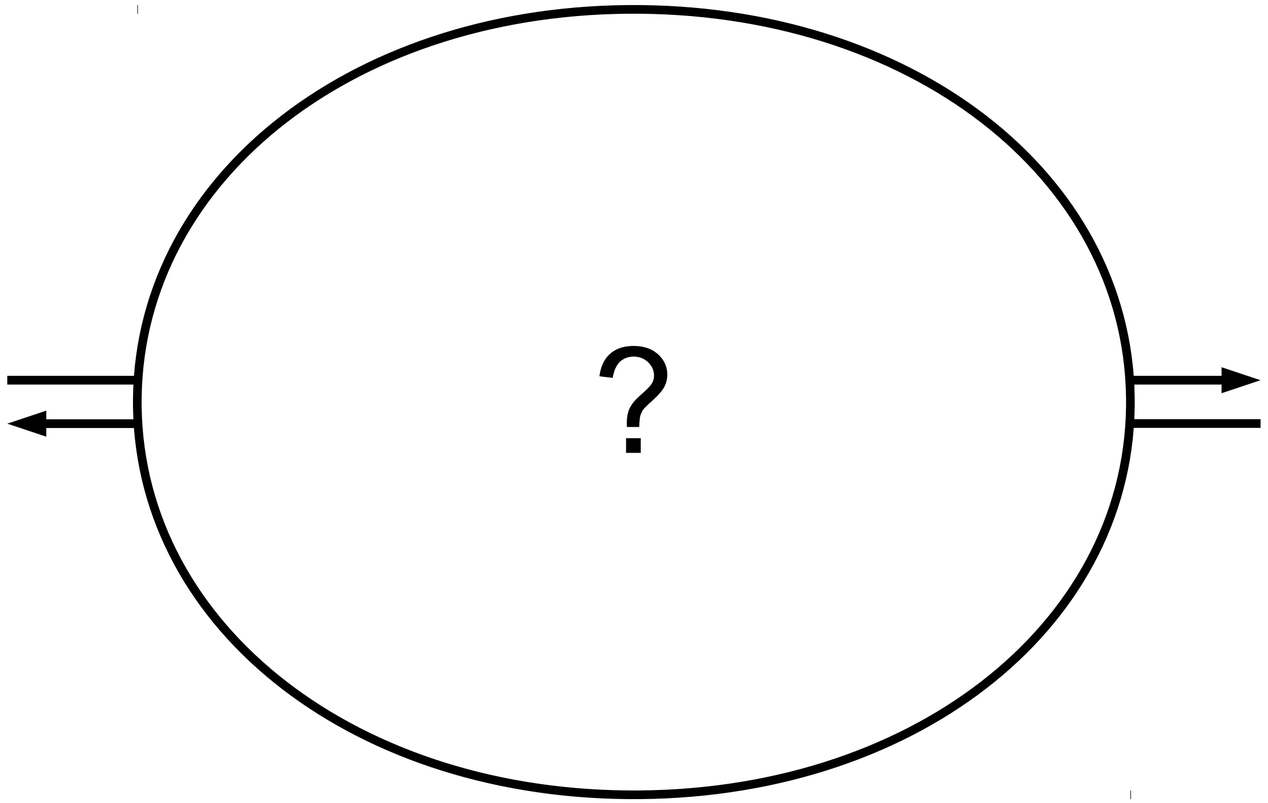
Protein Folding Pathways

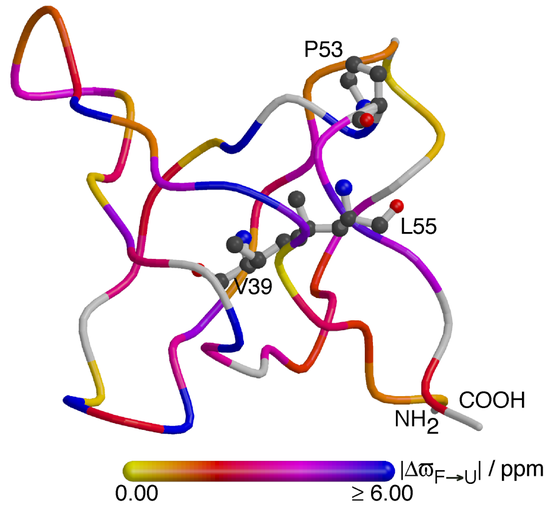
Protein folding is not a random combinatorial search
(Anfinsen, *Science* **181**, 223-230 (1973))

⇒ folding pathways with transient intermediates, rate-limiting transition states



native state

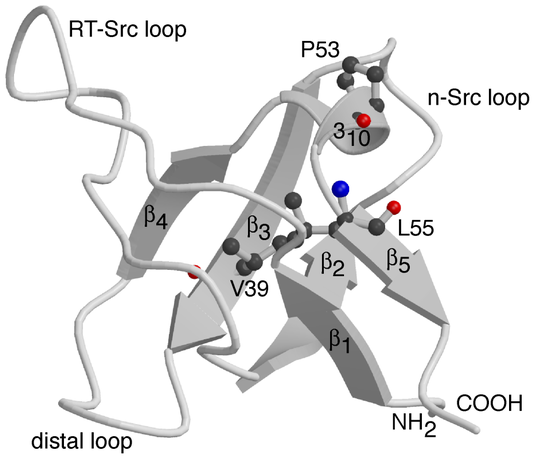




unfolded state

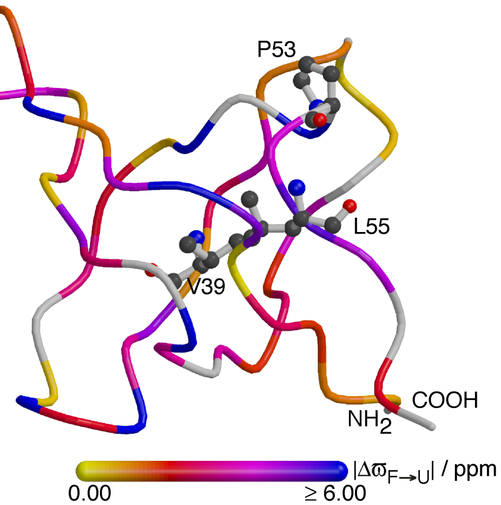
Folding Intermediates and Misfolding Diseases

Folding intermediates are suspected to be the precursors that initiate aggregation into the amyloid fibrils associated with many neurodegenerative diseases
(Dobson, *Nature* **426**, 884-890 (2003); Jahn et al. & Radford, *NSMB* **13**, 195-201 (2006))

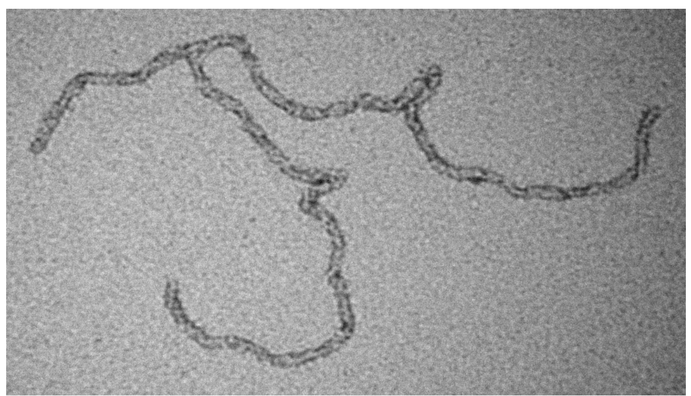


native state

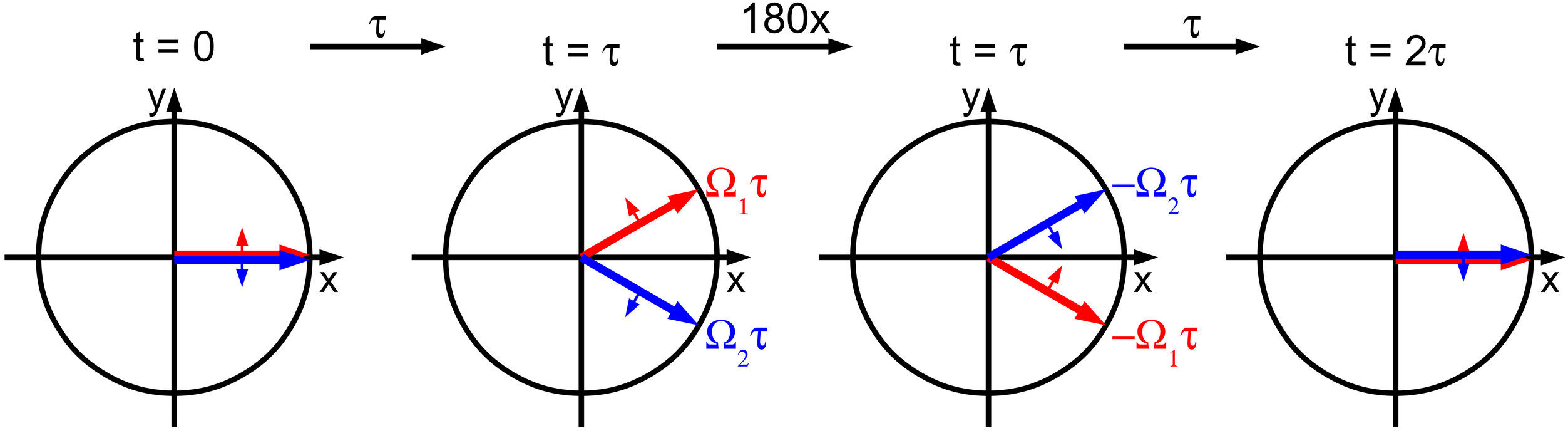
?



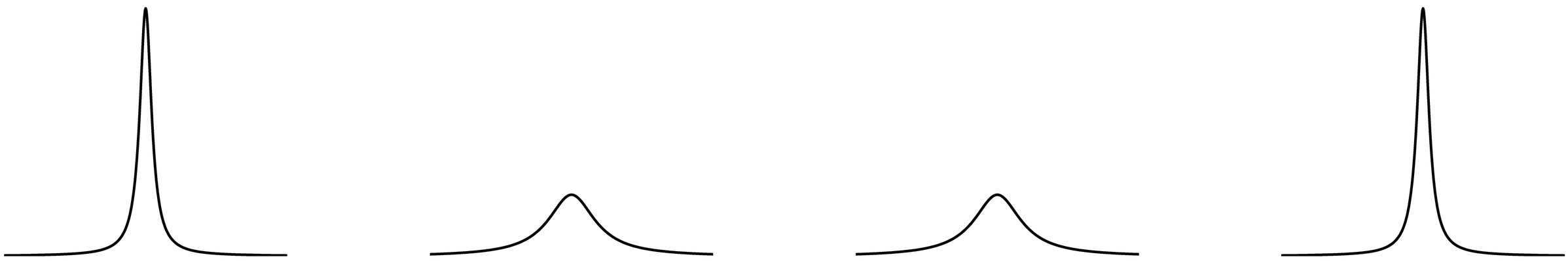
unfolded state



Hahn Echo

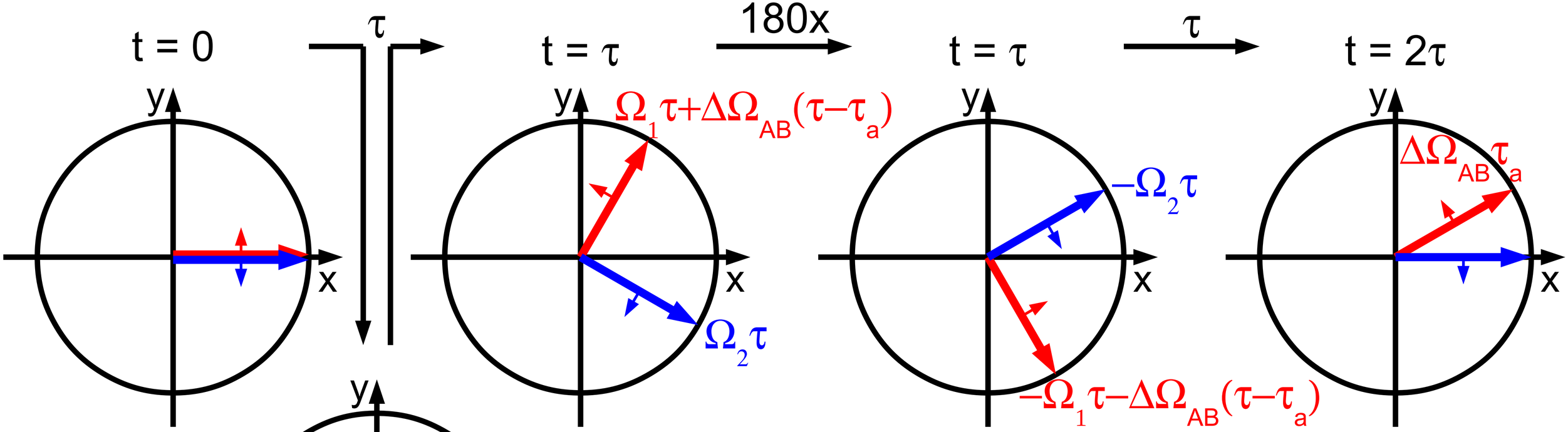


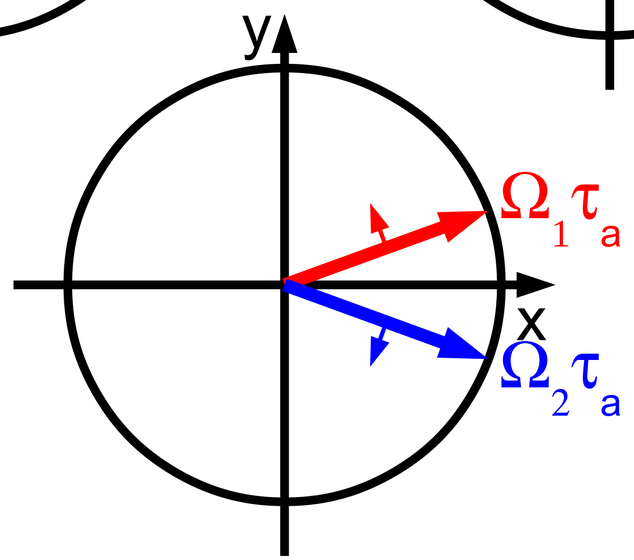
Spectrum:



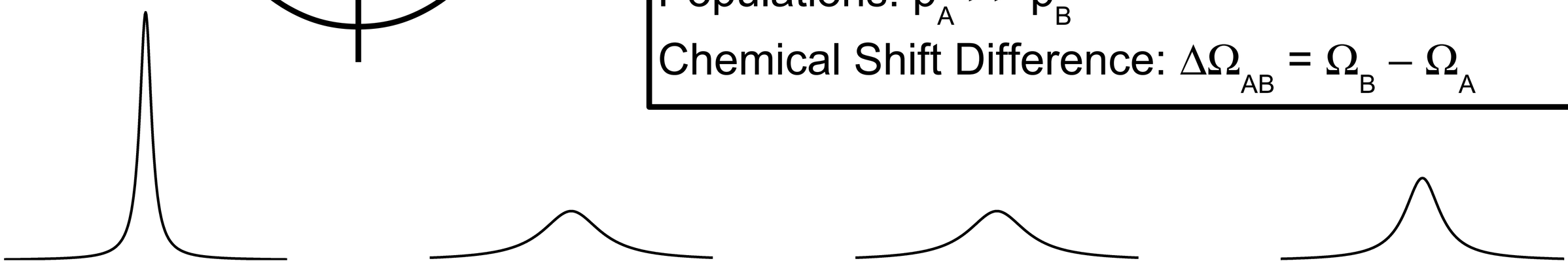
⇒Signal is refocused!

