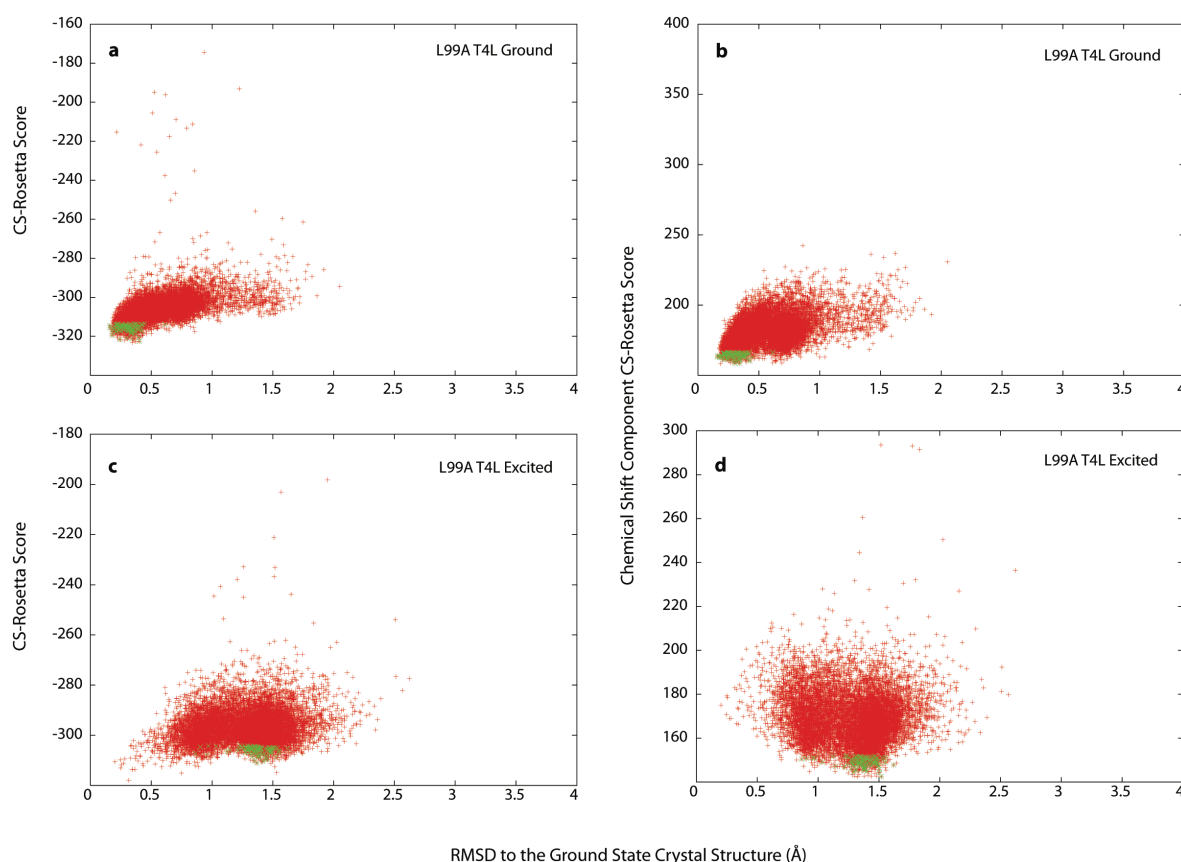


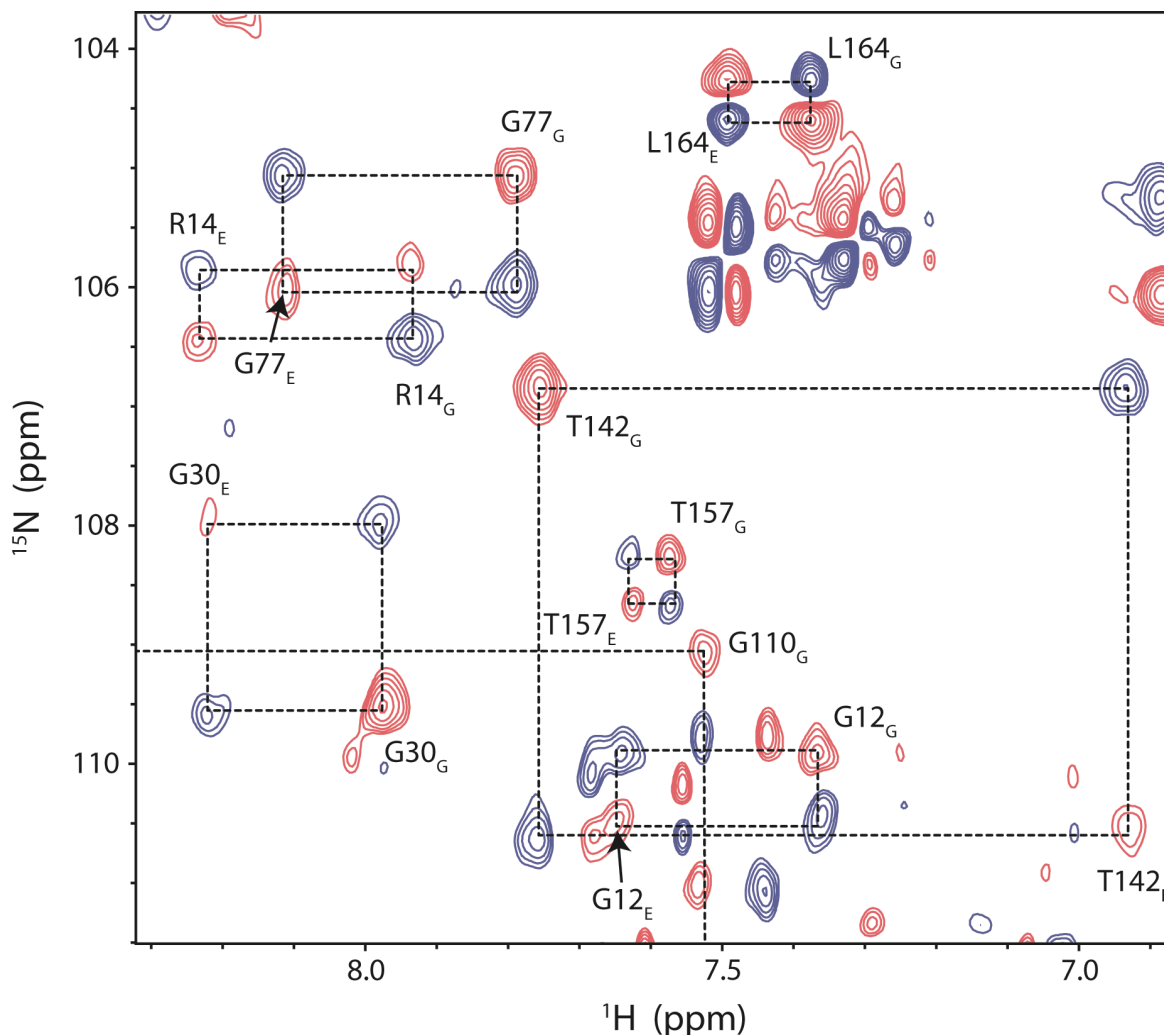
## Supplementary Figure 1 | Relaxation dispersion experiments indicate that L99A T4L exchanges between two states in solution.

**a-e**, Experimental relaxation dispersion profiles (circles) of backbone  $^{15}N$  (a),  $^1H^N$  (b),  $^{13}C'$  (c),  $^{13}C^\alpha$  (d) and  $^1H^\alpha$  (e) nuclei of residue Phe114 measured on L99A T4L at 25°C using 600 MHz (green) and 800 MHz (blue) spectrometers, along with the best fit curves (solid lines). Error bars indicate uncertainties in  $R_{2,eff}$  rates.



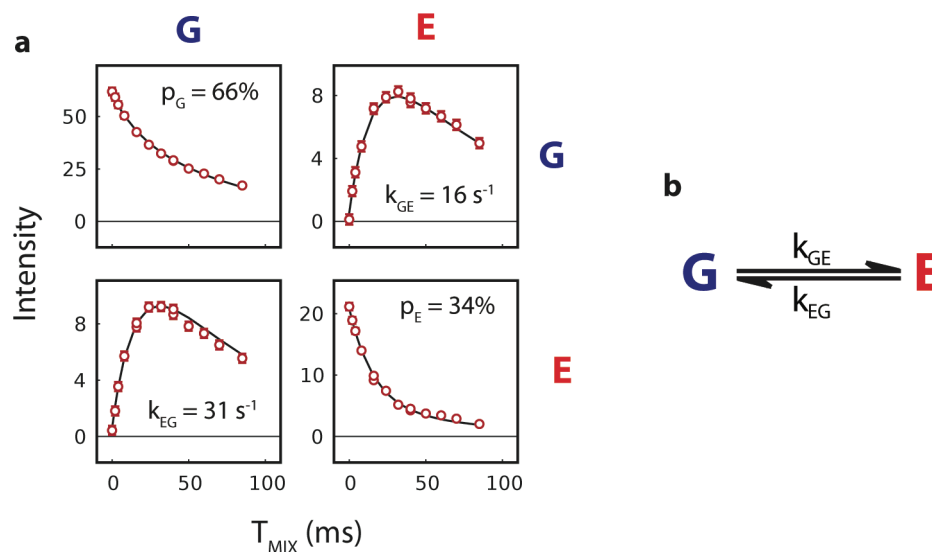
### Supplementary Figure 2 | CS-Rosetta modeling of the ground and excited state structures of L99A T4L.