Hahn Echo in the Presence of Chemical Exchange





Spectrum:



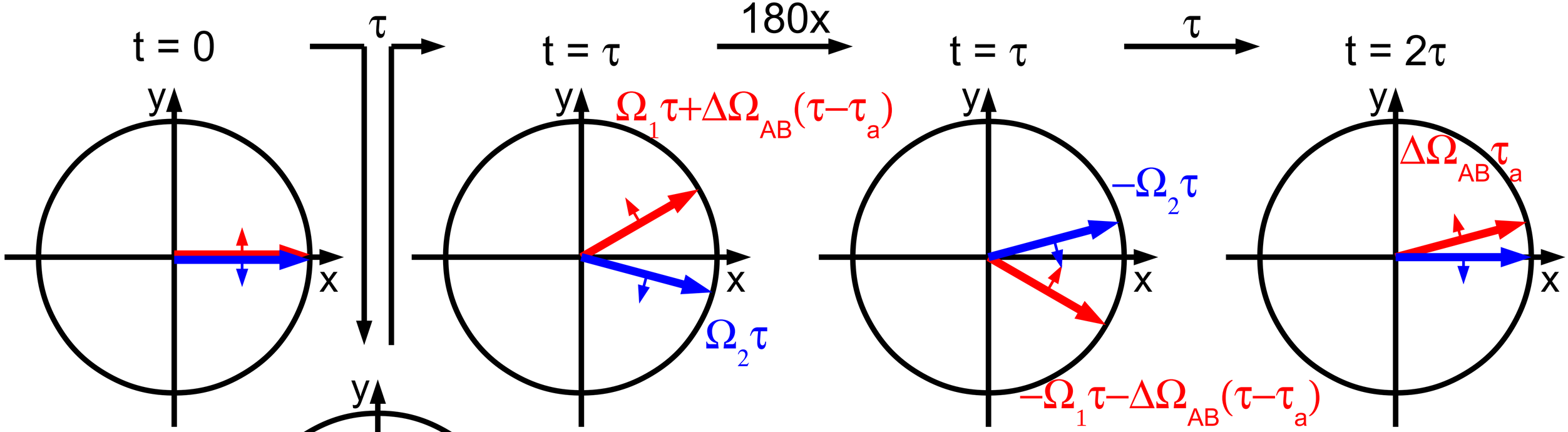
Chemical Exchange: $A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} B$, $k_{ex,AB} = k_{AB} + k_{BA}$

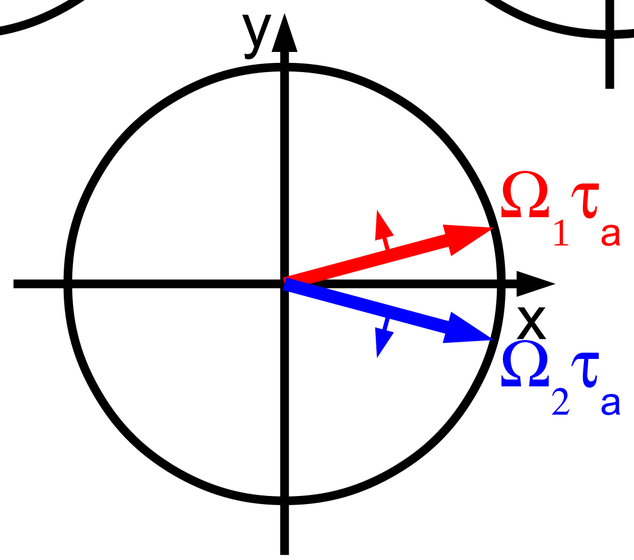
Populations: $p_A >> p_B$

Chemical Shift Difference: $\Delta\Omega_{AB} = \Omega_B - \Omega_A$

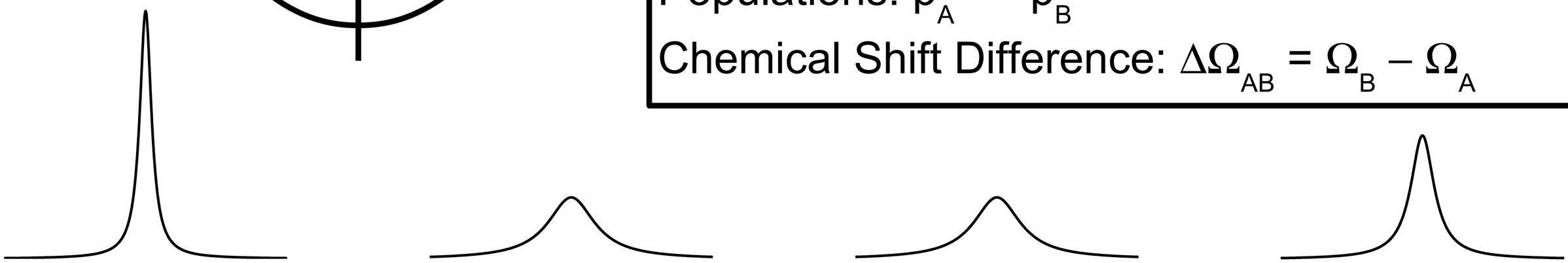
⇒Signal refocusing is incomplete!
Minor state B practically invisible!

Effects of Faster Pulsing (shorter delay τ)





Spectrum:



Chemical Exchange: $A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} B$, $k_{ex,AB} = k_{AB} + k_{BA}$

Populations: $p_A >> p_B$

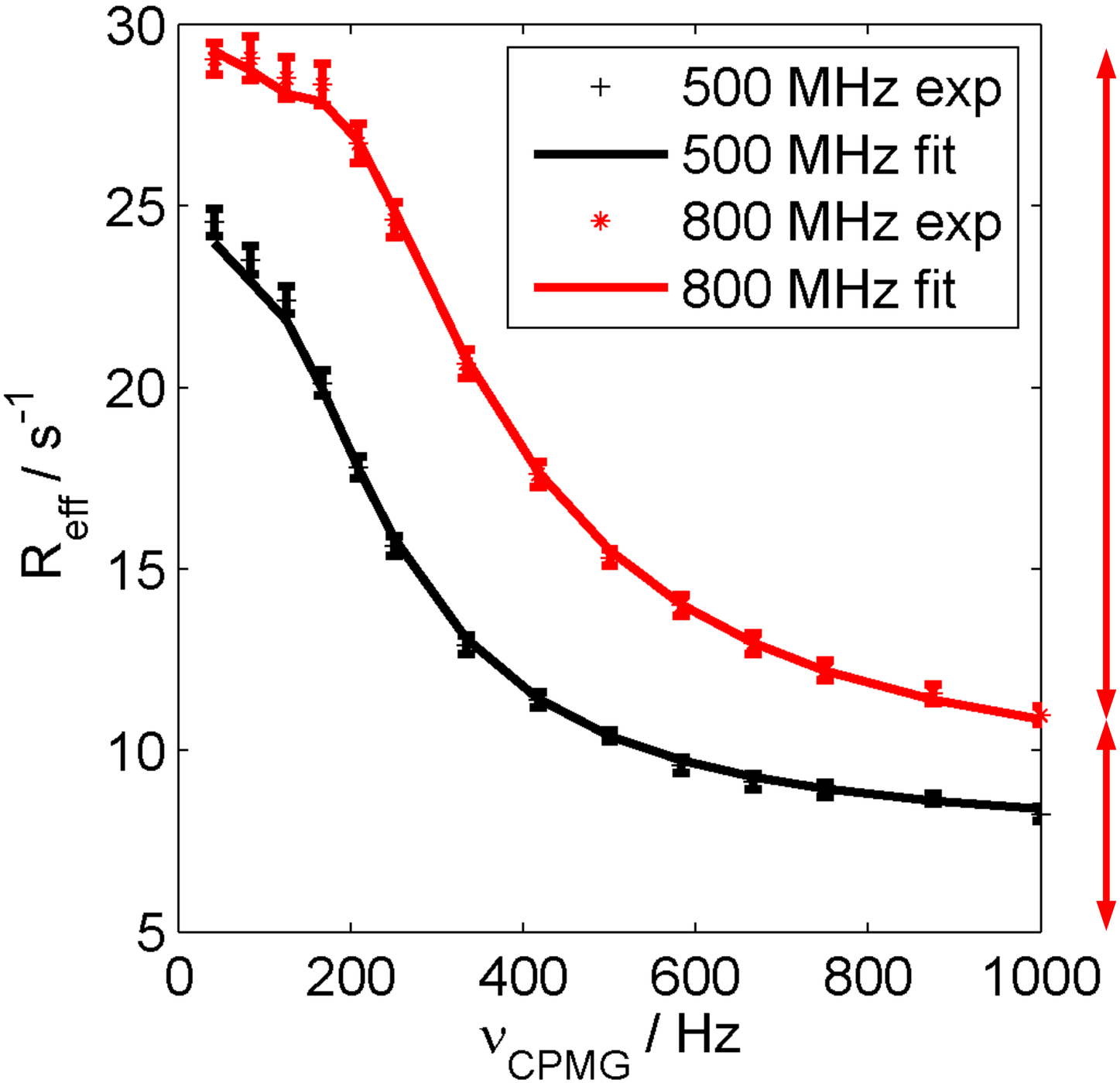
Chemical Shift Difference: $\Delta\Omega_{AB} = \Omega_B - \Omega_A$

⇒Signal refocusing is higher!

Minor state B practically invisible!

CPMG Relaxation Dispersion Spectroscopy

Effective transverse relaxation rate R_{eff} as a function of the CPMG pulsing frequency $\nu_{CPMG} = 1/4\tau$

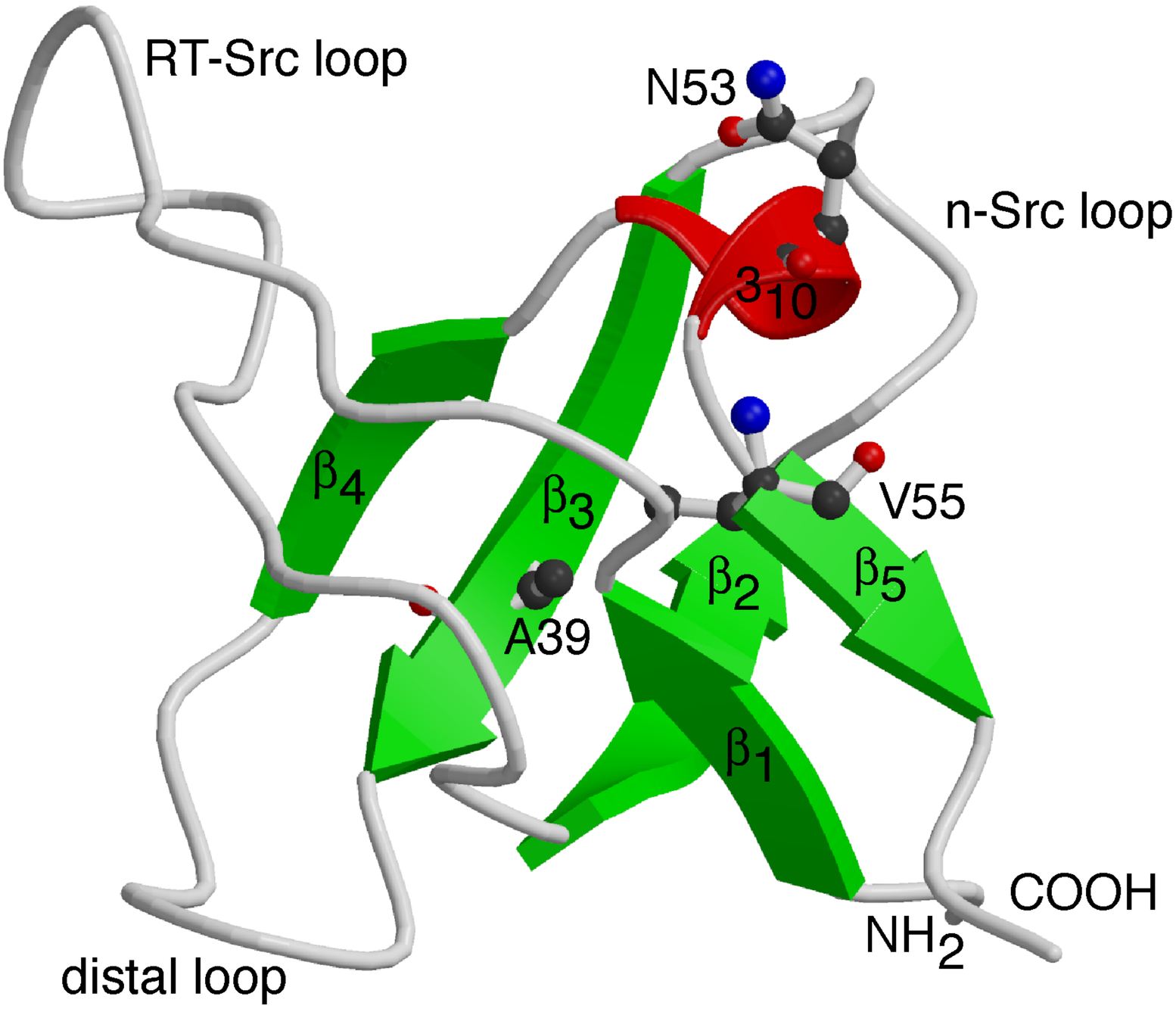


exchange contribution R_{ex}

intrinsic R_2

$R_{ex} = R_{ex}(k_{ex,AB}, p_B, \Delta\Omega_{AB}, \nu_{CPMG})$
(Bloch-McConnell equations)

The Fyn SH3 Domain Mutant A39V/N53P/V55L



Mutations:

N53P:
surface mutation to study 3_{10} helix propensities

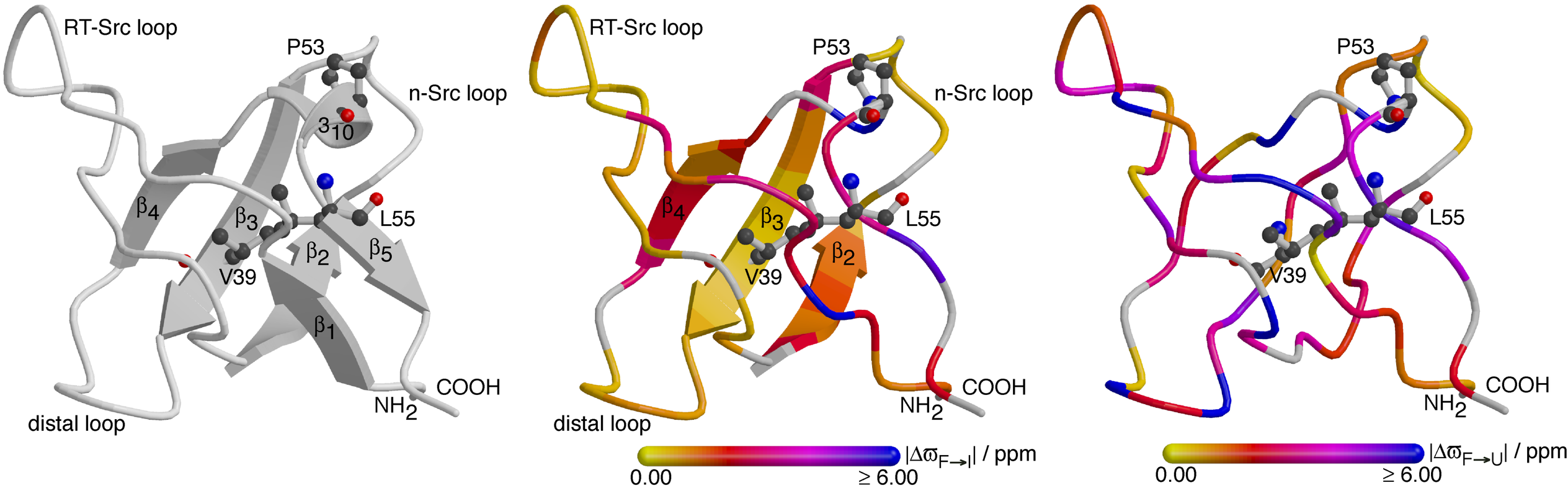
A39V/V55L:
hydrophobic core mutations that speed up folding by an order of magnitude:

wt:	≈ 30/s
A39V/N53P/V55L:	≈ 800/s

⇒ ideal for CPMG studies

Folding Pathway of the Fyn SH3 A39V/N53P/V55L

at 35°C



native state (ground state) 97.6%

$\rightleftharpoons$ 51/s / 2951/s

intermediate state (excited state) 1.7%

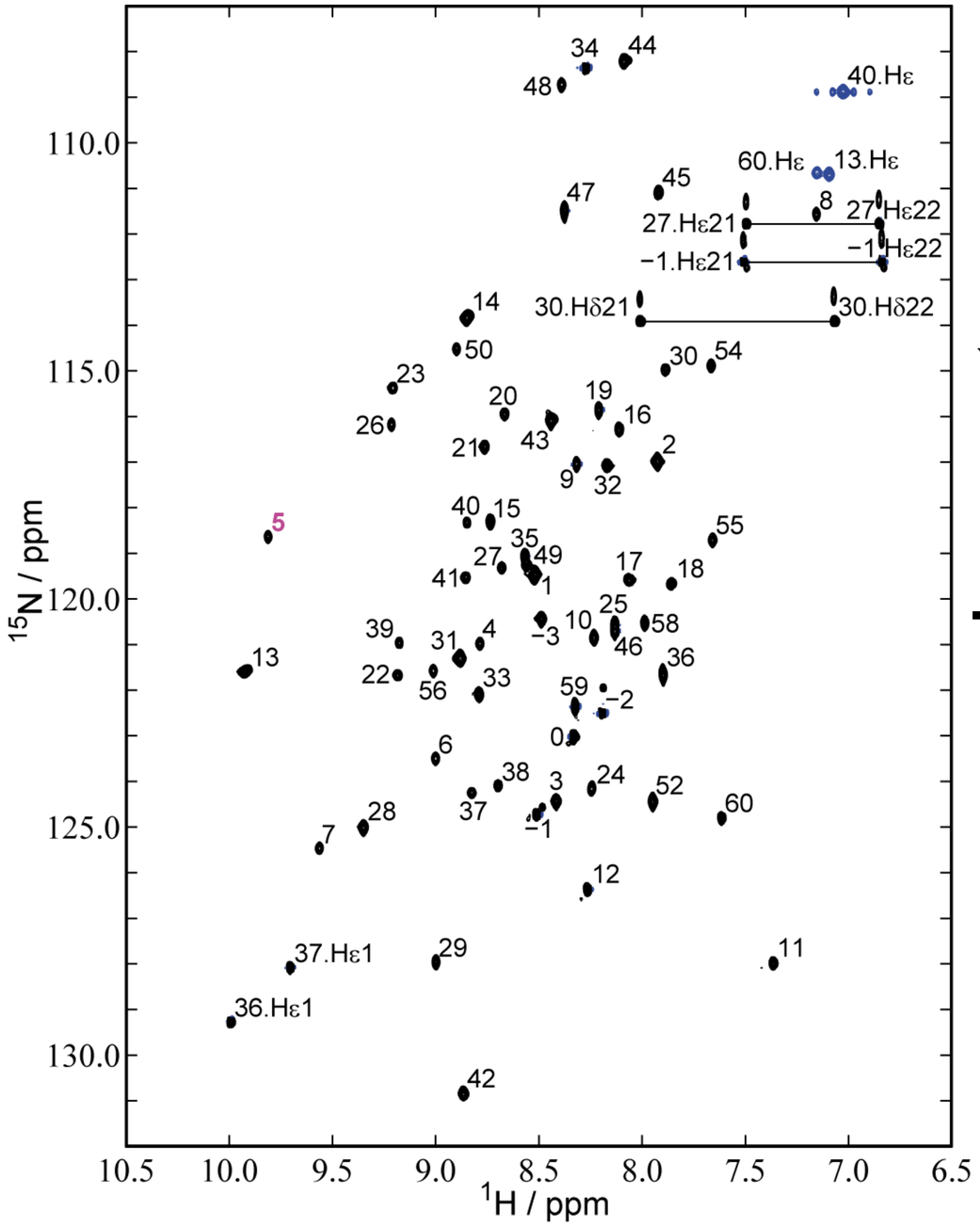
$\rightleftharpoons$ 389/s / 924/s

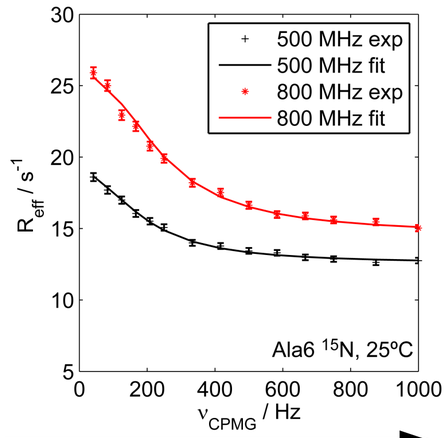
unfolded state (excited state) 0.7%

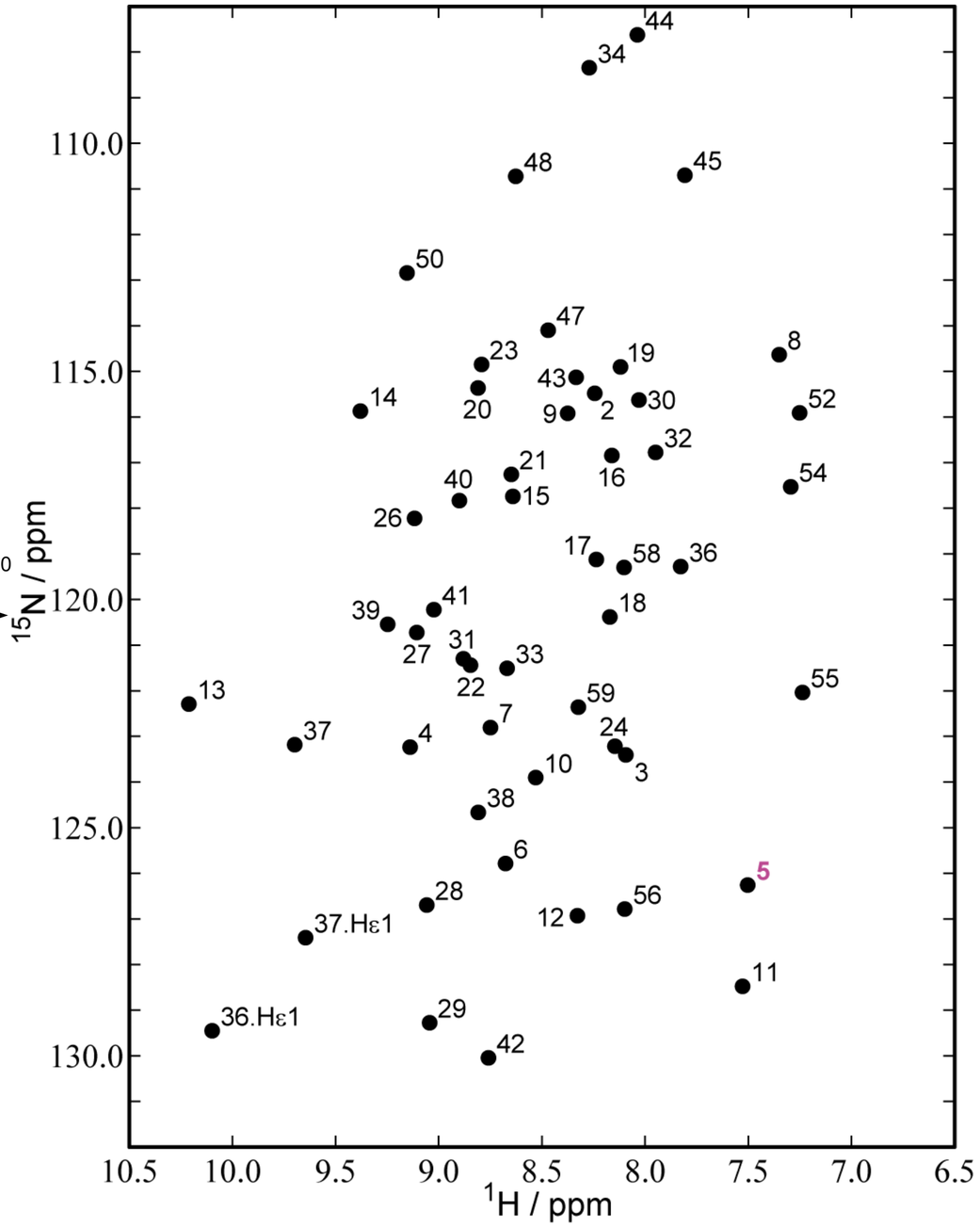
(Neudecker et al. & Kay, *J. Mol. Biol.* **363**, 958-976 (2006))

$\Rightarrow$ kinetics, thermodynamics, and structural changes of 3-state exchange even if the excited states are populated to only 1%.

Reconstruction of “Invisible” NMR Spectra from CPMG for the Intermediate of the Fyn SH3 A39V/N53P/V55L



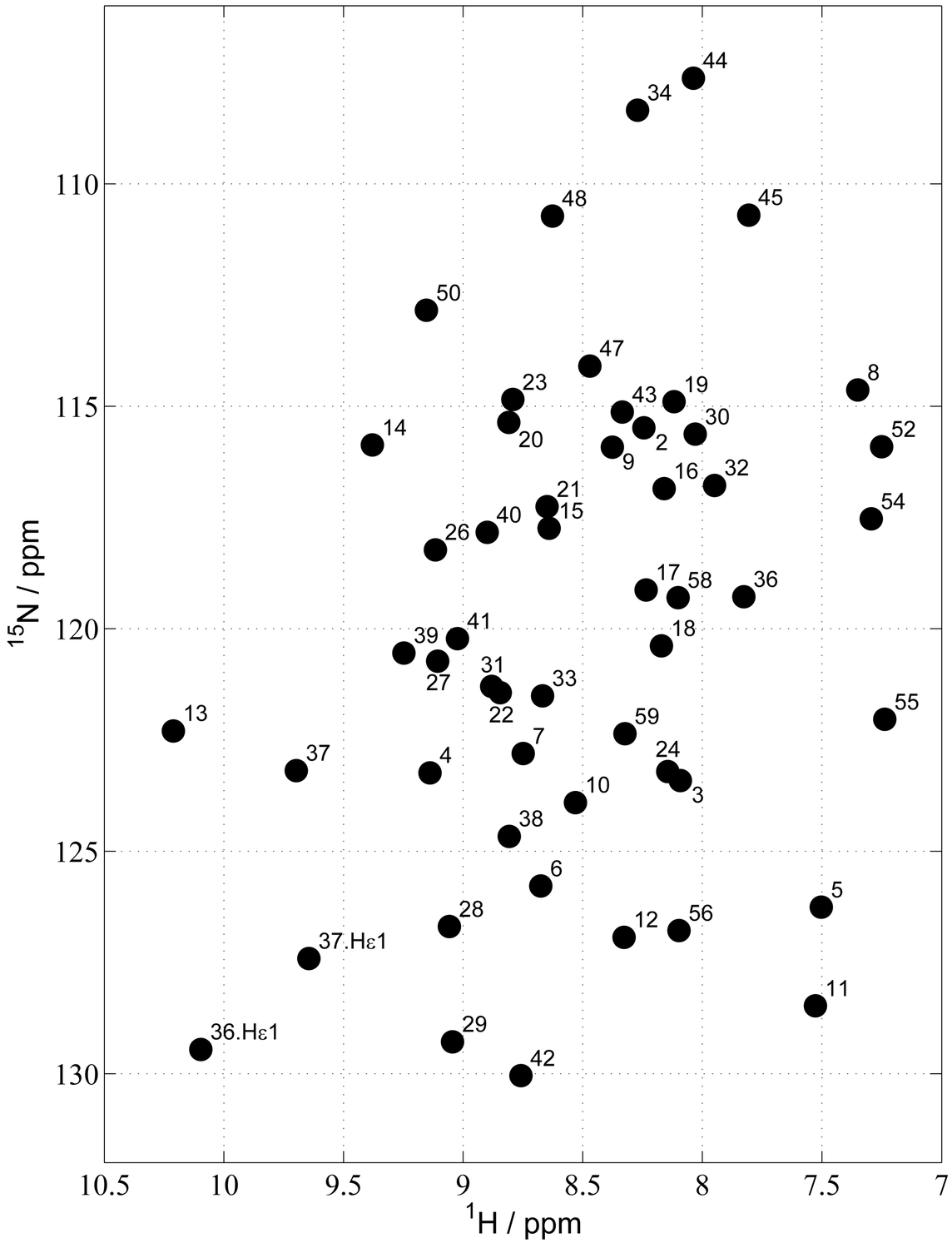




Native State F (98%)
ground state, directly visible

Intermediate State I (2%)
excited state, “invisible”, accessible via line broadening of ground state

High-Resolution Structural Information from CPMG for the Intermediate of the Fyn SH3 A39V/N53P/V55L



Reconstructed sequence-specific chemical shift assignments:

241 of 292 backbone ^{15}N, ^{1}HN, ^{13}CO, ^{13}Cα, ^{1}Hα (83% complete)

25 methyl group $^{13}CH_3$

Residual anisotropic interactions:

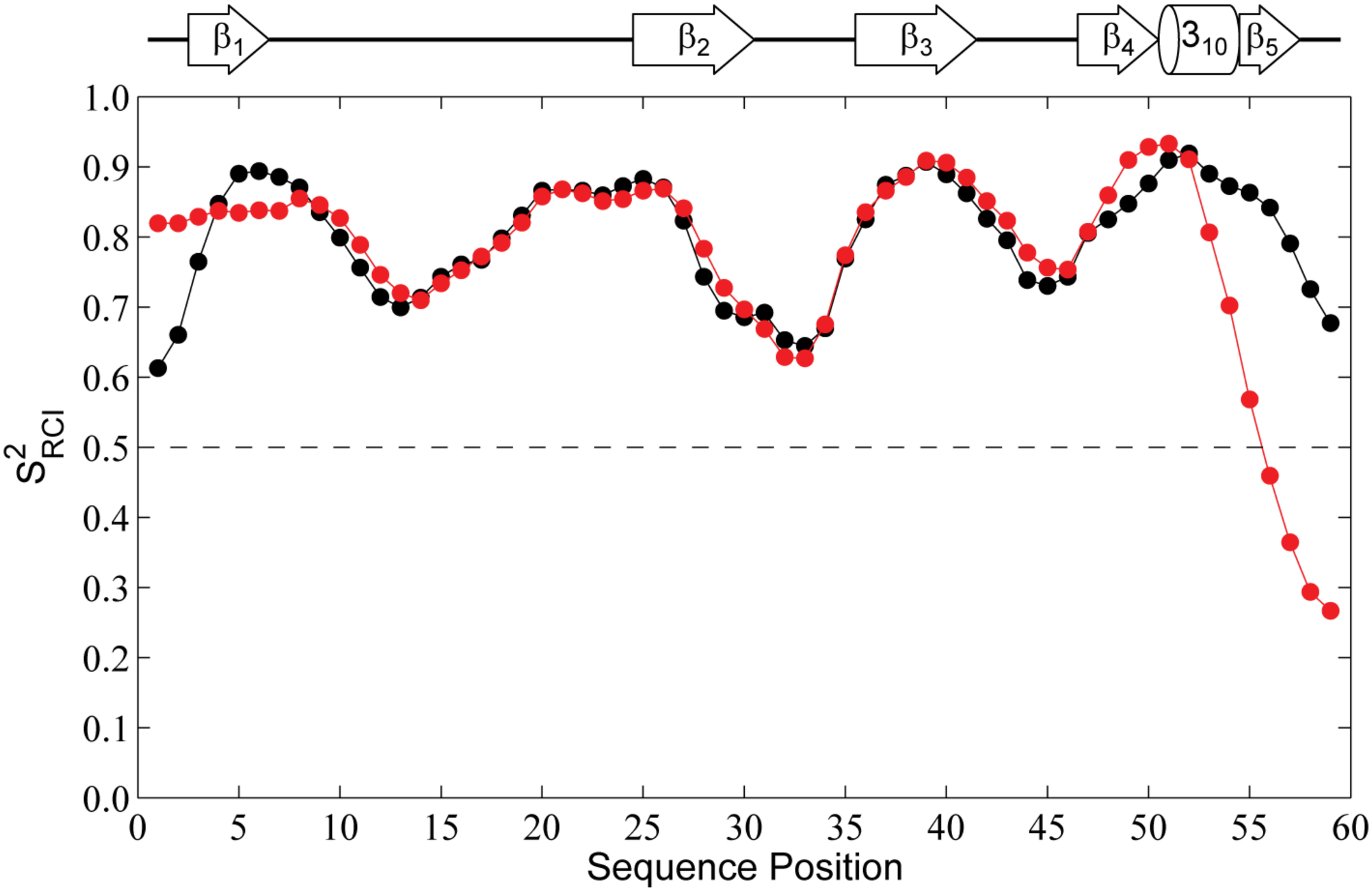
49 D_{NH} RDCs in 24 mg/ml Pf1

46 D_{NH} RDCs in PEG/hexanol

35 ^{13}CO RCSAs in 36 mg/ml Pf1

(43% higher alignment than the D_{NH} RDCs in 24 mg/ml Pf1)

Deviation of the Native State and the Intermediate from Random Coil Chemical Shifts

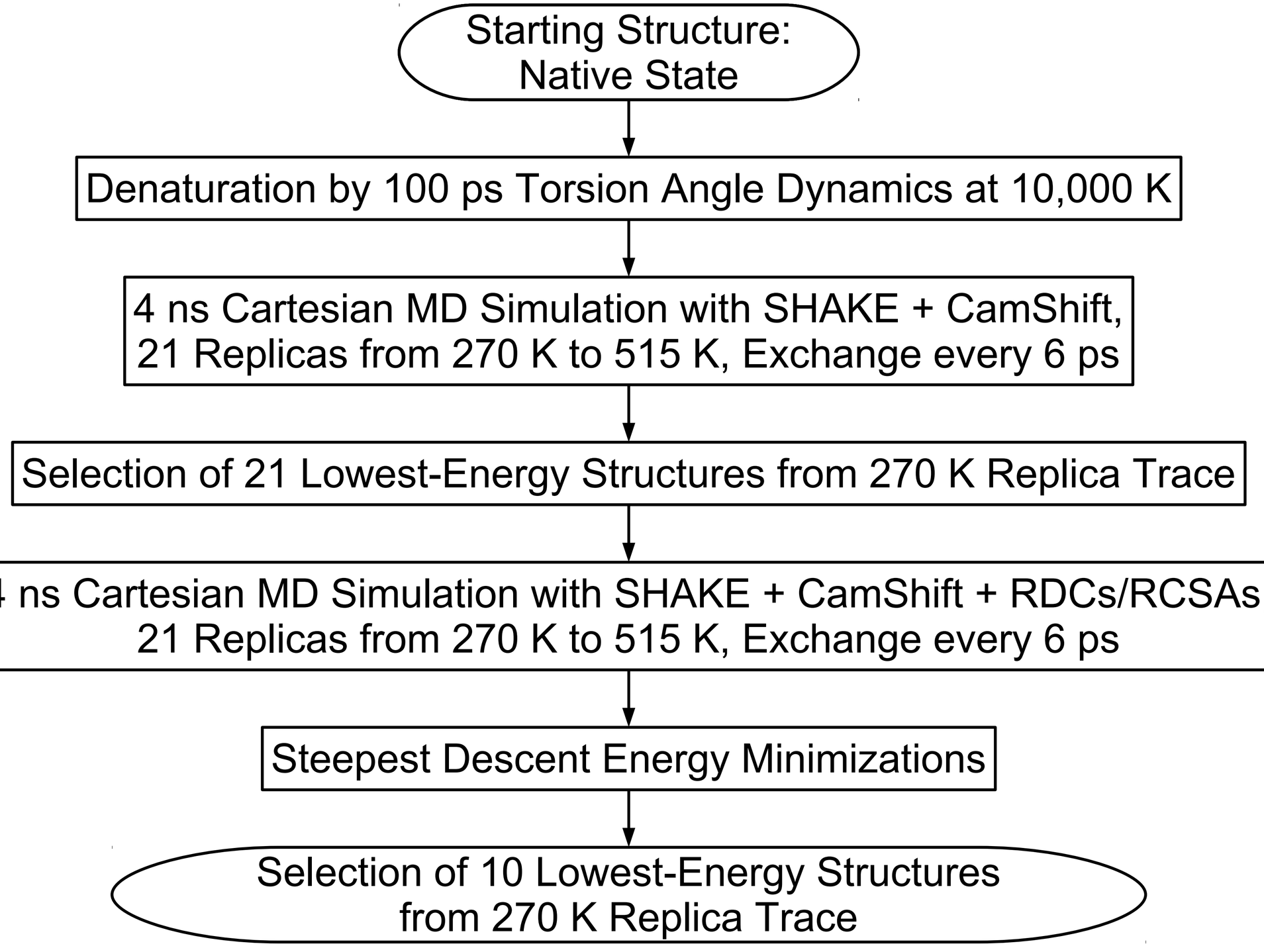


⇒ COOH-terminus is disordered in the intermediate, strand β_5 not yet formed

CamShift Structure Calculation Protocol

Strategy:
Replica Exchange Molecular Dynamics (MD) Simulation

Force Field:
+ AMBER-03
+ CamShift
+ X-PLOR-style flat-bottom harmonic potential wells for RDCs and RCSAs