**a**, Energy of 9600 models (red) generated from ground state L99A T4L chemical shifts vs. RMSD to the ground state X-ray structure of L99A T4L (3DMV)<sup>1</sup>. All backbone heavy atoms are included in the RMSD. **b**, As in (**a**) except that structures are scored according to the agreement between calculated and experimental chemical shifts. Shown in green are the 96 final structures generated using the two-stage selection scheme described in the text. **c**, **d**, As in (**a**, **b**) but for the excited state of L99A T4L. Differences in RMSD of the excited state ensemble to the ground state X-ray structure reflect (i) the rearrangement of helices F and G and (ii) reorientation of the side-chain of Phe 114 in the excited state, as described in the text.



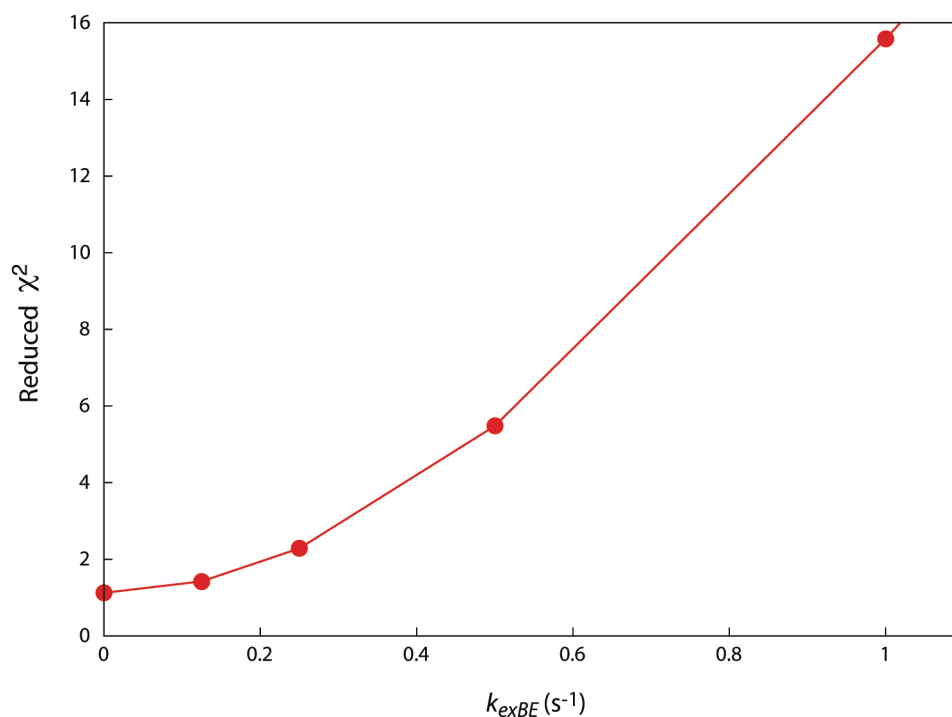
**Supplementary Figure 3 |  $^1\text{H}^{\text{N}}\text{-}^{15}\text{N}$  2D correlation map showing clusters of exchanging resonances in L99A,G113A T4L.**

A pair of  $^{15}\text{N}$  magnetization exchange experiments, recorded at  $1^\circ\text{C}$ , 800 MHz, were obtained in which the exchange mixing period,  $T_{\text{MIX}}$ , (50 ms) was placed before and after the indirect detection period, as described in Methods. Subtraction of the two data sets generates a 2D spectrum where correlations from ground (subscript 'G') and excited (subscript 'E') states (positive, red) are connected by cross-peaks (negative, blue), forming a 'rectangular' structure. Clusters of correlation peaks that are aliased in the  $^{15}\text{N}$  dimension, such as Arg14 and Leu164, have opposite signs. Only the diagonal peaks are labeled.



**Supplementary Figure 4 | Magnetization exchange experiments recorded on L99A,G113A T4L**

**a**, Intensity of auto- and cross-peaks for residue Met 102 from magnetization exchange experiments recorded on L99A,G113A T4L, 1°C, (no benzene), as a function of  $T_{MIX}$  (red circles), along with the best fit of the data (solid lines) to the exchange model of **(b)**. Values of rates of exchange,  $k_{ij}$ , and fractional populations of states,  $p_i$ , are indicated.



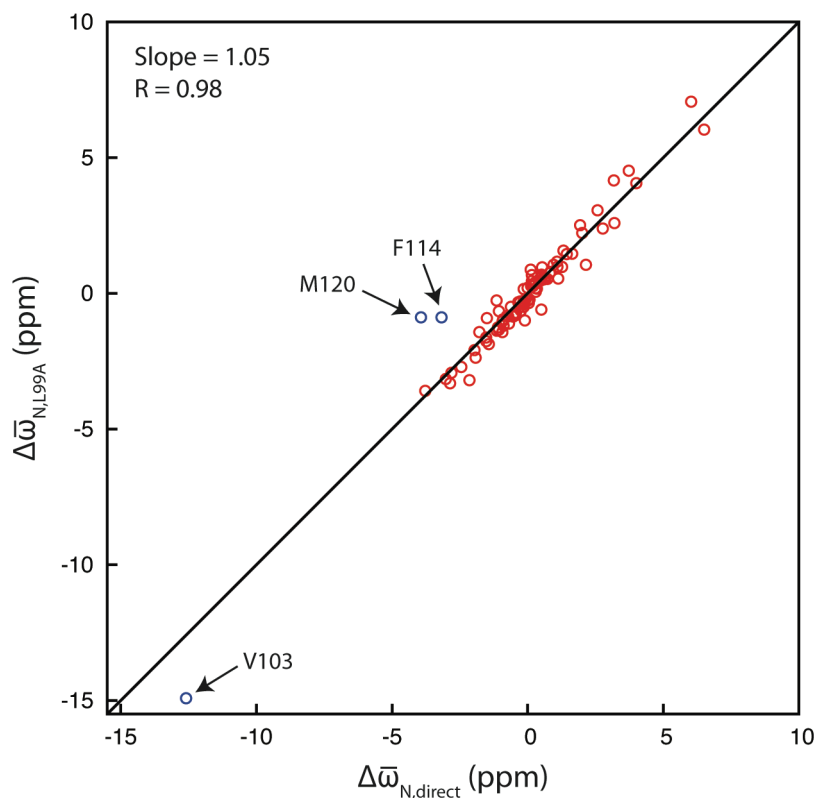
### Supplementary Figure 5 | Benzene does not bind to the excited state of L99A T4L

Reduced  $\chi^2$  obtained from fits of the time-dependencies of diagonal- and cross-peaks of Met 102 ( $^{13}\text{CH}_3^e$ ) in magnetization exchange spectra recorded on L99A,G113A T4L:benzene (1°C) vs  $k_{exBE} = k_{BE} + k_{EB}$  (see Figure 3d). The value of  $k_{exBE}$  was fixed to a given value (X-axis) and all the other parameters were optimized in the manner described in text. It is clear that  $k_{exBE}$  is very close to zero as even  $k_{exBE} = 0.5$  has a significantly higher reduced  $\chi^2$  compared to  $k_{exBE} = 0$ .



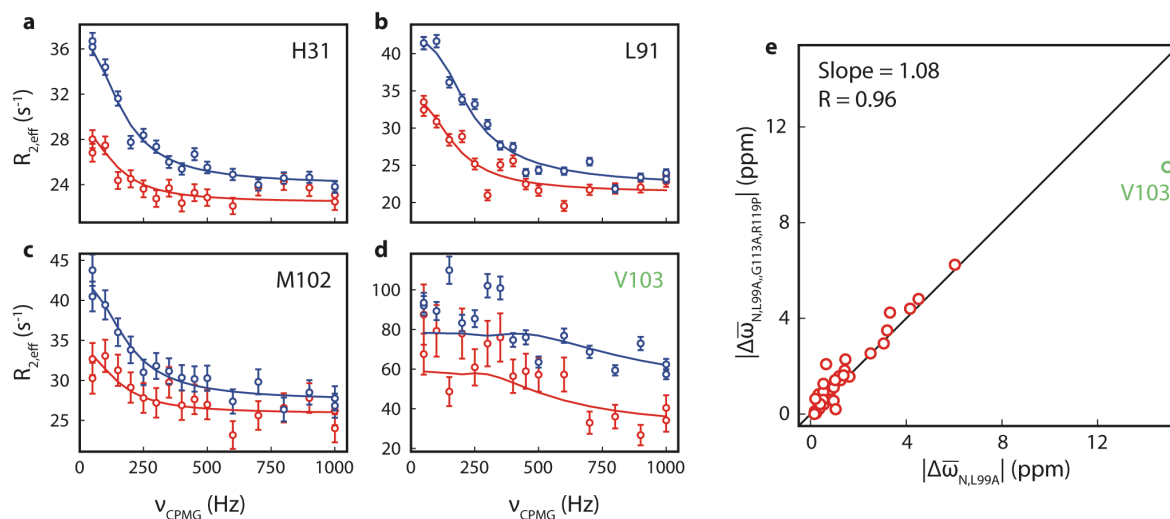
**Supplementary Figure 6 | L99A,G113A,R119P T4L exists in one predominant conformation in solution.**

<sup>1</sup>H-<sup>15</sup>N 2D HSQC correlation map of L99A,G113A,R119P T4L recorded at 500 MHz, 34°C. Correlation peak that are aliased in the <sup>15</sup>N dimension appear negative (blue).