(Kohlhoff, Robustelli, Cavalli et al. & Vendruscolo, *JACS* **131**, 13894-13895 (2009);
Robustelli, Kohlhoff, Cavalli & Vendruscolo, *Structure* **18**, 923-933 (2010))

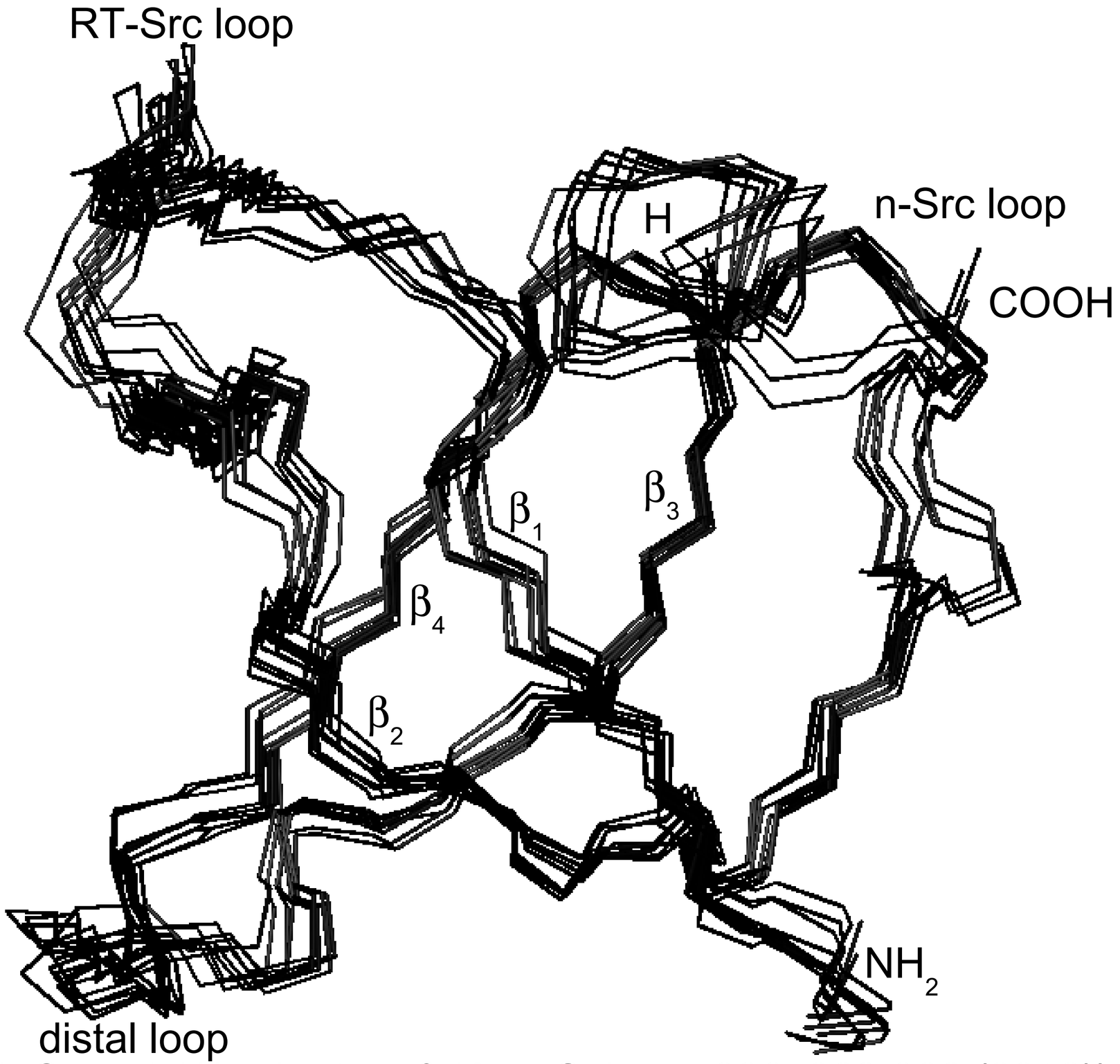
Structure of the Intermediate Determined by CPMG

Backbone overlay of the 10 accepted structures of the intermediate state calculated from CPMG relaxation dispersion experiments (PDB 2L2P)

RMSDs from the average structure for residues 2..55:

Backbone:
0.59 Å ± 0.17 Å

All heavy atoms:
1.12 Å ± 0.23 Å

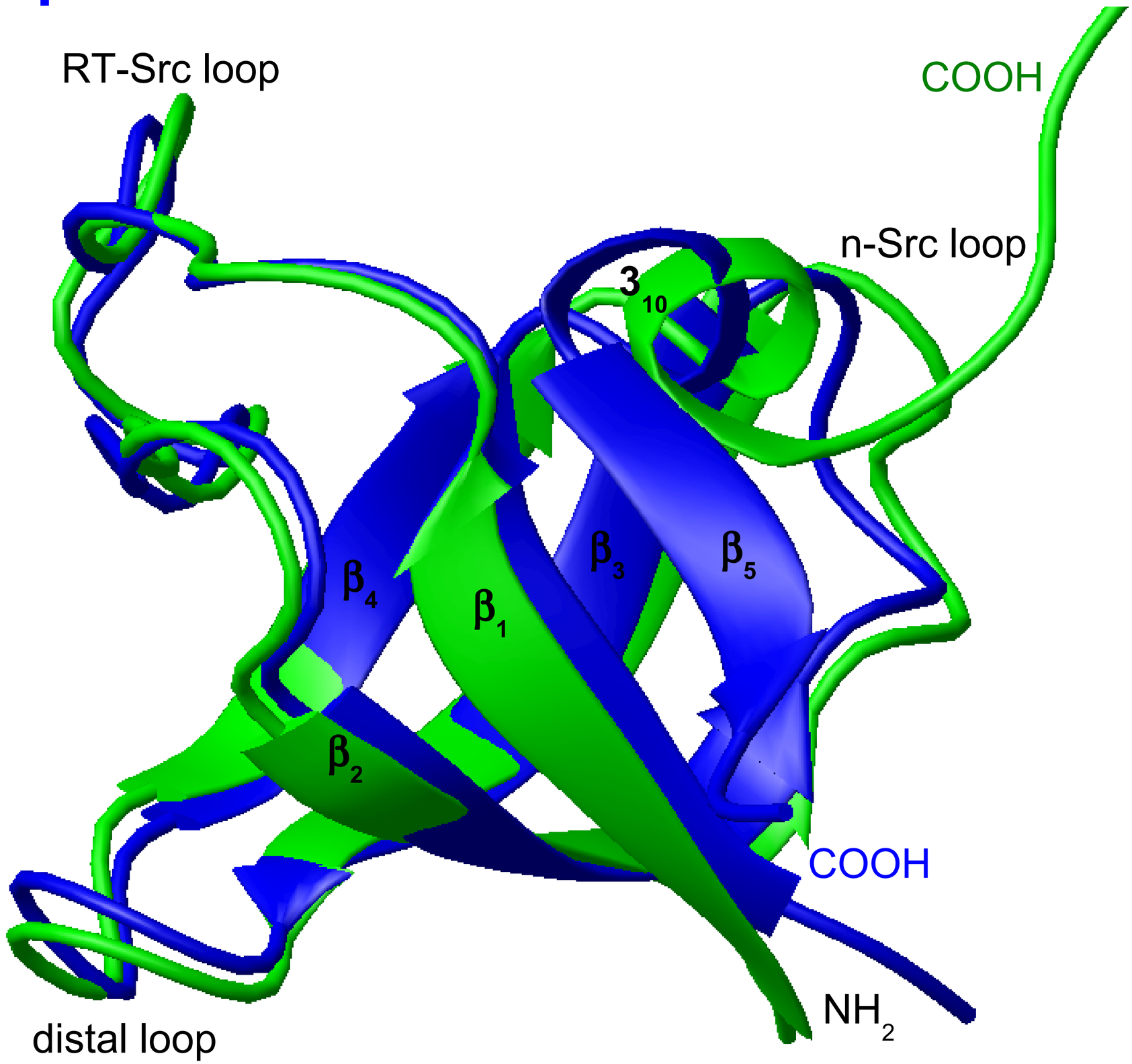


(Neudecker, Robustelli, Cavalli, Walsh et al. & Kay, *Science* **336**, 362-366 (2012))

Structure Comparison of Native State and Intermediate

Overlay of the high-resolution structure of the native state based on the X-ray structure of the Fyn SH3 N53I/V55L (PDB 3CQT) and a representative high-resolution structure of the intermediate state calculated from CPMG relaxation dispersion experiments (PDB 2L2P)

RMSDs from the folded state for residues 2..55:
Backbone:
1.17 Å ± 0.07 Å
All heavy atoms:
2.06 Å ± 0.19 Å



(Neudecker, Robustelli, Cavalli, Walsh et al. & Kay, *Science* **336**, 362-366 (2012))

Topology Validation of the Intermediate by Amide Hydrogen Exchange Measurements

- Folding equilibrium F $\leftrightarrow$ I $\leftrightarrow$ U with kinetics on millisecond time-scale
- Intrinsic exchange rates k_{int} on minute time-scale at pH = 6.0

$\Rightarrow$ k_{int} rate-limiting (EX2 limit)

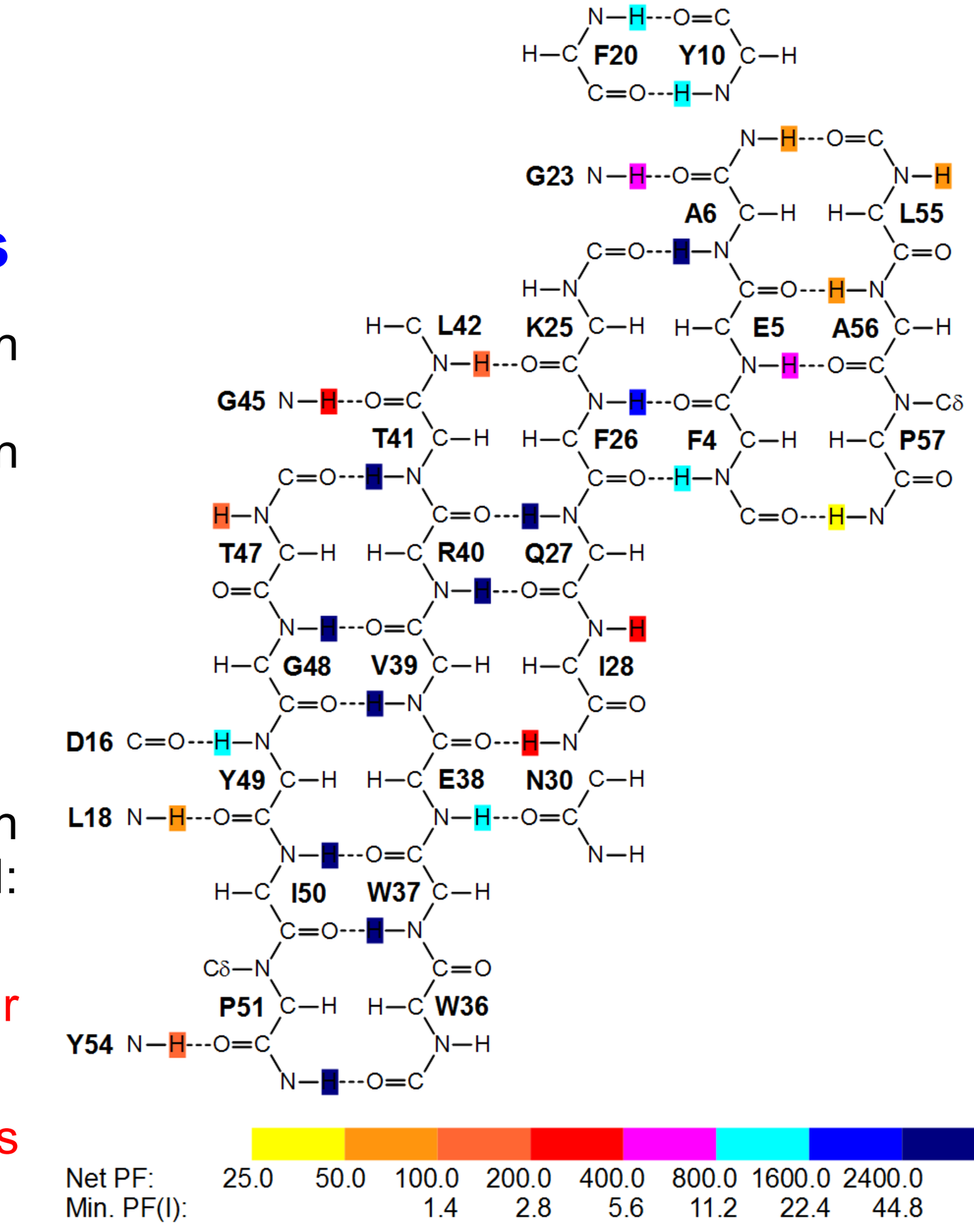
Net protection factor $PF = k_{int}/k_{ex}$

$k_{ex}/k_{int} = p_F/PF(F) + p_I/PF(I) + p_U$

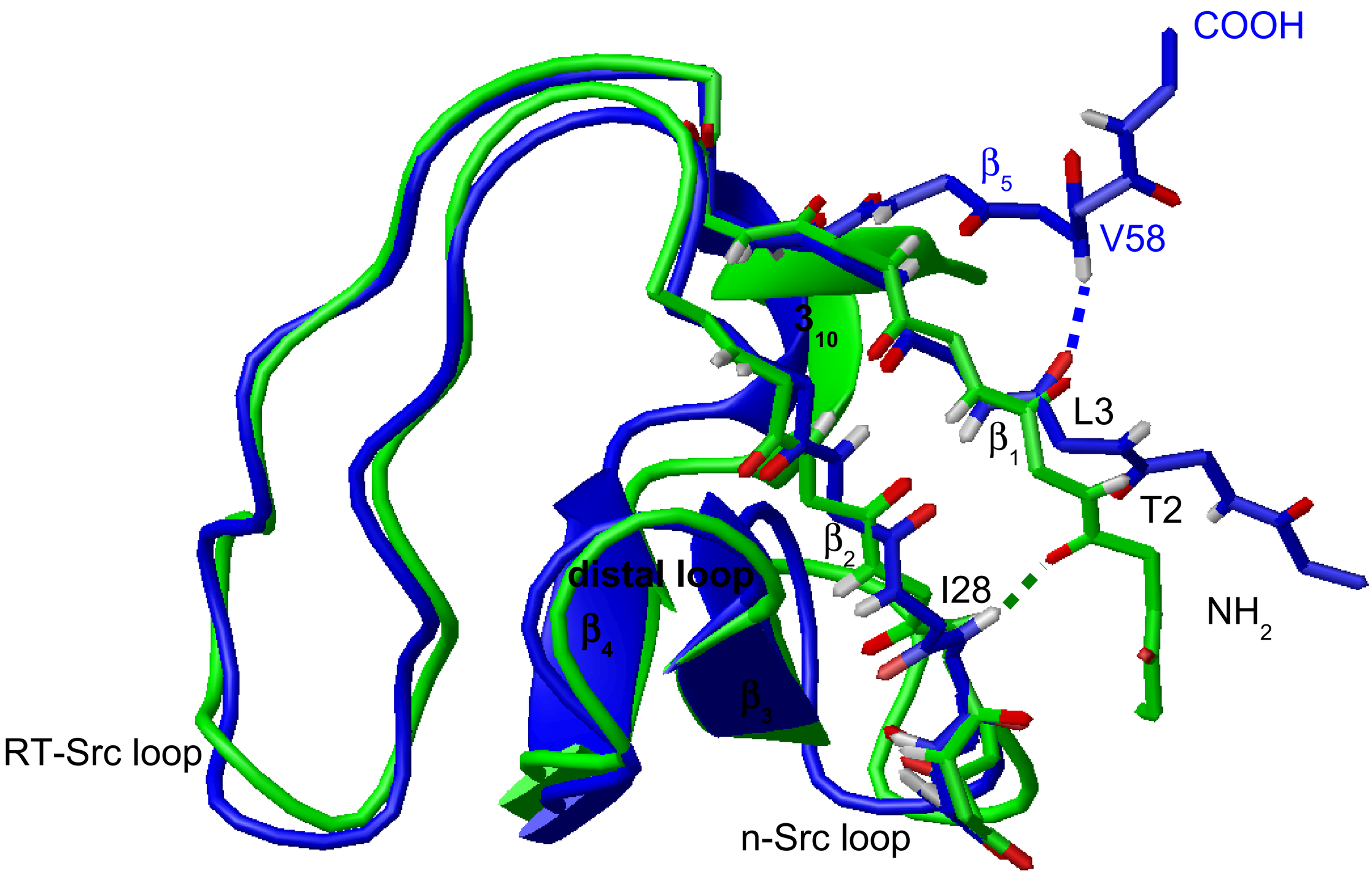
$\Rightarrow$ lower bound for the protection factor in the intermediate state I: $PF(I) \geq p_I \times PF \geq 0.014 \times PF$

H-bonding network between the four strands β_1-β_2-β_3-β_4 is conserved in I

H-bonding of the 3_{10}-helix and β_5 is not conserved in I

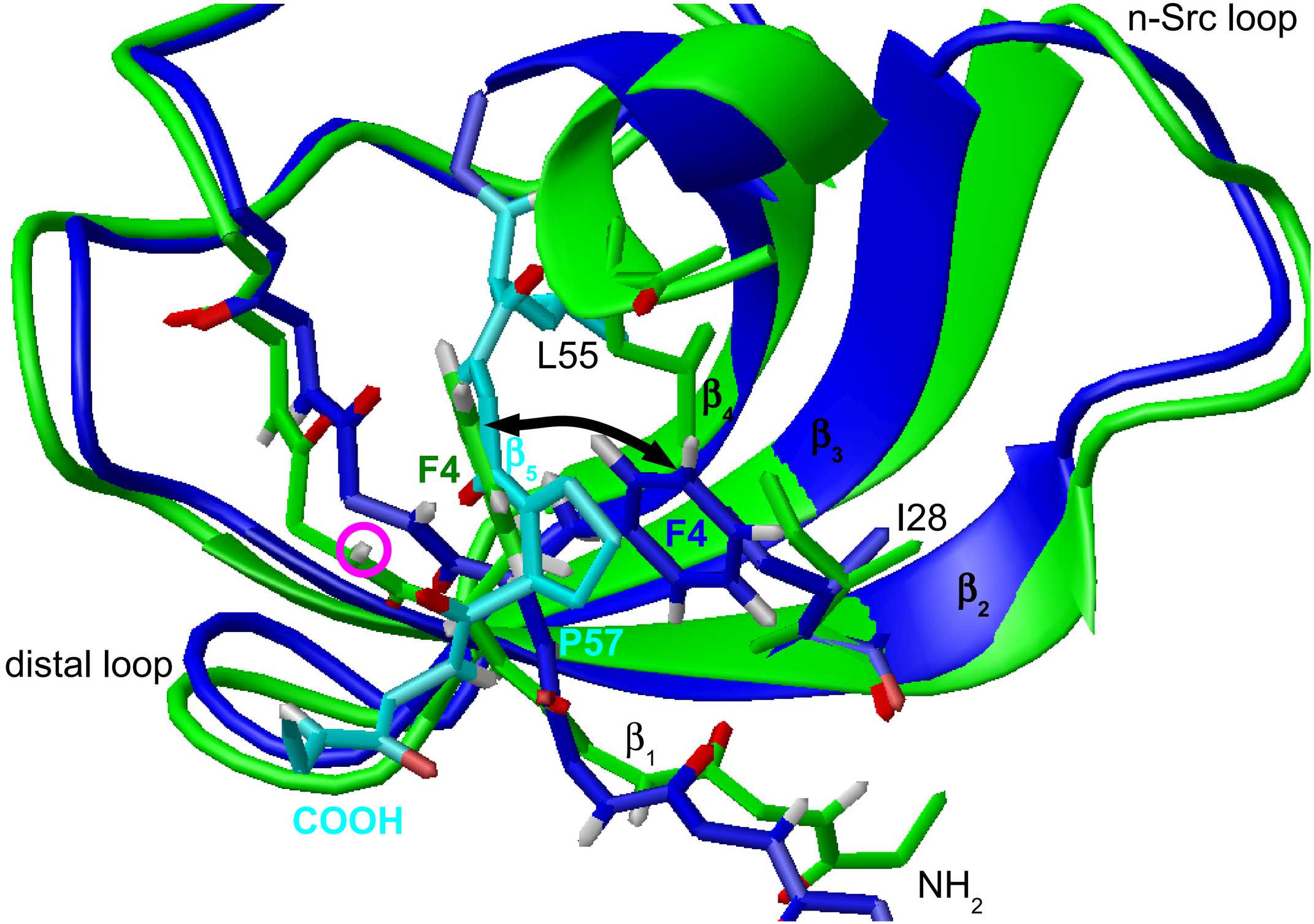


Non-native Long-Range Interactions I: β_1-β_2



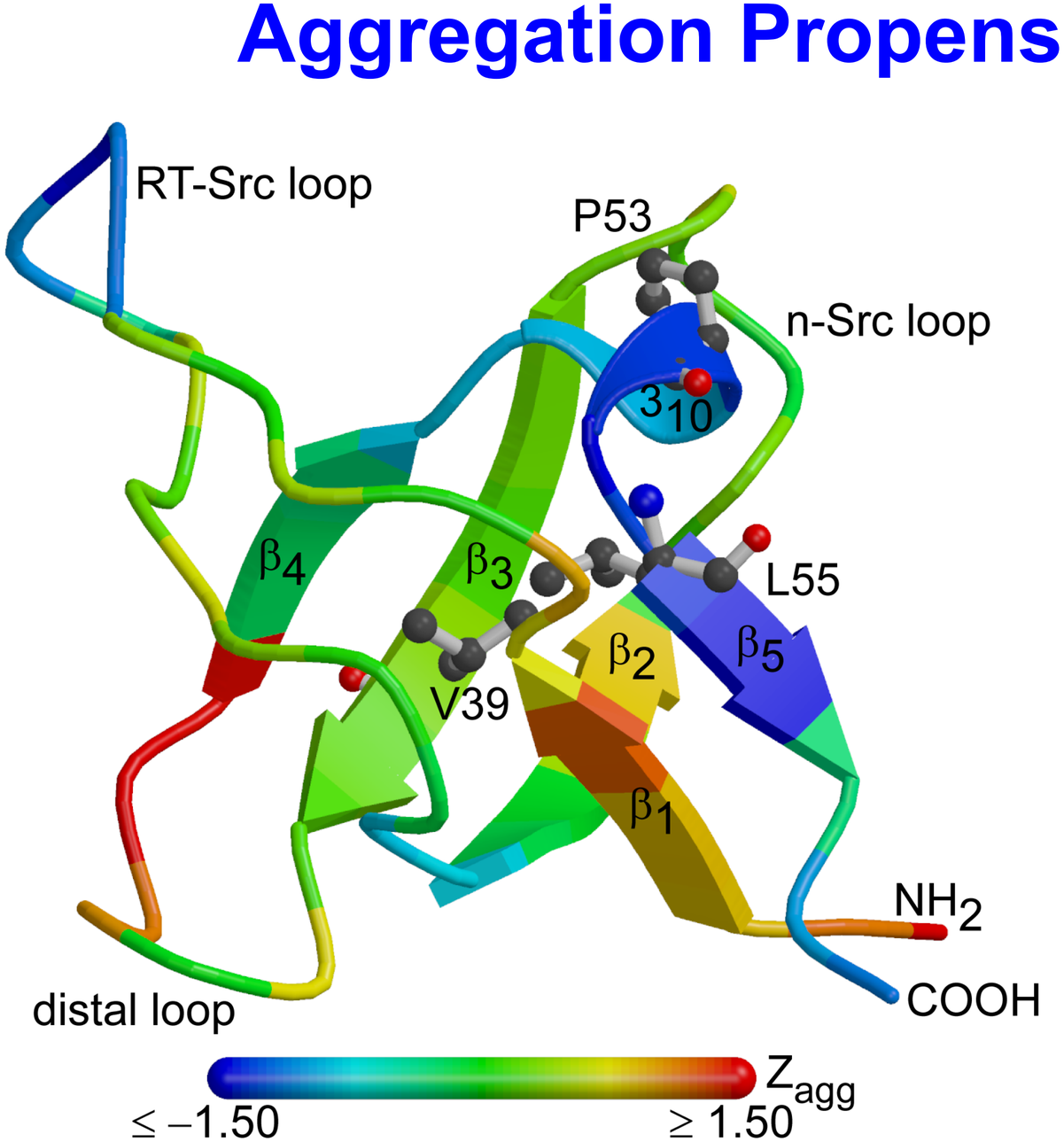
Experimental evidence: TALOS+ prediction for Leu3 Φ (chemical shift of Thr2 ^{13}CO)

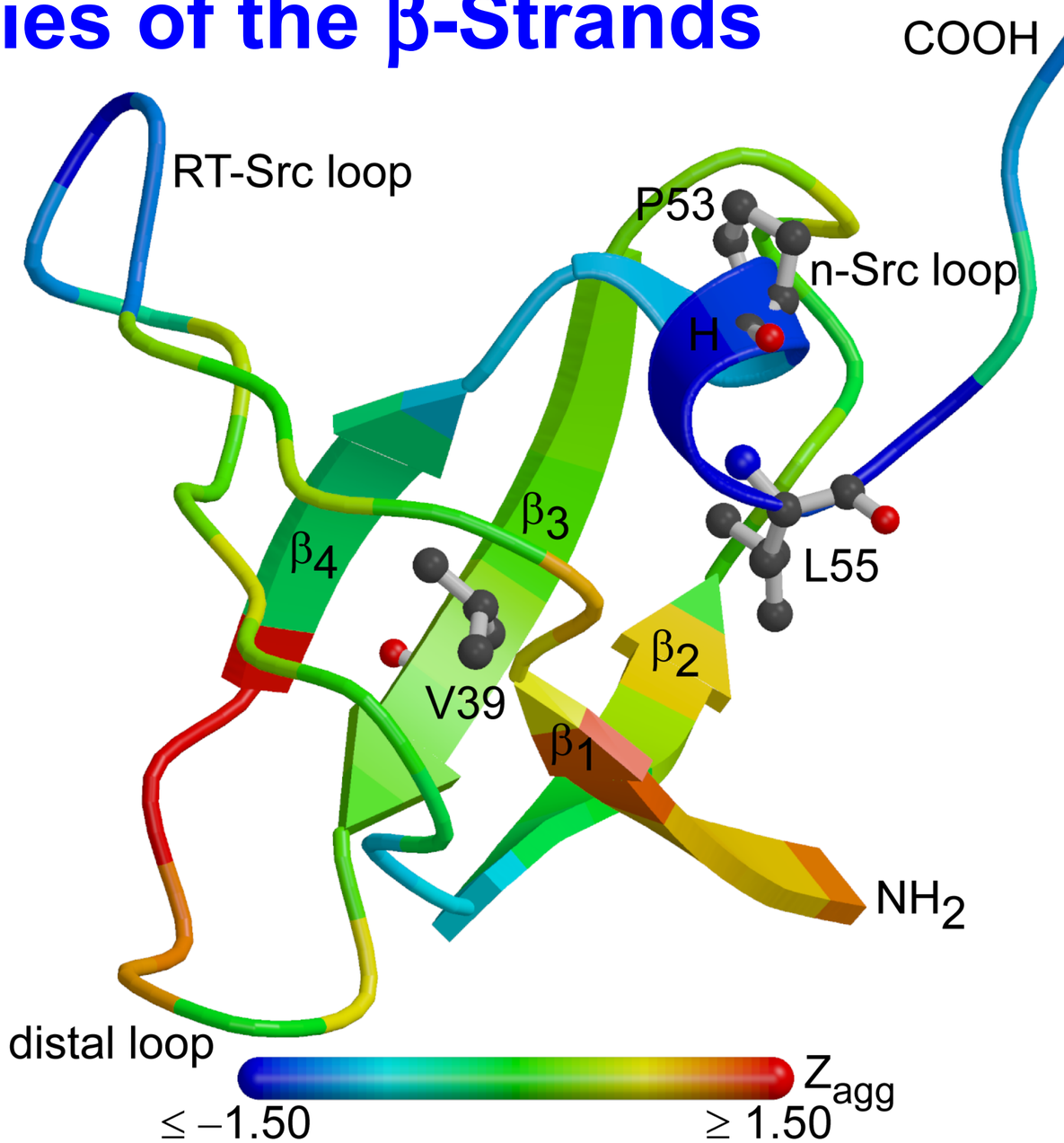
Non-native Long-Range Interactions II: Phe4



Experimental evidence: large ring current effects on chemical shift of Glu5 HN

Aggregation Propensities of the β-Strands

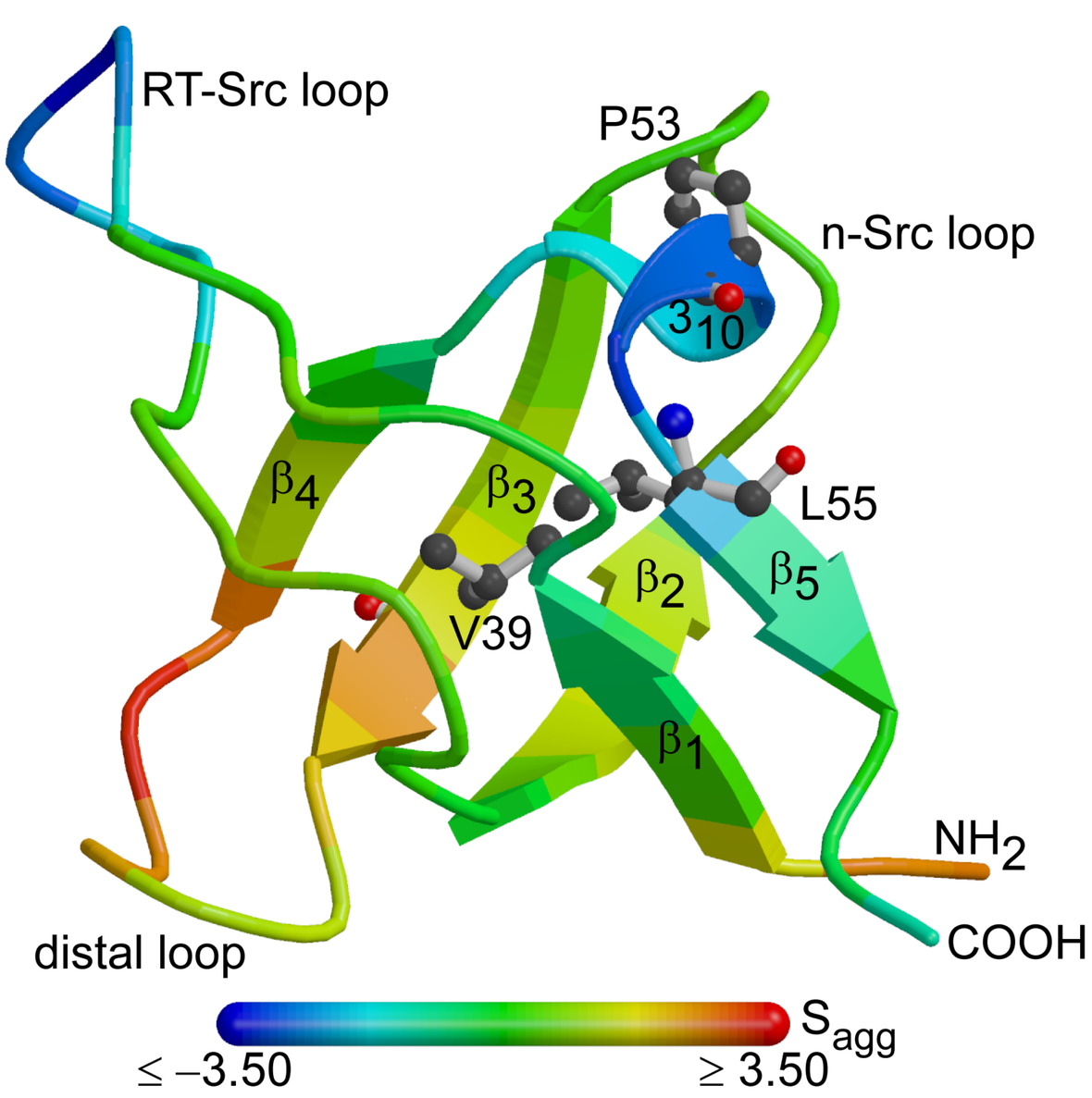


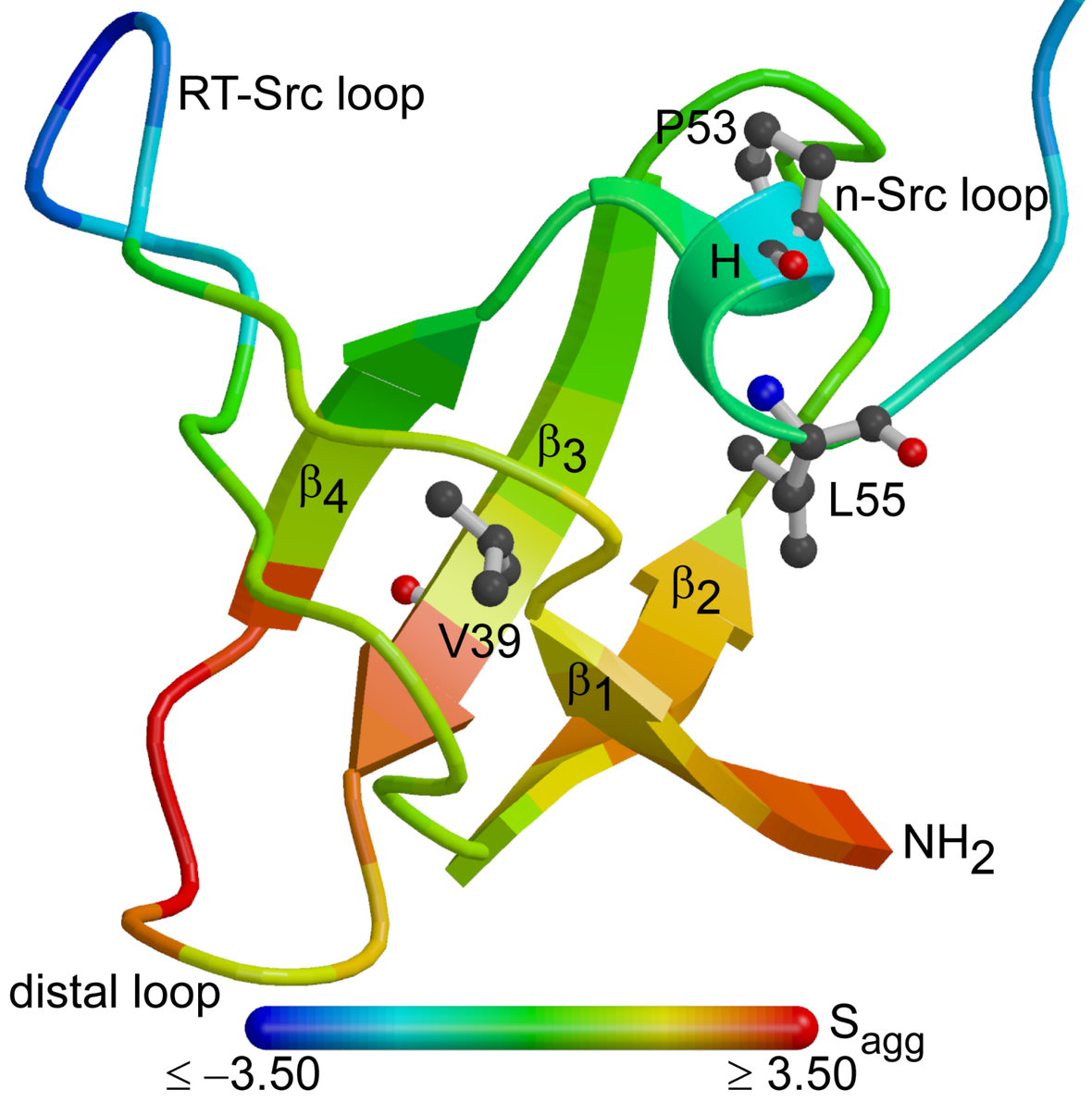


Aggregation propensities as predicted from the primary structure by Zyggregator (Tartaglia, Pawar, Campioni, Dobson, Chiti & Vendruscolo, *JMB* **380**, 425-436 (2008)):

The most aggregation-prone strand β_1, which is protected in the native state by the least aggregation-prone strand β_5 with its bulge caused by Pro57, becomes exposed in the intermediate state and readily available for aggregation.