**Supplementary Figure 7 | L99A,G113A,R119P T4L is an excellent mimic of the L99A T4L excited state, as predicted from Rosetta structure based design.**

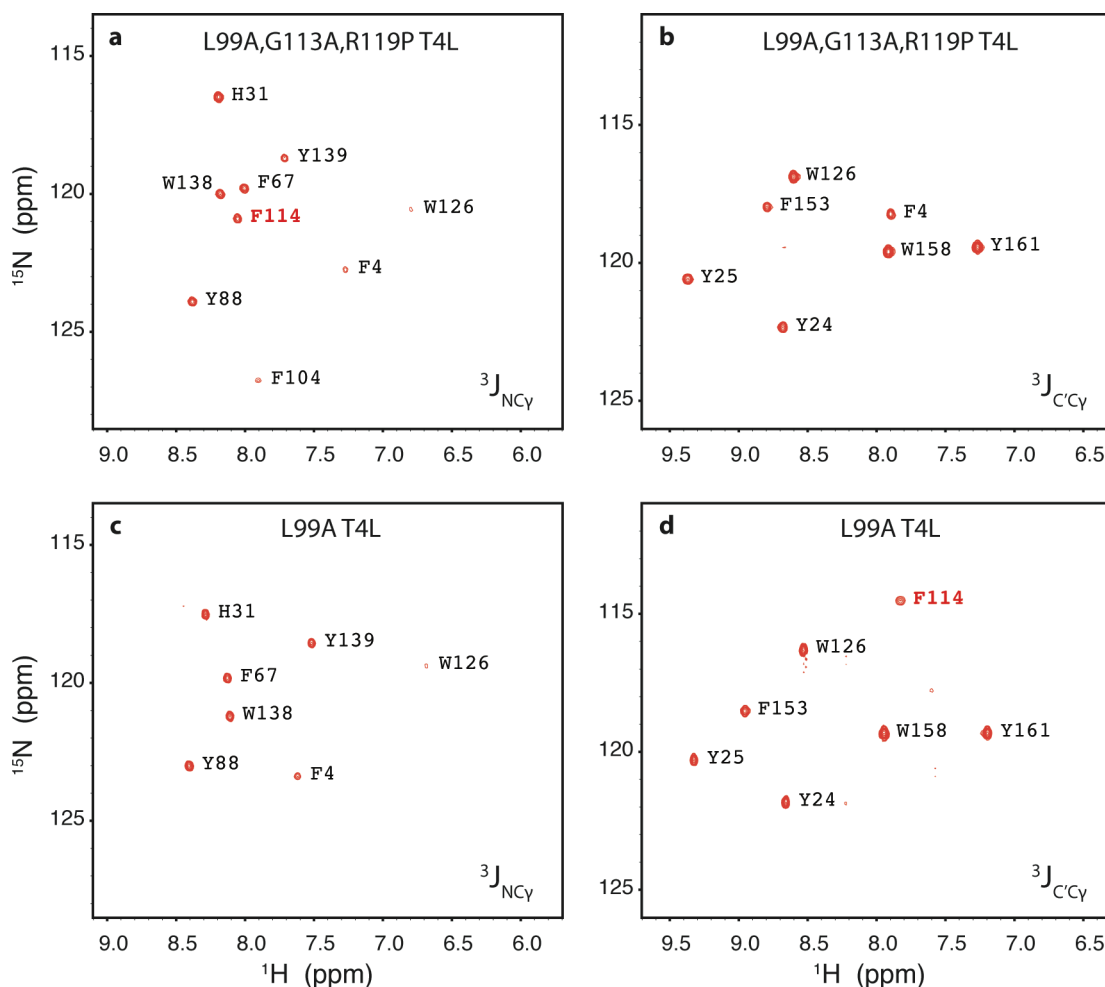
Correlation plot of the  $^{15}\text{N}$  chemical shift differences between the ground states of L99A T4L and L99A,G113A,R119P T4L,  $\Delta\bar{\omega}_{N,\text{direct}}$ , measured directly from spectra (X-axis) and between the major and the minor states of L99A T4L,  $\Delta\bar{\omega}_{N,L99A}$ , obtained via relaxation dispersion (Y-axis). Mutated residues 113 and 119 were excluded from analysis. Residues F114 and M120 (blue) directly follow the two point mutations in the sequence. The resonance of residue V103 (blue) is both highly broadened due to a very large  $\Delta\bar{\omega}$  and partially overlapped in spectra; dispersion profiles for this residue are thus difficult to quantify accurately. Consequently these three residues were removed from the calculation of the slope and Pearson correlation coefficient,  $R$ . The very strong correlation observed establishes that the structures of L99A,G113A,R119P T4L and the L99A T4L excited state are essentially identical.



**Supplementary Figure 8 | L99A,G113A,R119P T4L exchanges between two states: a ground state, corresponding to the excited state of L99A T4L and an excited state, corresponding to the ground state of L99A T4L.**

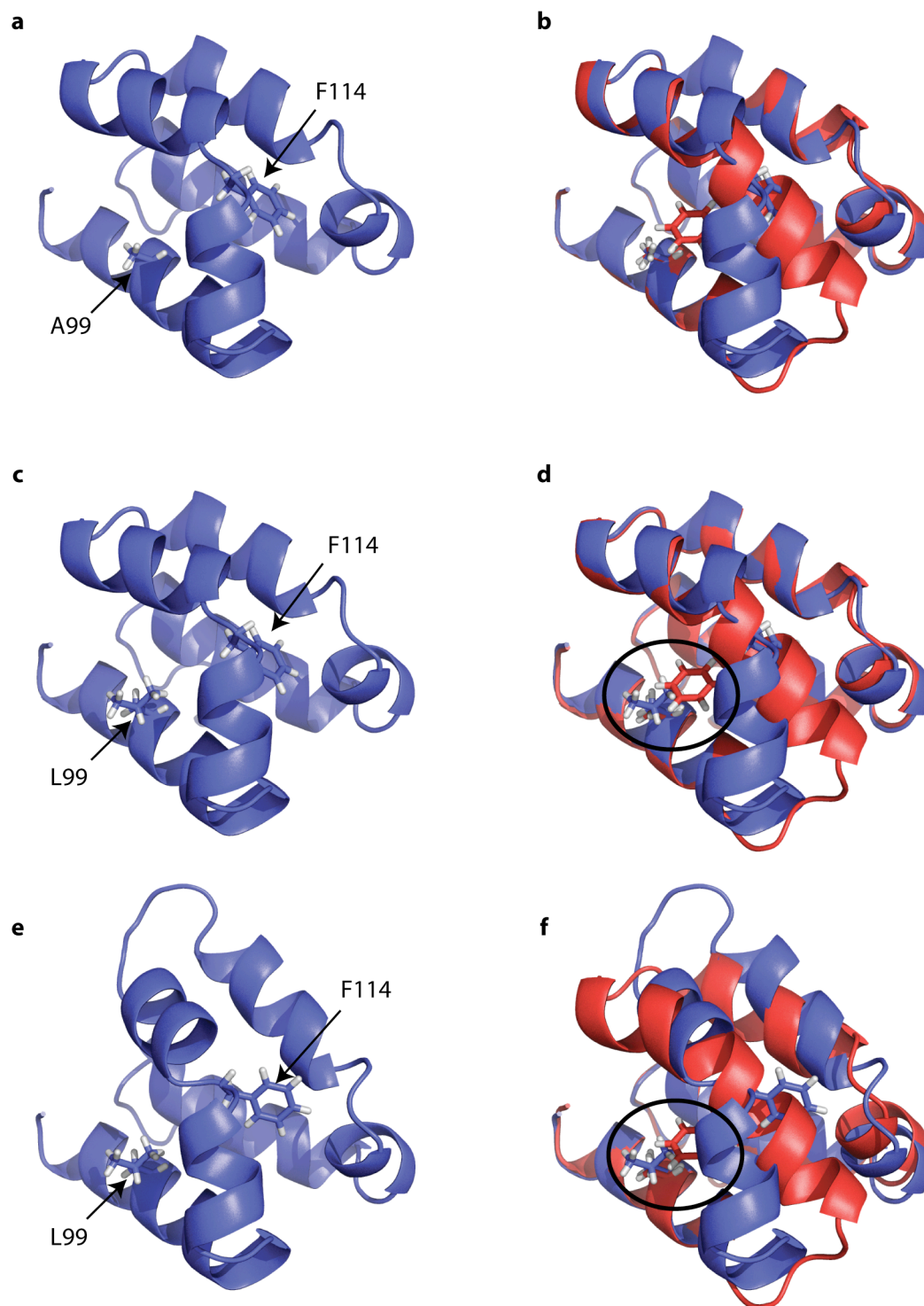
**a-d**, Experimental  $^{15}\text{N}$  relaxation dispersion profiles (circles) for H31 (**a**), L91 (**b**), M102 (**c**) and V103 (**d**) of L99A,G113A,R119P T4L measured at 500 MHz (red) and 800 MHz (blue), 1°C, along with the best fit profiles (solid lines). Error bars indicate uncertainties in  $R_{2,\text{eff}}$  rates. **e**, Correlation plot showing the absolute  $^{15}\text{N}$  chemical shift differences between the major and the minor states of L99A T4L, 25°C, and L99A,G113A,R119P T4L, 1°C. The resonance of residue V103 (green) is highly broadened due to a very large  $\Delta\omega$  and dispersion profiles for this residue are difficult to quantify accurately. Consequently it was removed from the calculation of the slope and Pearson correlation coefficient,  $R$ .