Surface Aggregation Propensities of the β-Strands

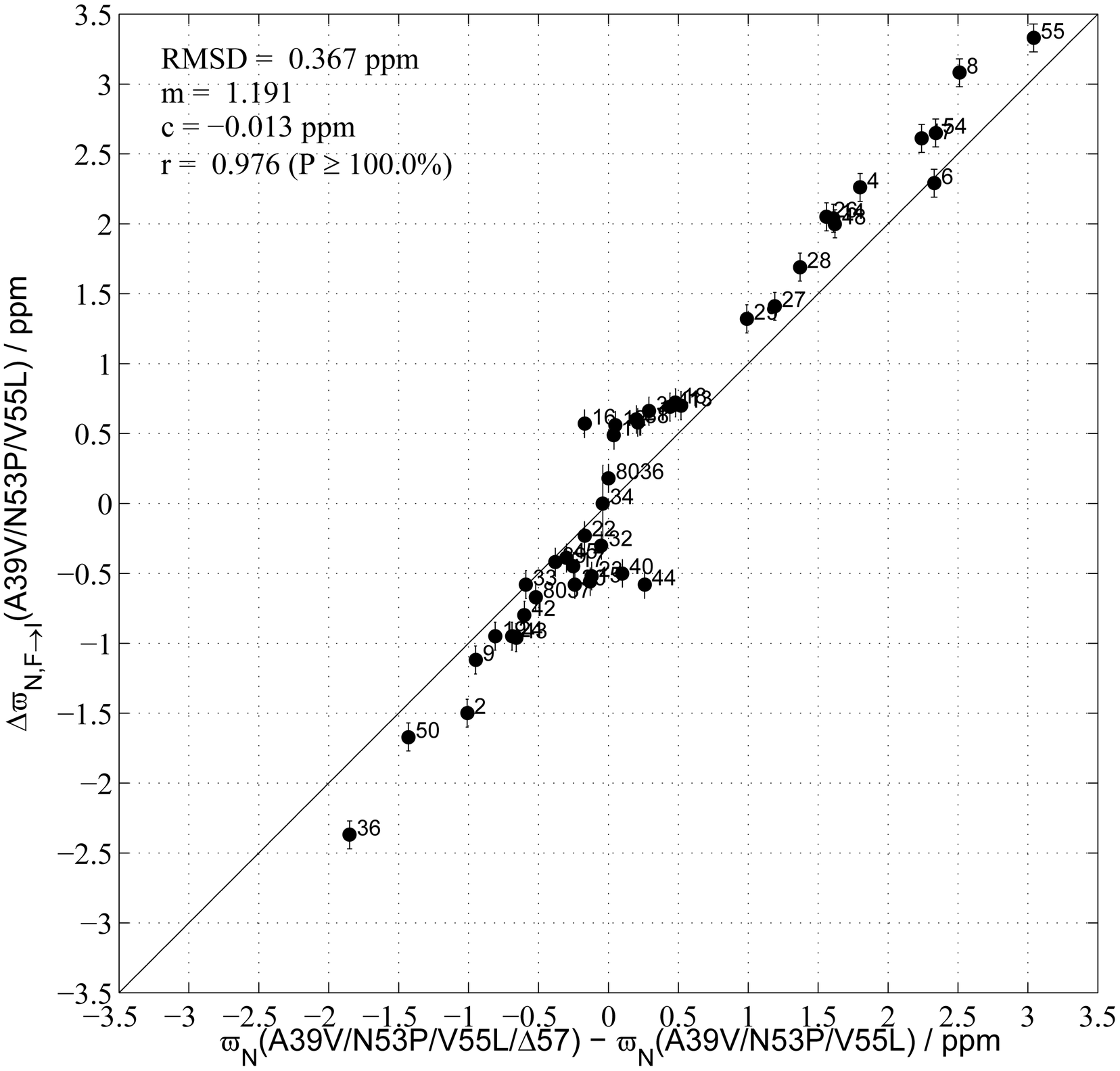




Surface aggregation propensities as predicted from the primary and tertiary structure by Zyggregator (Pechmann, Levy, Tartaglia & Vendruscolo, *PNAS* **106**, 10159–10164 (2009)):

The most aggregation-prone strand β_1, which is protected in the native state by the least aggregation-prone strand β_5 with its bulge caused by Pro57, becomes exposed in the intermediate state and readily available for aggregation.

Mutagenesis Mimicking the Intermediate



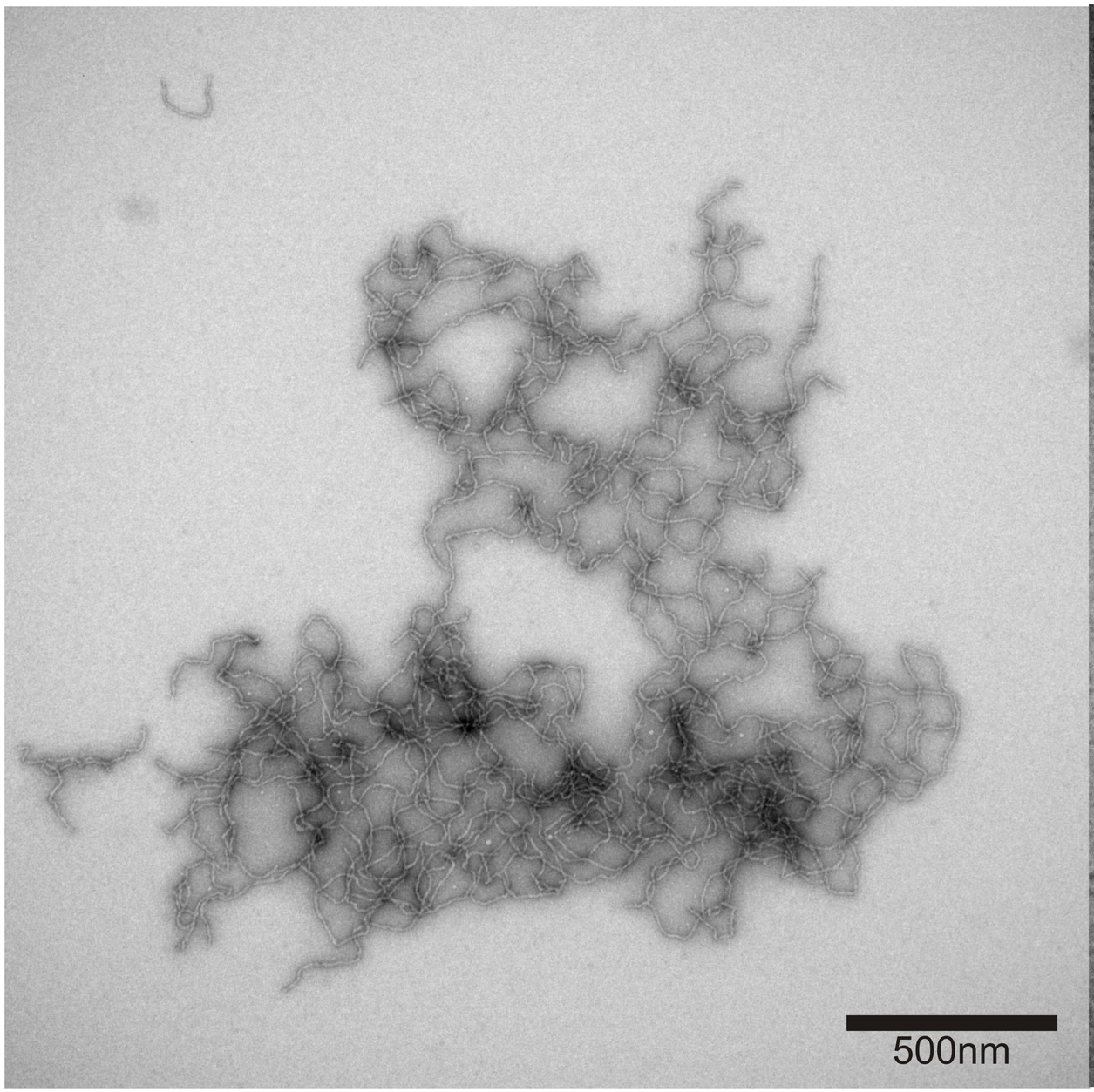
NMR spectra of the Fyn SH3 A39V/N53P/V55L/Δ(57-60) are severely line-broadened

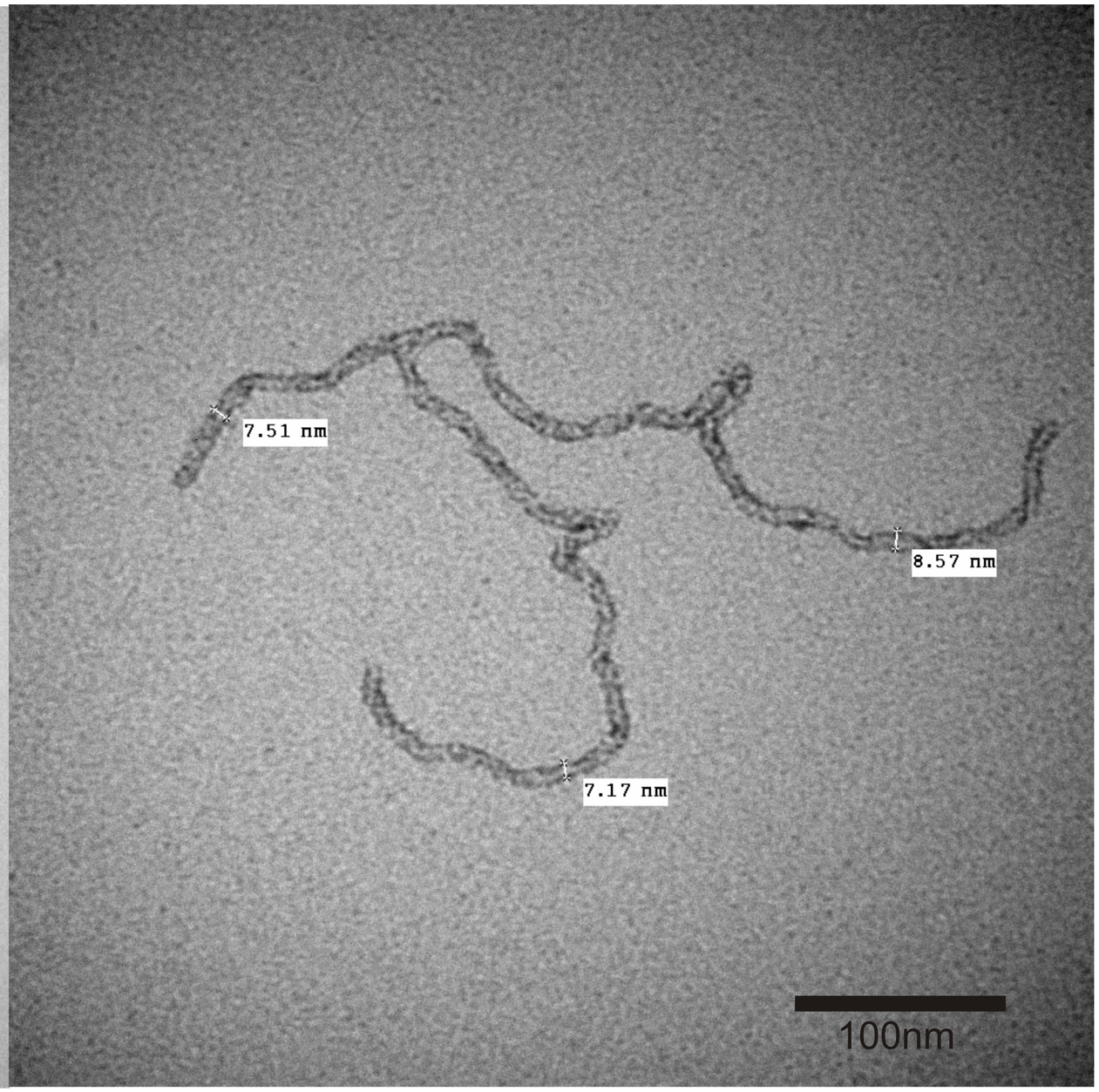
⇒ assignment incomplete, no NOEs in region of interest

All observable ^{15}N, ^{1}HN, ^{13}CO, $^{13}C\alpha$, $^{1}H\alpha$ resonances show chemical shifts that are virtually identical with those of the intermediate of the Fyn SH3 A39V/N53P/V55L

⇒ truncation exactly mimics the intermediate state structure

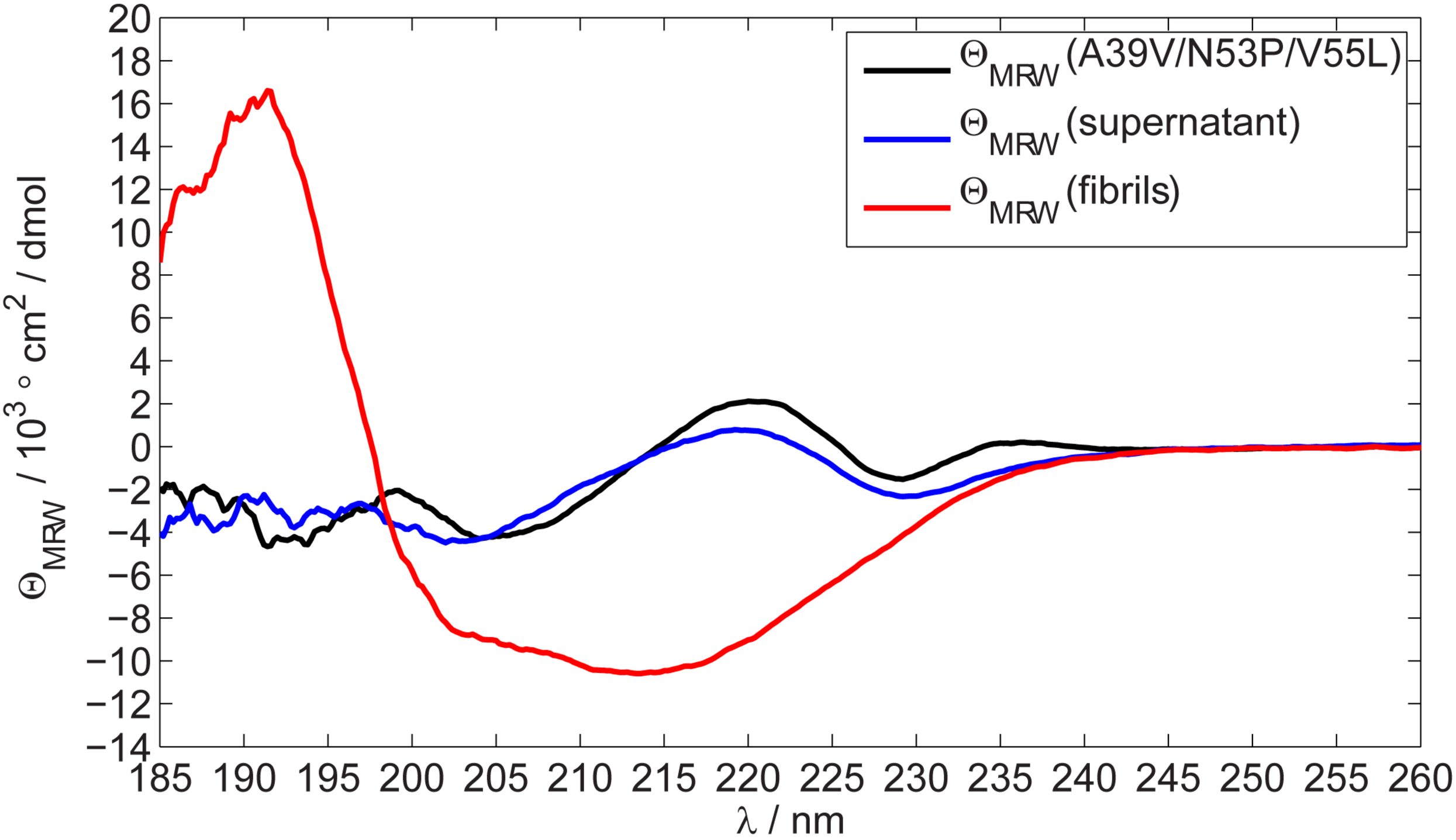
Mutagenesis Mimicking the Intermediate





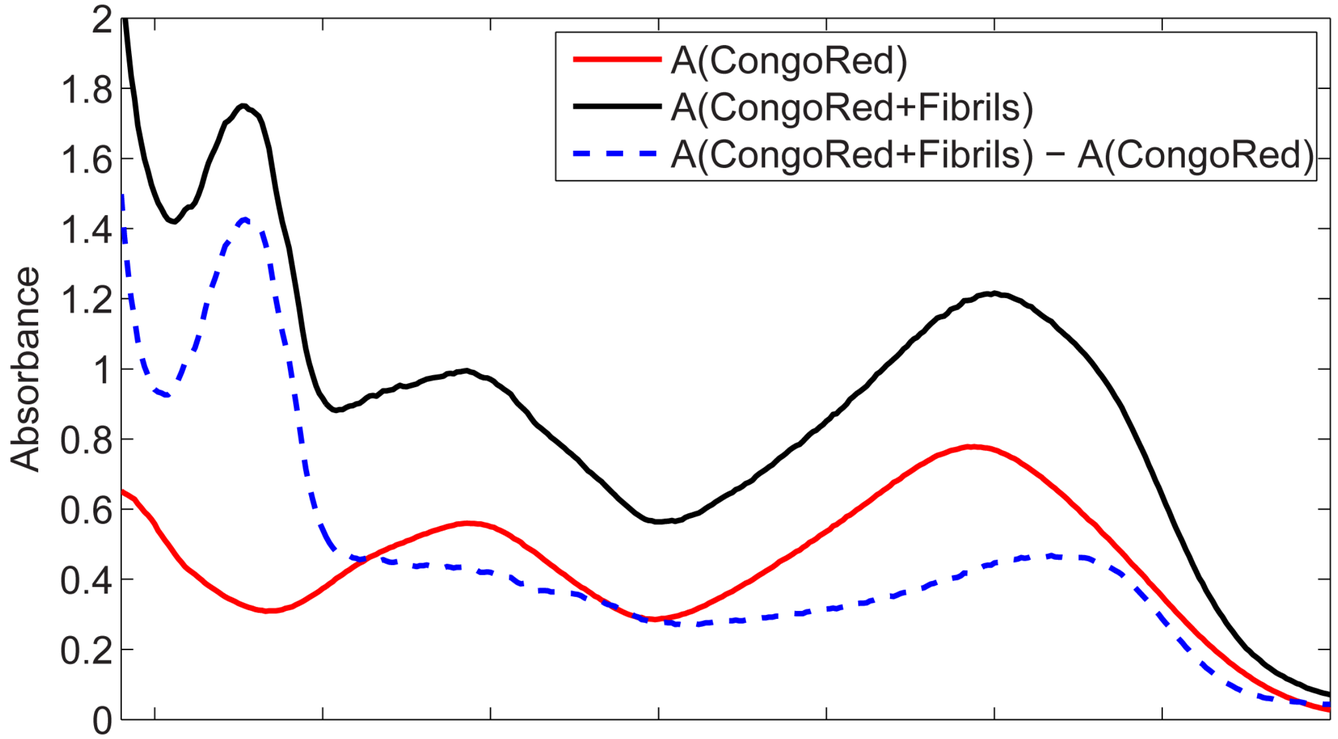
Fyn SH3 A39V/N53P/V55L/Δ(57-60) mimics the intermediate state structure, shows severe line broadening in CPMG experiments and aggregates on the time-scale of hours at room temperature and NMR sample concentrations into curly fibrils.

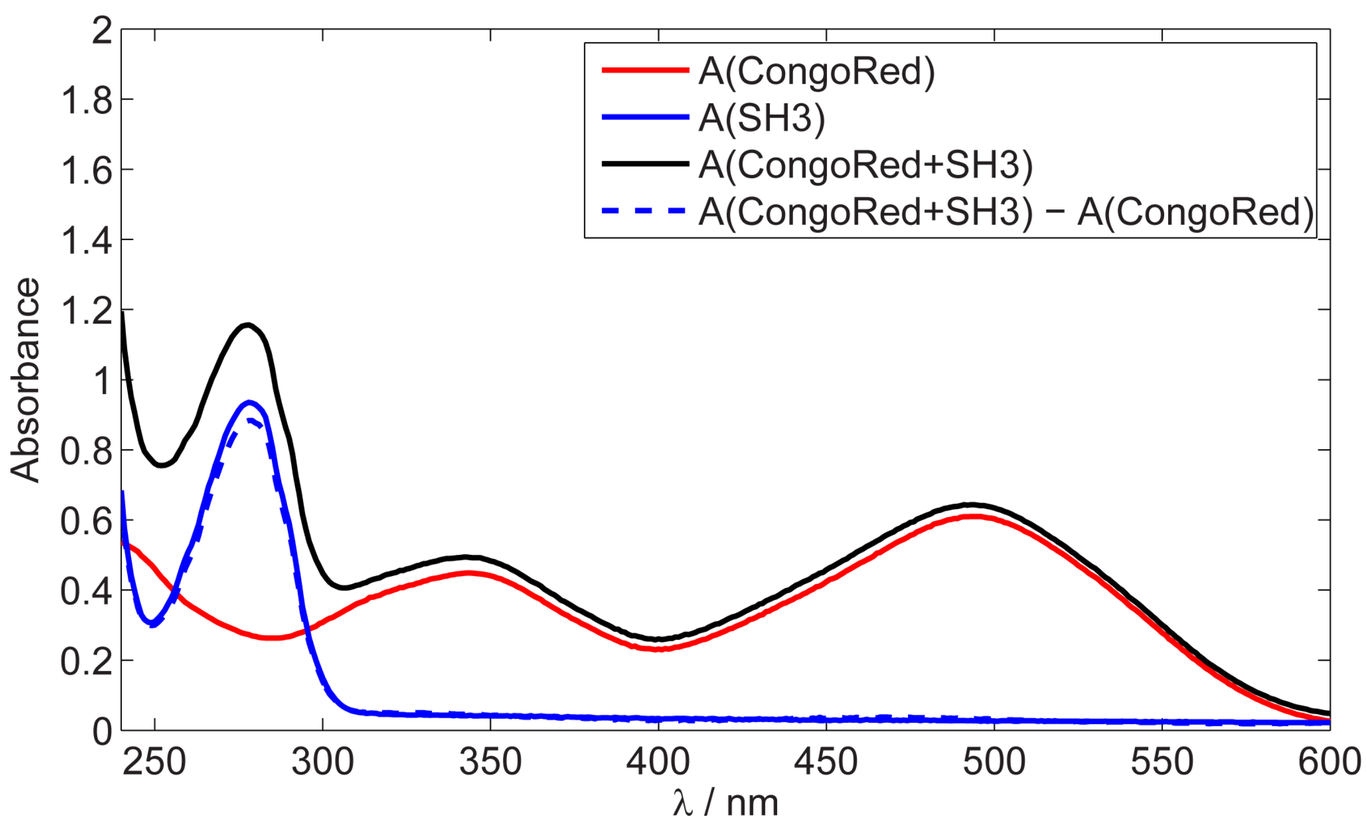
CD Spectroscopy of the Fyn SH3 Domain Fibrils



Upon fibril formation the CD spectrum changes from the SH3 domain spectral signature to a spectrum typical for proteins with very high β-sheet content (minimum at λ = 214 nm).

Congo Red Binding to the Fyn SH3 Domain Fibrils





Fyn SH3 A39V/N53P/V55L/Δ(57-60) fibrils bind Congo Red as evidenced by the typical hyperchromicity and red shift of the major absorption band of Congo Red, causing the fibril signature shoulder at $\lambda \approx 530$ nm above the fibril scattering baseline

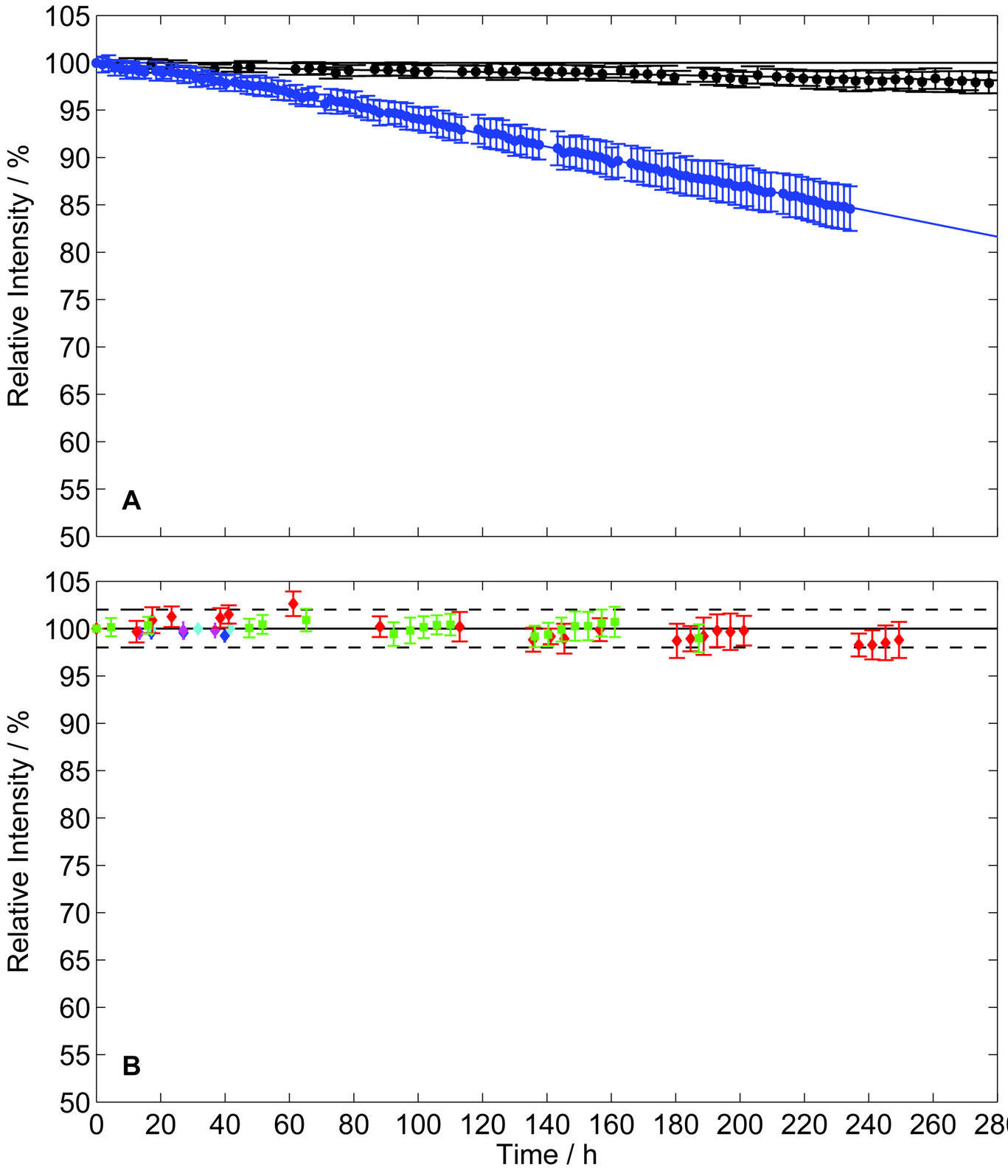
$\Rightarrow$ cross-β-sheet amyloid fibrils

(Klunk et al., *J. Histochem. Cytochem.* **37**, 1293-1297 (1989); Klunk et al., *Anal. Biochem.* **266**, 66-76 (1999))

Negative Control:

Fyn SH3 A39V/N53P/V55L does not bind Congo Red as evidenced by the fact that the absorption spectrum is simply the sum of the absorption spectra of free Congo Red and free SH3 domain

NMR Intensity Loss Associated with Aggregation



Freshly prepared samples of the Fyn SH3 A39V/N53P/V55L/Δ(57-60) show loss of NMR resonance intensity:

0.16%/day at 15°C

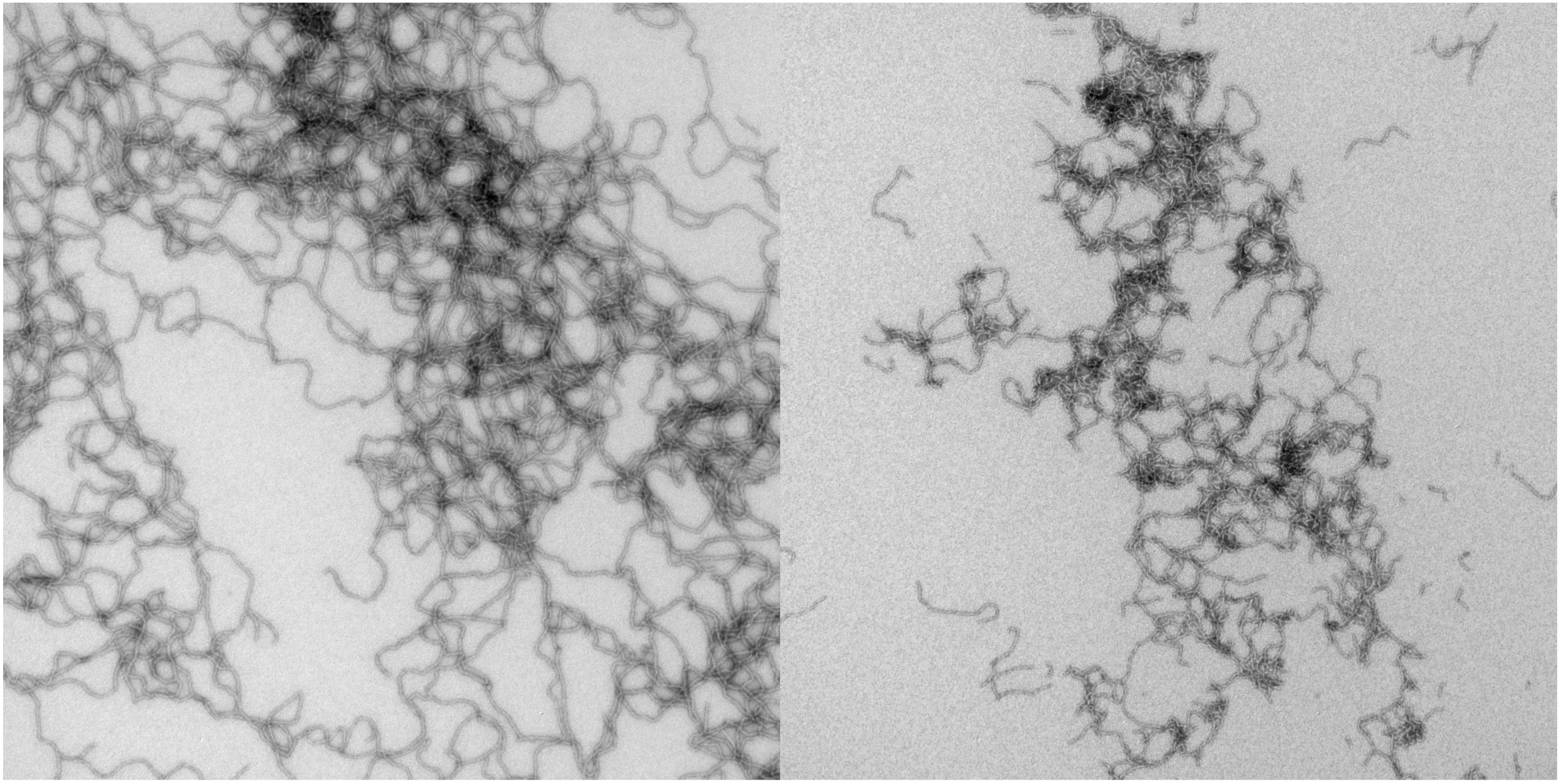
1.6%/day at 20°C

⇒ sizable rate-limiting energy barrier

Negative Control:

Fyn SH3 domain mutants not mimicking the folding intermediate show no sign of aggregation, even highly unstable ones such as Fyn SH3 L3A/A39V/N53P/V55L (diamonds) and F20L/A39V/N53P/V55L (squares).

Mutagenesis of the Fyn SH3 wt



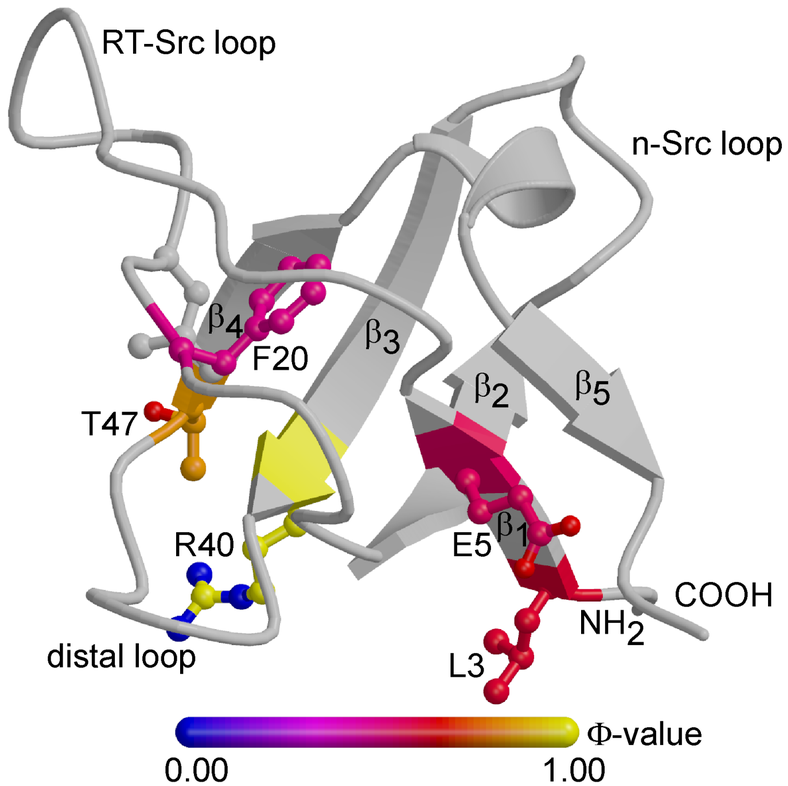
Fyn SH3 Δ(57-60) still forms the (rudimentary) native SH3 domain structure as verified by [^{1}H,^{15}N]-NOESY-HSQC and is not particularly aggregation prone at room temperature.