**Supplementary Figure 9 | Cross-validation of the Phe 114  $\chi_1$  angle of the excited state of L99A T4L.**

**a-d**,  $^{15}\text{N}\{-^{13}\text{C}_\gamma\}$  (**a**, **c**) and  $^{13}\text{C}'\{-^{13}\text{C}_\gamma\}$  (**b**, **d**) difference spectra <sup>2,3</sup> showing correlation peaks for L99A,G113A,R119P T4L (**a**, **b**) and L99A T4L (**c**, **d**). Only those aromatic residues with significant three-bond  $^{15}\text{N}\text{-}^{13}\text{C}_\gamma$  (**a**, **c**) and  $^{13}\text{C}'\text{-}^{13}\text{C}_\gamma$  (**b**, **d**) couplings (>1Hz) show correlations. The dephasing delay was 120 ms for the  $^{15}\text{N}\{-^{13}\text{C}_\gamma\}$  difference spectra and 100 ms for the  $^{13}\text{C}'\{-^{13}\text{C}_\gamma\}$  difference spectra.  $^3J_{\text{NC}_\gamma}$  and  $^3J_{\text{C}'\text{C}_\gamma}$  values are functions of the  $\chi_1$  dihedral angle: large  $^3J_{\text{NC}_\gamma}$  values (~2.6 Hz) are expected when  $\chi_1 \approx 180^\circ$ ; large  $^3J_{\text{C}'\text{C}_\gamma}$  values (~4.2 Hz) are expected when  $\chi_1 \approx -60^\circ$ . Note that the  $\chi_1$  of Phe114 (red) changes from gauche- to trans between the ground and excited states of L99A T4L. On the basis of the excited state structure, Phe 114 is the only aromatic residue that undergoes a  $\chi_1$  rotamer change. The quantitative-J experiments establish that this is indeed the case and provide a validation of the CS-Rosetta derived structure of the L99A T4L excited state.



**Supplementary Figure 10 | The L99A T4L excited state structure differs from previously published structures of T4L.**

**a-f**, Ribbon diagram backbone structures from residue 91 to the C-terminus of the ground states of L99A T4L (**a**, blue, 3DMV<sup>1</sup>), G113A T4L (**c**, blue, 1L60<sup>4</sup>) and R119-[A] T4L (**e**, blue, 260L<sup>5</sup>) superimposed with the structure of the excited state of L99A T4L, (red) as determined in the present study (**b, d, f**). Side-chains of residues 99 and 114 are shown using a stick representation. While the crystal structures of L99A T4L and G113A T4L are very similar (**a, c**), there are significant conformational rearrangements involving helices F and G in the crystal structure of R119-[A] T4L whereby these helices become ‘more’ collinear (**e**). The substantial differences in structures of the excited state of L99A T4L, G113A T4L, R119-[A] T4L are highlighted in **b,d,f**. The relative orientation of helices F and G are different in the set of three structures and the  $\chi_1$  angle of Phe 114 is unique in the excited state of L99A; Phe 114  $\chi_1$  torsion angles in the ground state of L99A T4L, G113A T4L, R119-[A] T4L and in the excited state are  $-80^\circ$ ,  $-74^\circ$ ,  $-86^\circ$  and  $-164^\circ$ , respectively. If G113A T4L or R119-[A] T4L were to adopt the excited state backbone conformation for helices F and G as well as a trans  $\chi_1$  rotameric state for Phe 114 this would lead to steric clashes involving Leu 99 and Phe 114, as highlighted by the black ellipses. These potential steric clashes strongly suggest that the L99A mutation is necessary for the excited state conformation.

## Supplementary Tables

### Supplementary Table 1 | Details of the CPMG experiments used to characterize the excited state of L99A T4L.

10% D<sub>2</sub>O refers to (90%H<sub>2</sub>O/10%D<sub>2</sub>O), CT=constant time, ZQ=zero-quantum, DQ=double-quantum. All the experiments were performed at 25°C at the specified fields.

| Site                      | CPMG Experiment                            | Sample (Solvent)  | Field (MHz) | $T_{Relax}$ (ms) | Ref. |
|---------------------------|--|---|-------------|------------------|------|
| Amide/Trp indole nitrogen | <sup>15</sup> N CT TROSY CPMG              | <sup>2</sup> H/ <sup>15</sup> N (10% D <sub>2</sub> O)                      | 500/800     | 25/25            | 6,7  |
| Amide/Trp indole proton   | <sup>1</sup> H CT CPMG                     | <sup>2</sup> H/ <sup>15</sup> N (10% D <sub>2</sub> O)                      | 500/800     | 25/25            | 8    |
| Amide/Trp indole proton   | <sup>1</sup> H/ <sup>15</sup> N ZQ/DQ CPMG | <sup>2</sup> H/ <sup>15</sup> N (10% D <sub>2</sub> O)                      | 500/800     | 25/25            | 9    |
| Carbonyl carbon           | <sup>13</sup> C' CT CPMG                   | <sup>13</sup> C/ <sup>15</sup> N (10% D <sub>2</sub> O)                     | 500/800     | 24/24            | 10   |
| C <sup>α</sup>            | <sup>13</sup> C <sup>α</sup> CT CPMG       | <sup>13</sup> C <sup>α</sup> / <sup>15</sup> N (100% D <sub>2</sub> O)      | 600/800     | 16/16            | 11   |
| H <sup>α</sup>            | <sup>1</sup> H <sup>α</sup> CT CPMG        | <sup>13</sup> C/ <sup>15</sup> N/50% <sup>2</sup> H (100% D <sub>2</sub> O) | 600/800     | 14/14            | 12   |
| Gly C <sup>α</sup>        | Gly <sup>13</sup> C <sup>α</sup> CT CPMG   | <sup>13</sup> C/ <sup>15</sup> N/50% <sup>2</sup> H (100% D <sub>2</sub> O) | 600         | 14               | 13   |
| Gly H <sup>α</sup>        | Gly <sup>1</sup> H <sup>α</sup> CT CPMG    | <sup>13</sup> C/ <sup>15</sup> N/50% <sup>2</sup> H (100% D <sub>2</sub> O) | 600/800     | 16/15            | 13   |

**Supplementary Table 2 | Specific experiments used to obtain the signs of  $\Delta\varpi_{GE}$  and hence the  $\varpi$  values of the L99A T4L excited state.**

H(S/S)QC refers to comparison of peak positions between a pair of HSQC experiments recorded at different static magnetic fields, H(S/M)QC denotes a comparison of peak positions in HSQC and HMQC spectra recorded at the same field. 10%D<sub>2</sub>O = (90%H<sub>2</sub>O/10%D<sub>2</sub>O). All experiments were performed at 25°C at the specified fields.

| Site                      | Sign Experiments                                       | Sample (Solvent)  | Field (MHz) | Reference |
|---------------------------|--|---|-------------|-----------|
| Amide/Trp indole nitrogen | H(S/S)QC<br>H(S/M)QC                                   | <sup>2</sup> H/ <sup>15</sup> N (10% D <sub>2</sub> O)                      | 500/800     | 14        |
| Amide/Trp indole proton   | H(S/S)QC<br><sup>1</sup> H/ <sup>15</sup> N ZQ/DQ CPMG | <sup>2</sup> H/ <sup>15</sup> N (10% D <sub>2</sub> O)                      | 500/800     | 14,15 9   |
| Carbonyl carbon           | H(S/S)QC<br>H(S/M)QC                                   | <sup>13</sup> C/ <sup>15</sup> N (10% D <sub>2</sub> O)                     | 500/800     | 14,16     |
| C <sup>α</sup>            | H(S/S)QC   | <sup>13</sup> C <sup>α</sup> / <sup>15</sup> N (100% D <sub>2</sub> O)      | 600/800     | 14        |
| H <sup>α</sup>            | H(S/S)QC   | <sup>13</sup> C/ <sup>15</sup> N/50% <sup>2</sup> H (100% D <sub>2</sub> O) | 600/800     | 14,15     |
| Gly C <sup>α</sup>        | H(S/S)QC   | <sup>13</sup> C/ <sup>15</sup> N/50% <sup>2</sup> H (100% D <sub>2</sub> O) | 600         | 14        |
| Gly H <sup>α</sup>        | H(S/S)QC   | <sup>13</sup> C/ <sup>15</sup> N/50% <sup>2</sup> H (100% D <sub>2</sub> O) | 600/800     | 14,15     |

**Supplementary Table 3 | Summary of the numbers of chemical shifts of the excited state of L99A T4L that are obtained from CPMG relaxation dispersion experiments.**

The total number of sites in L99A is given in parenthesis in the first column.

| Site                             | Signed $ \Delta\varpi_{GE} $ /Total $ \Delta\varpi_{GE} $ |
|----------------------------------|---|
| Amide nitrogen (164)             | 153/155   |
| Amide proton (161)               | 158/159   |
| Carbonyl carbon (164)            | 154/157   |
| C <sup>α</sup> (164)             | 94/118  |
| Non glycine H <sup>α</sup> (153) | 100/123   |
| Gly H <sup>α</sup> (22 11×2)     | 9/22  |

**Supplementary Table 4 | Chemical shifts of the ground state of L99A T4L, 25°C.**

$^1\text{H}^\alpha$  shifts of glycine residues are indicated as follows:  $\left[\varpi_{^1\text{H}_2^\alpha}\right]/\left[\varpi_{^1\text{H}_3^\alpha}\right]$ .

| Residue | $\varpi_{^{15}\text{N}}$ (ppm) | $\varpi_{^1\text{H}}$ (ppm) | $\varpi_{^{13}\text{C}'}$ (ppm) | $\varpi_{^{13}\text{C}^\alpha}$ (ppm) | $\varpi_{^1\text{H}^\alpha}$ (ppm) |
|---------|--------------------------------|-----------------------------|---------------------------------|---------------------------------------|------------------------------------|
| M1      | --                             | --                          | 170.2                           | 53.3                                  | 3.73                               |
| N2      | 115.7                          | 6.88                        | 174.3                           | 51.7                                  | 4.28                               |
| I3      | 117.4                          | 8.66                        | 174.9                           | 64.1                                  | 4.00                               |
| F4      | 122.9                          | 7.75                        | 176.3                           | 61.0                                  | 3.60                               |
| E5      | 117.7                          | 7.97                        | 178.3                           | 58.1                                  | 3.63                               |
| M6      | 118.2                          | 7.72                        | 177.0                           | 58.7                                  | 3.24                               |
| L7      | 118.5                          | 7.89                        | 178.9                           | 56.0                                  | 3.95                               |
| R8      | 123.8                          | 8.46                        | 178.8                           | 60.2                                  | 3.30                               |
| I9      | 119.7                          | 7.54                        | 177.7                           | 65.0                                  | 3.49                               |
| D10     | 117.7                          | 8.01                        | 177.3                           | 57.8                                  | 4.49                               |
| E11     | 118.4                          | 9.00                        | 177.0                           | 56.4                                  | 4.55                               |
| G12     | 109.3                          | 7.29                        | 171.0                           | 44.1                                  | 4.20/3.76                          |
| L13     | 120.0                          | 8.04                        | 175.0                           | 54.0                                  | 4.38                               |
| R14     | 127.2                          | 7.99                        | 175.9                           | 54.9                                  | 4.67                               |
| L15     | 123.0                          | 8.81                        | 173.7                           | 54.4                                  | 4.37                               |
| K16     | 115.0                          | 7.12                        | 176.1                           | 53.6                                  | 5.42                               |
| I17     | 120.7                          | 7.56                        | 174.8                           | 63.9                                  | 4.13                               |
| Y18     | 127.7                          | 9.39                        | 172.7                           | 55.3                                  | 5.11                               |
| K19     | 121.1                          | 8.33                        | 176.1                           | 55.0                                  | 4.56                               |
| D20     | 124.9                          | 8.51                        | 178.1                           | 52.7                                  | 4.57                               |
| T21     | 110.3                          | 7.98                        | 175.8                           | 65.1                                  | 3.98                               |
| E22     | 120.1                          | 7.97                        | 175.5                           | 56.0                                  | 4.16                               |
| G23     | 106.8                          | 7.83                        | 173.5                           | 44.8                                  | 3.32/3.96                          |
| Y24     | 120.9                          | 8.13                        | 177.6                           | 54.7                                  | 4.95                               |
| Y25     | 122.6                          | 8.73                        | 175.2                           | 59.8                                  | 4.89                               |
| T26     | 120.9                          | 9.33                        | 170.3                           | 61.4                                  | 5.03                               |
| I27     | 120.8                          | 8.77                        | 172.6                           | 60.9                                  | 4.63                               |
| G28     | 112.0                          | 8.48                        | 175.1                           | 45.7                                  | 4.31/3.59                          |
| I29     | 130.1                          | 9.54                        | 176.8                           | 59.3                                  | 4.72                               |
| G30     | 109.1                          | 7.95                        | 173.7                           | 47.5                                  | 4.02/3.79                          |
| H31     | 116.8                          | 8.39                        | 173.2                           | 56.0                                  | 4.64                               |
| L32     | 132.3                          | 8.12                        | 176.4                           | 56.2                                  | 4.04                               |
| L33     | 125.4                          | 9.13                        | 176.2                           | 55.4                                  | 4.50                               |
| T34     | 109.7                          | 7.27                        | 171.6                           | 60.0                                  | 4.46                               |
| K35     | 124.9                          | 8.58                        | 176.7                           | 56.3                                  | 4.26                               |
| S36     | 119.5                          | 8.75                        | --                              | --                                    | --                                 |
| P37     | --                             | --                          | 177.1                           | 63.2                                  | 4.88                               |
| S38     | 116.3                          | 8.23                        | 174.4                           | 56.3                                  | 4.73                               |
| L39     | 133.1                          | 8.96                        | 178.5                           | 57.2                                  | 3.33                               |
| N40     | 116.8                          | 8.28                        | 178.3                           | 56.2                                  | 4.28                               |
| A41     | 123.9                          | 7.75                        | 179.9                           | 54.7                                  | 4.21                               |
| A42     | 121.0                          | 7.59                        | 178.8                           | 55.4                                  | 3.74                               |
| K43     | 117.1                          | 8.40                        | 177.7                           | 60.6                                  | 3.66                               |
| S44     | 113.8                          | 7.93                        | 178.0                           | 61.7                                  | 4.26                               |
| E45     | 120.1                          | 7.99                        | 179.4                           | 58.5                                  | 4.12                               |
| L46     | 122.7                          | 8.30                        | 177.3                           | 57.6                                  | 3.98                               |