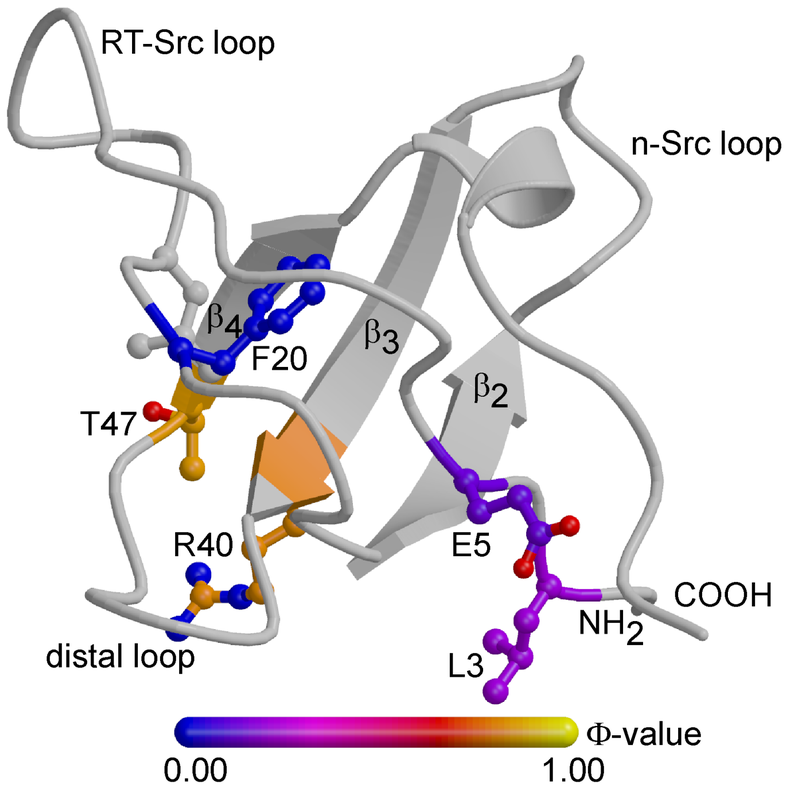
Fyn SH3 Δ(56-60) shows line broadening in CPMG experiments and aggregates on the time-scale of hours at room temperature and NMR sample concentrations into curly fibrils

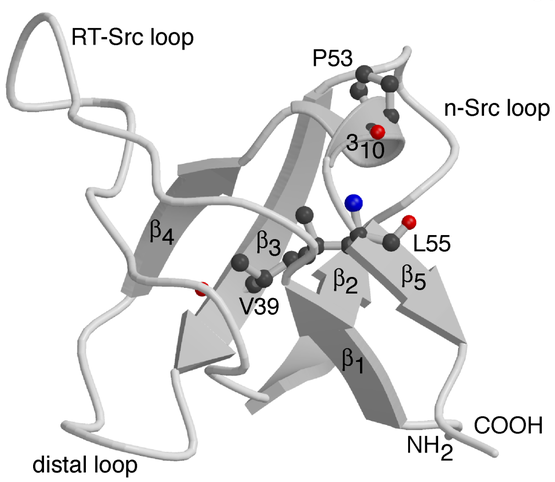
⇒ amyloid fibril formation independent of the mutations A39V/N53P/V55L

Structure of Rate-Limiting Transition States

CPMG-based Φ-value analysis of the Fyn SH3 A39V/N53P/V55L:

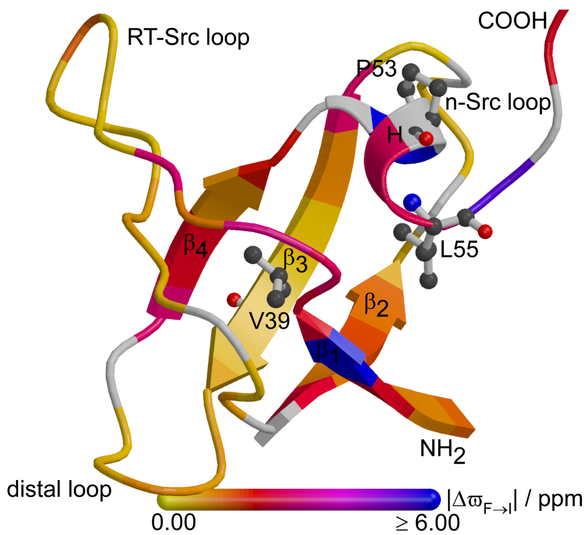






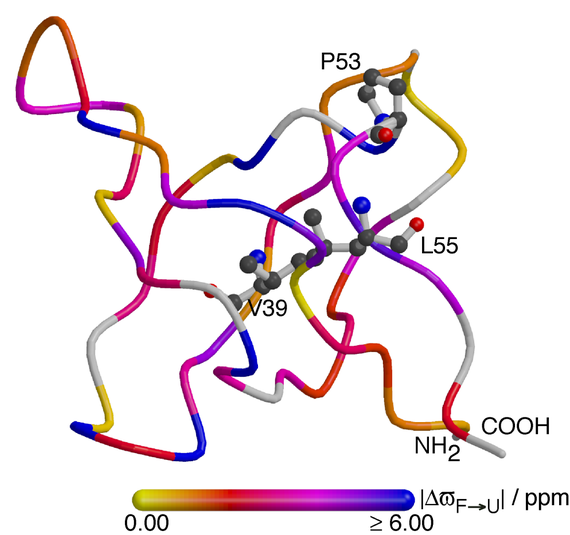
51/s

2951/s



389/s

924/s

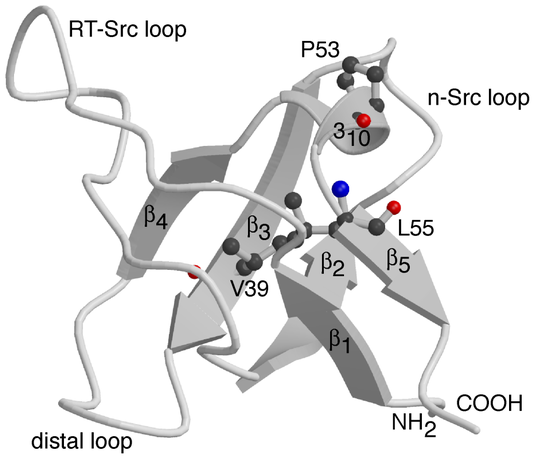


(Neudecker, Zarrine-Afsar, Davidson & Kay, *PNAS* **104**, 15717-15722 (2007))

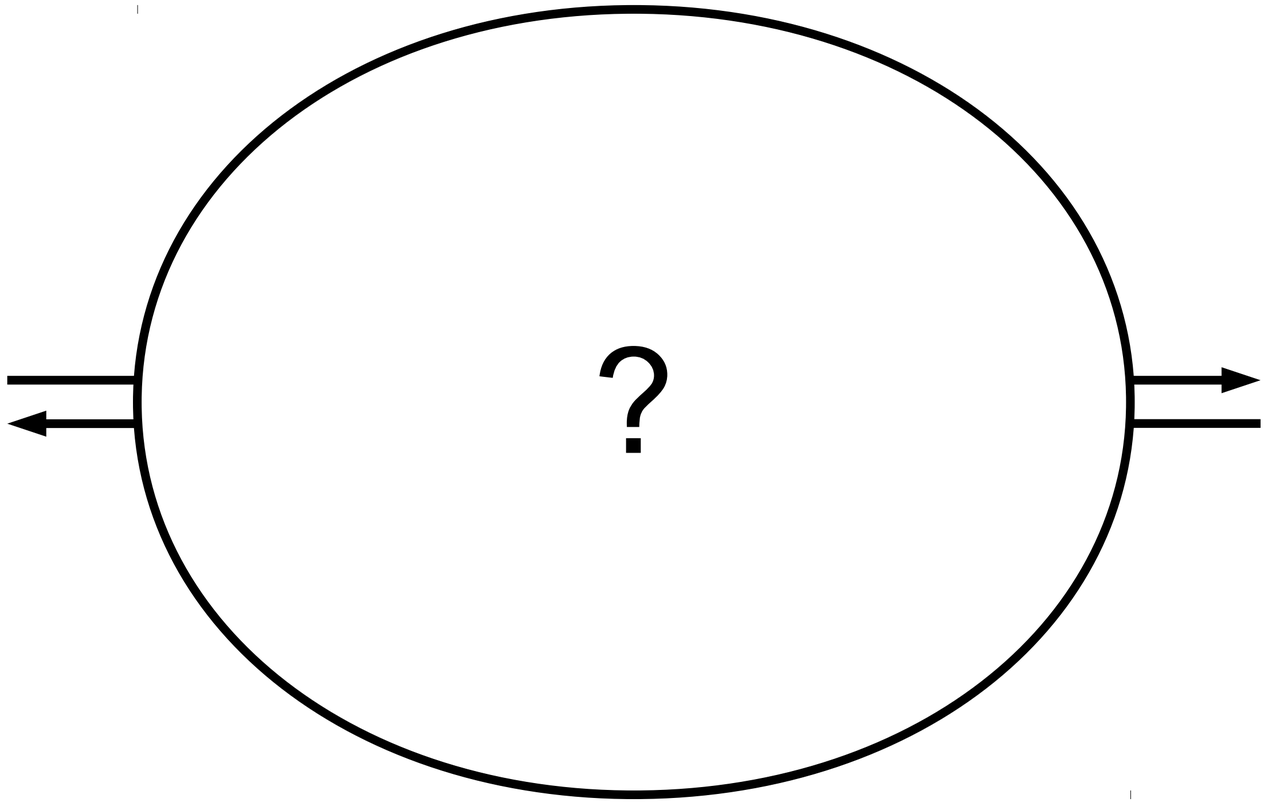
Protein Folding Pathways

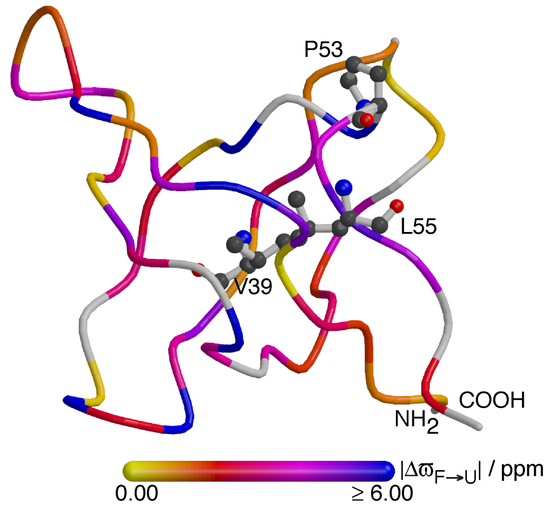
Protein folding is not a random combinatorial search
(Anfinsen, *Science* **181**, 223-230 (1973))

⇒ folding pathways with transient intermediates, rate-limiting transition states



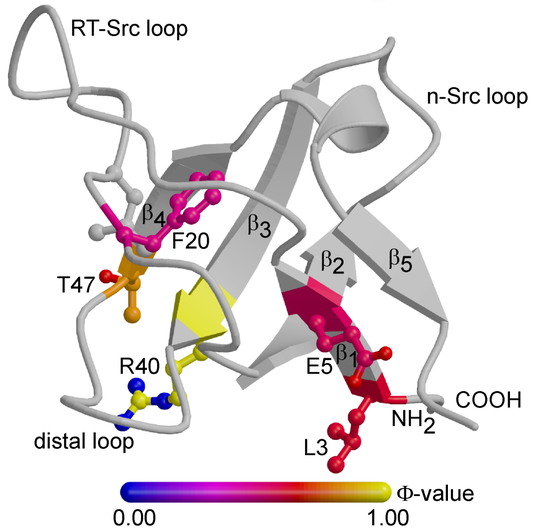
native state

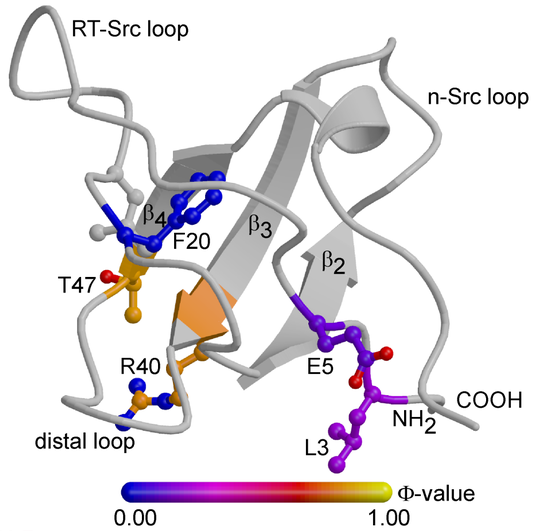


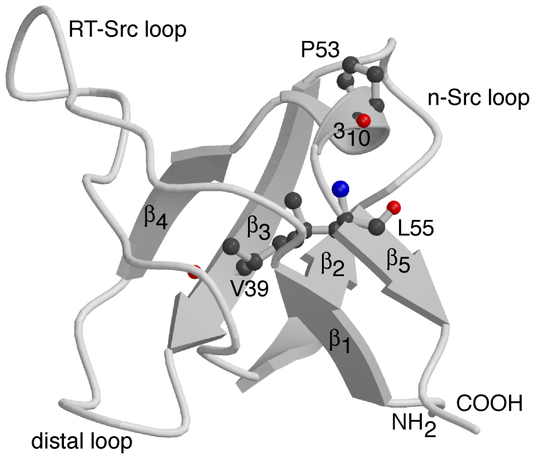


unfolded state

Folding Pathway of the Fyn SH3 A39V/N53P/V55L







51/s

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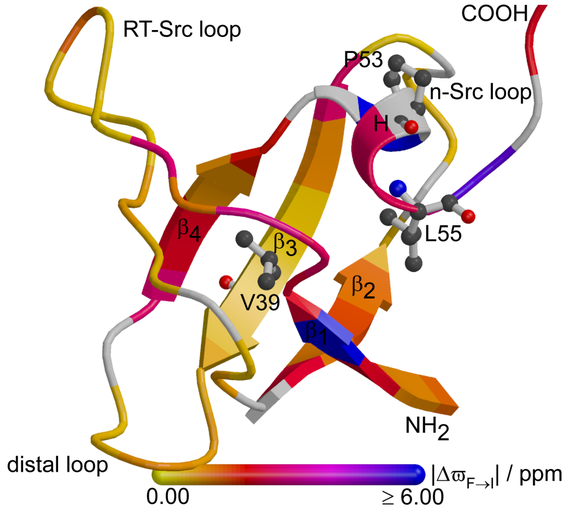

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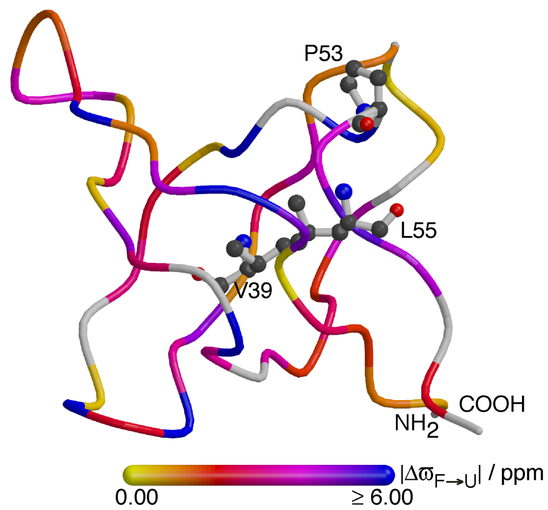

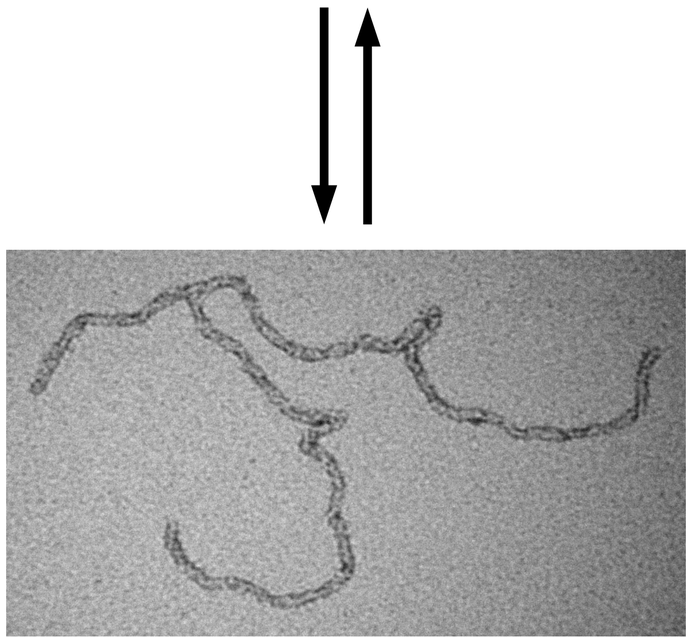

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