| Residue | $\varpi_{15\text{N}}$ (ppm) | $\varpi_{1\text{H}}$ (ppm) | $\varpi_{13\text{C}}$ (ppm) | $\varpi_{13\text{C}^\alpha}$ (ppm) | $\varpi_{1\text{H}^\alpha}$ (ppm) |
|---------|-----------------------------|----------------------------|-----------------------------|------------------------------------|-----------------------------------|
| D47     | 120.2                       | 8.65                       | 181.0                       | 57.3                               | 4.78                              |
| K48     | 120.9                       | 7.79                       | 178.2                       | 59.1                               | 4.01                              |
| A49     | 121.1                       | 7.93                       | 179.5                       | 54.4                               | 4.17                              |
| I50     | 114.8                       | 8.38                       | 177.3                       | 59.0                               | 4.08                              |
| G51     | 109.2                       | 8.38                       | 173.7                       | 45.7                               | 3.78/4.16                         |
| R52     | 115.5                       | 7.65                       | 172.3                       | 53.9                               | 4.57                              |
| N53     | 115.4                       | 8.27                       | 175.4                       | 52.9                               | 4.72                              |
| T54     | 119.6                       | 9.08                       | 177.3                       | 63.7                               | 4.30                              |
| N55     | 120.1                       | 9.48                       | 175.8                       | 54.3                               | 4.38                              |
| G56     | 102.9                       | 8.69                       | 172.1                       | 46.1                               | 4.16/3.30                         |
| V57     | 120.3                       | 7.47                       | 174.9                       | 61.1                               | 5.12                              |
| I58     | 116.6                       | 8.61                       | 175.4                       | 58.6                               | 4.88                              |
| T59     | 109.2                       | 8.69                       | 176.4                       | 59.8                               | 4.62                              |
| K60     | 121.5                       | 9.15                       | 178.1                       | 60.9                               | 3.87                              |
| D61     | 117.2                       | 8.25                       | 179.6                       | 57.2                               | 4.35                              |
| E62     | 121.7                       | 7.71                       | 178.8                       | 58.9                               | 3.88                              |
| A63     | 121.2                       | 8.56                       | 180.4                       | 55.1                               | 3.89                              |
| E64     | 121.2                       | 8.42                       | 177.3                       | 59.6                               | 3.70                              |
| K65     | 121.1                       | 7.66                       | 179.9                       | 60.0                               | 4.07                              |
| L66     | 118.3                       | 7.70                       | 178.0                       | 57.5                               | 3.87                              |
| F67     | 119.2                       | 8.23                       | 177.3                       | 60.8                               | 4.94                              |
| N68     | 118.2                       | 8.78                       | 178.8                       | 56.2                               | 4.29                              |
| Q69     | 119.3                       | 7.60                       | 179.0                       | 58.8                               | 4.24                              |
| D70     | 123.4                       | 8.34                       | 179.9                       | 57.4                               | 4.61                              |
| V71     | 125.1                       | 9.19                       | 177.2                       | 67.6                               | 3.59                              |
| D72     | 119.5                       | 7.76                       | 179.0                       | 57.6                               | 4.35                              |
| A73     | 120.4                       | 7.97                       | 180.4                       | 55.1                               | 4.03                              |
| A74     | 123.4                       | 8.20                       | 180.4                       | 54.7                               | 4.17                              |
| V75     | 119.9                       | 8.73                       | 177.3                       | 67.2                               | 3.21                              |
| R76     | 117.8                       | 8.07                       | 179.5                       | 60.2                               | 3.81                              |
| G77     | 105.2                       | 7.80                       | 176.4                       | 47.2                               | 3.70/3.89                         |
| I78     | 123.5                       | 8.01                       | 177.4                       | 66.1                               | 3.28                              |
| L79     | 114.2                       | 7.96                       | 178.4                       | 56.8                               | 3.83                              |
| R80     | 116.4                       | 7.38                       | 174.8                       | 55.9                               | 4.33                              |
| N81     | 121.7                       | 7.62                       | 174.9                       | 52.9                               | 4.73                              |
| A82     | 128.9                       | 8.96                       | 178.9                       | 54.8                               | 4.08                              |
| K83     | 113.6                       | 8.10                       | 177.7                       | 57.3                               | 4.29                              |
| L84     | 115.7                       | 7.78                       | 177.6                       | 55.5                               | 4.38                              |
| K85     | 122.5                       | 8.80                       | --                          | --                                 | --                                |
| P86     | --                          | --                         | 180.0                       | --                                 | --                                |
| V87     | 115.6                       | 6.83                       | 177.9                       | 65.5                               | 3.51                              |
| Y88     | 122.5                       | 8.52                       | 177.7                       | 63.2                               | 3.73                              |
| D89     | 117.2                       | 9.10                       | 177.8                       | 56.9                               | 4.32                              |
| S90     | 112.8                       | 7.29                       | 173.9                       | 59.7                               | 4.42                              |
| L91     | 121.9                       | 7.26                       | 176.3                       | 54.9                               | 4.42                              |
| D92     | 115.5                       | 7.40                       | 175.6                       | 52.2                               | 4.62                              |
| A93     | 119.8                       | 8.72                       | 179.9                       | 56.3                               | 3.80                              |
| V94     | 118.8                       | 7.70                       | 177.2                       | 65.9                               | 3.04                              |
| R95     | 118.3                       | 7.61                       | 178.9                       | 60.4                               | 3.46                              |
| R96     | 118.3                       | 8.71                       | 177.9                       | 60.7                               | 3.66                              |
| A97     | 120.4                       | 7.17                       | 179.1                       | 55.4                               | 4.07                              |
| A98     | 119.1                       | 6.97                       | 177.5                       | 55.3                               | 3.67                              |

| Residue | $\varpi_{15\text{N}}$ (ppm) | $\varpi_{1\text{H}}$ (ppm) | $\varpi_{13\text{C'}}$ (ppm) | $\varpi_{13\text{C}^\alpha}$ (ppm) | $\varpi_{1\text{H}^\alpha}$ (ppm) |
|---------|-----------------------------|----------------------------|------------------------------|------------------------------------|-----------------------------------|
| A99     | 119.7                       | 7.64                       | 178.9                        | 55.6                               | 4.13                              |
| I100    | 118.3                       | 8.55                       | 177.3                        | 65.5                               | 3.45                              |
| N101    | 119.4                       | 8.42                       | 177.0                        | 57.5                               | 4.08                              |
| M102    | 115.3                       | 7.64                       | 178.7                        | 60.3                               | 4.37                              |
| V103    | 120.4                       | 8.16                       | 179.5                        | 66.9                               | 3.30                              |
| F104    | 123.4                       | 9.17                       | 177.7                        | 61.5                               | 4.17                              |
| Q105    | 117.5                       | 7.94                       | 177.8                        | 59.0                               | 4.35                              |
| M106    | 113.2                       | 8.67                       | 176.6                        | 55.6                               | 4.84                              |
| G107    | 111.6                       | 8.46                       | 173.3                        | 44.5                               | 4.48/4.00                         |
| E108    | 120.4                       | 9.07                       | 178.3                        | 60.6                               | 3.67                              |
| T109    | 111.8                       | 8.39                       | 177.2                        | 65.6                               | 3.85                              |
| G110    | 109.1                       | 7.54                       | 176.3                        | 46.8                               | 3.82/3.57                         |
| V111    | 122.3                       | 7.66                       | 177.1                        | 66.6                               | 3.64                              |
| A112    | 118.0                       | 8.08                       | 177.1                        | 54.0                               | 3.90                              |
| G113    | 102.2                       | 7.32                       | 175.9                        | 45.4                               | 4.14/3.79                         |
| F114    | 123.3                       | 7.93                       | 176.4                        | 56.7                               | 4.91                              |
| T115    | 114.6                       | 7.80                       | 176.6                        | 67.2                               | 3.67                              |
| N116    | 119.1                       | 8.97                       | --                           | --                                 | --                                |
| S117    | --                          | --                         | 176.0                        | --                                 | --                                |
| L118    | 120.1                       | 8.59                       | 178.4                        | 58.3                               | 3.86                              |
| R119    | 118.5                       | 7.44                       | 178.5                        | 59.2                               | 4.05                              |
| M120    | 116.4                       | 7.61                       | 178.4                        | 59.0                               | 3.97                              |
| L121    | 118.6                       | 8.17                       | 179.9                        | 57.6                               | 3.85                              |
| Q122    | 120.7                       | 8.22                       | 176.6                        | 58.9                               | 3.94                              |
| Q123    | 113.6                       | 7.25                       | 174.5                        | 55.2                               | 4.07                              |
| K124    | 114.6                       | 7.39                       | 174.9                        | --                                 | --                                |
| R125    | 120.0                       | 7.71                       | 177.2                        | 53.6                               | 4.24                              |
| W126    | 118.7                       | 6.76                       | 178.9                        | 59.0                               | 4.35                              |
| D127    | 116.5                       | 8.55                       | 178.9                        | 57.7                               | 4.42                              |
| E128    | 120.6                       | 8.31                       | 179.9                        | 59.6                               | 3.95                              |
| A129    | 122.3                       | 8.65                       | 178.7                        | 55.4                               | 4.01                              |
| A130    | 118.8                       | 8.16                       | 180.1                        | 55.7                               | 4.08                              |
| V131    | 118.2                       | 7.79                       | 178.3                        | 66.1                               | 3.58                              |
| N132    | 118.3                       | 7.73                       | 179.0                        | 57.4                               | 4.33                              |
| L133    | 121.8                       | 8.97                       | 178.8                        | 58.1                               | 4.21                              |
| A134    | 119.3                       | 7.33                       | 176.7                        | 52.7                               | 3.66                              |
| K135    | 118.3                       | 7.17                       | 176.1                        | 55.3                               | 4.41                              |
| S136    | 113.2                       | 7.55                       | 174.7                        | 57.9                               | 4.66                              |
| R137    | 123.5                       | 9.10                       | 178.5                        | 59.3                               | 4.25                              |
| W138    | 120.8                       | 8.30                       | 176.1                        | 59.6                               | 4.55                              |
| Y139    | 117.8                       | 7.62                       | 175.2                        | 60.6                               | 3.00                              |
| N140    | 110.9                       | 7.24                       | 177.0                        | 55.3                               | 4.36                              |
| Q141    | 118.4                       | 8.32                       | 177.5                        | 58.3                               | 4.21                              |
| T142    | 106.9                       | 7.63                       | --                           | --                                 | --                                |
| P143    | --                          | --                         | 178.5                        | 65.2                               | 3.78                              |
| N144    | 116.2                       | 7.99                       | 177.5                        | 56.5                               | 4.28                              |
| R145    | 120.3                       | 7.78                       | 176.9                        | 58.7                               | 3.90                              |
| A146    | 118.6                       | 8.00                       | 179.4                        | 55.4                               | 3.71                              |
| K147    | 115.6                       | 8.30                       | 180.1                        | 60.6                               | 3.94                              |
| R148    | 119.3                       | 7.29                       | 179.7                        | 60.9                               | 3.77                              |
| V149    | 125.0                       | 8.61                       | 178.5                        | 68.3                               | 3.39                              |
| I150    | 120.3                       | 9.59                       | 178.5                        | 67.5                               | 3.89                              |



| Residue | $\varpi_{^{15}\text{N}}$ (ppm) | $\varpi_{^1\text{H}}$ (ppm) | $\varpi_{^{13}\text{C}'}$ (ppm) | $\varpi_{^{13}\text{C}^\alpha}$ (ppm) | $\varpi_{^1\text{H}^\alpha}$ (ppm) |
|---------|--------------------------------|-----------------------------|---------------------------------|---------------------------------------|------------------------------------|
| T151    | 117.4                          | 8.56                        | 176.4                           | 67.4                                  | 3.94                               |
| T152    | 122.5                          | 7.90                        | 176.9                           | 67.4                                  | 3.98                               |
| F153    | 120.1                          | 8.30                        | 178.2                           | 61.5                                  | 4.15                               |
| R154    | 118.8                          | 9.04                        | 178.0                           | 60.2                                  | 4.10                               |
| T155    | 104.1                          | 8.14                        | 176.7                           | 62.2                                  | 4.24                               |
| G156    | 111.7                          | 8.50                        | 172.9                           | 46.0                                  | 3.66/3.13                          |
| T157    | 108.1                          | 7.57                        | 174.0                           | 58.7                                  | 4.65                               |
| W158    | 117.9                          | 8.60                        | 178.1                           | 56.1                                  | 5.02                               |
| D159    | 119.5                          | 7.97                        | 177.4                           | 58.7                                  | 4.20                               |
| A160    | 119.9                          | 9.14                        | 177.3                           | 53.6                                  | 4.17                               |
| Y161    | 113.6                          | 8.07                        | 175.1                           | 58.9                                  | 4.23                               |
| K162    | 119.6                          | 7.18                        | 176.1                           | 57.6                                  | 4.08                               |
| N163    | 116.7                          | 8.62                        | 173.7                           | 54.0                                  | 4.53                               |
| L164    | 125.6                          | 7.35                        | --                              | --                                    | --                                 |

**Supplementary Table 5 | Chemical shift differences between the ground and the excited states of L99A T4L ( $\Delta\varpi = \varpi_E - \varpi_G$ ) as quantified by relaxation dispersion experiments (25°C).**

Values that could not be ‘signed’ are indicated by ‘±’.  $^1\text{H}^\alpha$  shift differences of glycine residues are indicated as follows:  $\left[\Delta\varpi_{\text{H}_2^\alpha}\right]/\left[\Delta\varpi_{\text{H}_3^\alpha}\right]$ .

| Residue | $\Delta\varpi_{\text{N}} \text{ (ppm)}$ | $\Delta\varpi_{\text{H}} \text{ (ppm)}$ | $\Delta\varpi_{\text{C}'} \text{ (ppm)}$ | $\Delta\varpi_{\text{C}^\alpha} \text{ (ppm)}$ | $\Delta\varpi_{\text{H}^\alpha} \text{ (ppm)}$ |
|---------|---|---|--|--|--|
| M1      | --                                      | --                                      | --                                       | ±0.2   | ±0.03  |
| N2      | ±0.7                                    | ±0.04                                   | --                                       | 0.7  | 0.23   |
| I3      | -0.9                                    | 0.23                                    | -0.9                                     | --   | ±0.12  |
| F4      | -1.2                                    | -0.46                                   | 0.8                                      | -0.4   | 0.48   |
| E5      | 0.5                                     | -0.09                                   | ±0.1                                     | 0.7  | 0.21   |
| M6      | ±0.4                                    | ±0.24                                   | 0.1                                      | ±0.4   | ±0.01  |
| L7      | -2.4                                    | 0.14                                    | -0.4                                     | --   | ±0.09  |
| R8      | -3.2                                    | -0.34                                   | 1.3                                      | ±0.5   | 0.26   |
| I9      | 1.2                                     | 0.08                                    | -0.3                                     | --   | ±0.11  |
| D10     | ±0.4                                    | ±0.00                                   | 0.6                                      | 0.5  | ±0.11  |
| E11     | -3.3                                    | -0.16                                   | 1.3                                      | -1.0   | ±0.04  |
| G12     | ±0.7                                    | --                                      | -0.9                                     | ±0.5   | ±0.21/±0.08                                    |
| L13     | -2.7                                    | -0.27                                   | 0.6                                      | --   | ±0.12  |
| R14     | -0.7                                    | 0.28                                    | ±0.0                                     | -0.8   | ±0.02  |
| L15     | ±0.1                                    | ±0.05                                   | ±0.2                                     | --   | --   |
| K16     | ±0.2                                    | ±0.02                                   | ±0.0                                     | ±0.4   | ±0.03  |
| I17     | ±0.1                                    | ±0.00                                   | ±0.1                                     | --   | ±0.04  |
| Y18     | ±0.1                                    | ±0.01                                   | ±0.2                                     | ±0.1   | ±0.01  |
| K19     | 1.4                                     | 0.02                                    | ±0.2                                     | ±0.2   | ±0.03  |
| D20     | ±0.5                                    | ±0.00                                   | ±0.2                                     | ±0.3   | ±0.05  |
| T21     | ±0.6                                    | ±0.04                                   | ±0.2                                     | ±0.4   | ±0.00  |
| E22     | ±0.0                                    | --                                      | ±0.0                                     | ±0.4   | ±0.03  |
| G23     | ±0.2                                    | ±0.01                                   | ±0.1                                     | ±0.4   | ±0.05/±0.05                                    |
| Y24     | -0.3                                    | 0.06                                    | ±0.2                                     | ±0.4   | ±0.06  |
| Y25     | ±0.3                                    | ±0.00                                   | ±0.1                                     | ±0.3   | ±0.06  |
| T26     | -0.3                                    | 0.07                                    | ±0.4                                     | -0.5   | ±0.16  |
| I27     | -0.3                                    | 0.08                                    | ±0.2                                     | --   | ±0.07  |
| G28     | ±0.3                                    | 0.11                                    | -0.4                                     | ±0.4   | ±0.10/±0.13                                    |
| I29     | 4.2                                     | 0.72                                    | -0.1                                     | --   | ±0.13  |
| G30     | -1.4                                    | 0.21                                    | -0.6                                     | ±0.9   | ±0.10/±0.14                                    |
| H31     | -1.3                                    | -0.11                                   | ±0.3                                     | 0.7  | -0.30  |
| L32     | -0.3                                    | -0.21                                   | ±0.0                                     | --   | ±0.11  |
| L33     | 0.4                                     | -0.05                                   | ±0.1                                     | --   | ±0.00  |
| T34     | ±0.3                                    | ±0.01                                   | ±0.1                                     | ±0.2   | ±0.01  |
| K35     | ±0.1                                    | -0.00                                   | ±0.1                                     | --   | --   |
| S36     | ±0.1                                    | ±0.05                                   | --                                       | --   | --   |
| P37     | --                                      | --                                      | ±0.2                                     | ±0.3   | ±0.02  |
| S38     | ±0.1                                    | ±0.00                                   | ±0.1                                     | ±0.1   | ±0.03  |
| L39     | ±0.4                                    | ±0.00                                   | ±0.2                                     | --   | ±0.11  |
| N40     | ±0.1                                    | ±0.00                                   | ±0.0                                     | --   | --   |

| Residue | $\Delta\varpi_{^{15}\text{N}}$ (ppm) | $\Delta\varpi_{^1\text{H}}$ (ppm) | $\Delta\varpi_{^{13}\text{C}'}$ (ppm) | $\Delta\varpi_{^{13}\text{C}^\alpha}$ (ppm) | $\Delta\varpi_{^1\text{H}^\alpha}$ (ppm) |
|---------|--------------------------------------|-----------------------------------|---------------------------------------|---|--|
| A41     | $\pm 0.2$                            | $\pm 0.00$                        | $\pm 0.2$                             | $\pm 0.2$                                   | $\pm 0.00$                               |
| A42     | $\pm 0.0$                            | $\pm 0.01$                        | $\pm 0.2$                             | $\pm 0.1$                                   | $\pm 0.02$                               |
| K43     | $\pm 0.2$                            | $\pm 0.00$                        | $\pm 0.2$                             | --  | --                                       |
| S44     | $\pm 0.1$                            | $\pm 0.00$                        | $\pm 0.2$                             | $\pm 0.2$                                   | $\pm 0.11$                               |
| E45     | $\pm 0.5$                            | --                                | $\pm 0.2$                             | $\pm 0.3$                                   | $\pm 0.06$                               |
| L46     | $\pm 0.0$                            | $\pm 0.00$                        | $\pm 0.1$                             | --  | $\pm 0.10$                               |
| D47     | $\pm 0.1$                            | $\pm 0.09$                        | $\pm 0.0$                             | $\pm 0.1$                                   | $\pm 0.11$                               |
| K48     | $\pm 0.0$                            | $\pm 0.04$                        | $\pm 0.2$                             | $\pm 0.3$                                   | $\pm 0.02$                               |
| A49     | $\pm 0.0$                            | $\pm 0.00$                        | $\pm 0.2$                             | $\pm 0.1$                                   | $\pm 0.03$                               |
| I50     | $\pm 0.0$                            | $\pm 0.06$                        | $\pm 0.1$                             | --  | $\pm 0.05$                               |
| G51     | $\pm 0.1$                            | $\pm 0.07$                        | $\pm 0.0$                             | $\pm 0.3$                                   | $\pm 0.09/\pm 0.05$                      |
| R52     | $\pm 0.7$                            | 0.01                              | $\pm 0.1$                             | $\pm 0.5$                                   | $\pm 0.01$                               |
| N53     | $\pm 0.0$                            | 0.00                              | $\pm 0.1$                             | --  | --                                       |
| T54     | $\pm 0.0$                            | $\pm 0.01$                        | $\pm 0.2$                             | $\pm 0.4$                                   | $\pm 0.04$                               |
| N55     | $\pm 0.1$                            | $\pm 0.00$                        | $\pm 0.0$                             | $\pm 0.5$                                   | --                                       |
| G56     | $\pm 0.1$                            | $\pm 0.00$                        | $\pm 0.2$                             | $\pm 0.4$                                   | $\pm 0.05/\pm 0.04$                      |
| V57     | $\pm 0.1$                            | $\pm 0.01$                        | $\pm 0.1$                             | -0.5  | $\pm 0.00$                               |
| I58     | 0.3                                  | -0.06                             | $\pm 0.1$                             | --  | $\pm 0.06$                               |
| T59     | $\pm 0.2$                            | $\pm 0.00$                        | $\pm 0.0$                             | $\pm 0.2$                                   | $\pm 0.00$                               |
| K60     | $\pm 0.2$                            | $\pm 0.00$                        | $\pm 0.3$                             | $\pm 0.3$                                   | $\pm 0.07$                               |
| D61     | $\pm 0.1$                            | $\pm 0.00$                        | $\pm 0.2$                             | $\pm 0.3$                                   | $\pm 0.01$                               |
| E62     | -0.2                                 | $\pm 0.05$                        | $\pm 0.3$                             | $\pm 0.6$                                   | $\pm 0.02$                               |
| A63     | $\pm 0.2$                            | $\pm 0.00$                        | 0.4                                   | $\pm 0.1$                                   | $\pm 0.08$                               |
| E64     | -1.3                                 | 0.00                              | 0.6                                   | 0.7   | 0.48                                     |
| K65     | 1.1                                  | 0.23                              | $\pm 0.3$                             | -0.3  | $\pm 0.11$                               |
| L66     | $\pm 0.3$                            | $\pm 0.04$                        | $\pm 0.2$                             | --  | $\pm 0.14$                               |
| F67     | -0.3                                 | -0.17                             | $\pm 0.3$                             | -1.2  | 0.33                                     |
| N68     | -0.5                                 | -0.31                             | $\pm 0.0$                             | --  | --                                       |
| Q69     | -0.8                                 | 0.19                              | $\pm 0.2$                             | --  | --                                       |
| D70     | -0.3                                 | 0.52                              | $\pm 0.3$                             | $\pm 0.5$                                   | $\pm 0.07$                               |
| V71     | 0.6                                  | -0.17                             | $\pm 0.1$                             | 0.8   | $\pm 0.06$                               |
| D72     | -0.6                                 | -0.15                             | -0.4                                  | $\pm 0.3$                                   | $\pm 0.01$                               |
| A73     | -0.5                                 | 0.37                              | $\pm 0.3$                             | $\pm 0.2$                                   | $\pm 0.01$                               |
| A74     | -0.4                                 | -0.33                             | -0.7                                  | $\pm 0.3$                                   | $\pm 0.02$                               |
| V75     | $\pm 1.7$                            | -0.56                             | $\pm 0.7$                             | $\pm 0.6$                                   | --                                       |
| R76     | $\pm 0.3$                            | 0.35                              | -0.7                                  | $\pm 0.6$                                   | $\pm 0.03$                               |
| G77     | 0.6                                  | 0.21                              | -0.3                                  | -1.2  | $\pm 0.09/\pm 0.08$                      |
| I78     | -1.8                                 | -0.51                             | -0.2                                  | --  | --                                       |
| L79     | 1.0                                  | -0.05                             | 0.4                                   | --  | $\pm 0.00$                               |
| R80     | -1.6                                 | 0.32                              | 0.3                                   | $\pm 0.5$                                   | $\pm 0.09$                               |
| N81     | -0.8                                 | -0.12                             | -0.3                                  | --  | --                                       |
| A82     | -1.0                                 | -0.11                             | $\pm 0.2$                             | $\pm 0.2$                                   | $\pm 0.04$                               |
| K83     | 0.5                                  | -0.04                             | 0.2                                   | $\pm 0.2$                                   | $\pm 0.02$                               |
| L84     | 0.7                                  | -0.25                             | 0.8                                   | --  | $\pm 0.13$                               |
| K85     | -1.4                                 | -0.33                             | --                                    | --  | --                                       |
| P86     | --                                   | --                                | $\pm 0.2$                             | --  | --                                       |
| V87     | $\pm 0.0$                            | 0.06                              | 0.6                                   | $\pm 0.2$                                   | 0.24                                     |
| Y88     | $\pm 0.0$                            | -0.06                             | $\pm 0.4$                             | -0.3  | 0.17                                     |
| D89     | $\pm 0.3$                            | $\pm 0.02$                        | $\pm 0.3$                             | $\pm 0.4$                                   | $\pm 0.05$                               |
| S90     | 0.5                                  | 0.08                              | 0.5                                   | $\pm 0.3$                                   | $\pm 0.01$                               |
| L91     | 1.5                                  | 0.20                              | $\pm 0.1$                             | --  | $\pm 0.02$                               |
| D92     | $\pm 0.6$                            | $\pm 0.03$                        | $\pm 0.2$                             | $\pm 0.4$                                   | $\pm 0.05$                               |

| Residue | $\Delta\varpi_{^{15}\text{N}}$ (ppm) | $\Delta\varpi_{^1\text{H}}$ (ppm) | $\Delta\varpi_{^{13}\text{C}'}$ (ppm) | $\Delta\varpi_{^{13}\text{C}^\alpha}$ (ppm) | $\Delta\varpi_{^1\text{H}^\alpha}$ (ppm) |
|---------|--------------------------------------|-----------------------------------|---------------------------------------|---|--|
| A93     | $\pm 0.0$                            | -0.03                             | $\pm 0.2$                             | $\pm 0.4$                                   | $\pm 0.02$                               |
| V94     | $\pm 0.0$                            | 0.12                              | 0.2                                   | $\pm 0.3$                                   | --                                       |
| R95     | 0.7                                  | 0.15                              | -0.4                                  | $\pm 0.5$                                   | $\pm 0.12$                               |
| R96     | -0.3                                 | -0.08                             | $\pm 0.2$                             | --  | --                                       |
| A97     | -0.4                                 | -0.15                             | -0.4                                  | $\pm 0.2$                                   | $\pm 0.06$                               |
| A98     | -0.2                                 | 0.23                              | $\pm 0.3$                             | $\pm 0.1$                                   | $\pm 0.15$                               |
| A99     | 0.1                                  | 0.16                              | $\pm 0.8$                             | $\pm 0.0$                                   | -0.60                                    |
| I100    | -2.1                                 | -0.46                             | -0.3                                  | --  | $\pm 0.11$                               |
| N101    | -2.9                                 | -0.32                             | 0.5                                   | $\pm 0.7$                                   | $\pm 0.18$                               |
| M102    | -1.4                                 | 0.08                              | $\pm 0.8$                             | 0.8   | -0.71                                    |
| V103    | -14.9                                | -0.84                             | -4.0                                  | -5.2  | --                                       |
| F104    | 3.1                                  | -1.29                             | -0.8                                  | --  | --                                       |
| Q105    | 2.4                                  | 0.60                              | -2.8                                  | --  | --                                       |
| M106    | 4.1                                  | 0.20                              | 0.5                                   | -0.8  | -0.70                                    |
| G107    | -3.6                                 | -0.64                             | 1.6                                   | 1.6   | $\pm 0.53/\pm 0.07$                      |
| E108    | 2.6                                  | -0.29                             | 1.2                                   | --  | --                                       |
| T109    | 0.2                                  | 0.22                              | 1.0                                   | -0.6  | 0.35                                     |
| G110    | 7.1                                  | 1.09                              | -0.5                                  | $\pm 0.5$                                   | $\pm 0.62/\pm 0.34$                      |
| V111    | -1.9                                 | 0.41                              | 2.0                                   | 0.5   | $\pm 0.11$                               |
| A112    | 6.0                                  | 0.22                              | 3.0                                   | 1.4   | 0.32                                     |
| G113    | 3.7                                  | 0.90                              | -0.2                                  | 1.6   | $\pm 0.21\pm 0.23$                       |
| F114    | -0.9                                 | 0.18                              | 1.0                                   | 3.2   | -0.70                                    |
| T115    | $\pm 0.4$                            | 0.58                              | $\pm 0.1$                             | -0.6  | $\pm 0.15$                               |
| N116    | $\pm 0.3$                            | -0.69                             | --                                    | --  | --                                       |
| S117    | --                                   | --                                | -1.1                                  | --  | --                                       |
| L118    | 1.0                                  | -1.25                             | -0.5                                  | --  | 0.31                                     |
| R119    | -1.2                                 | 0.89                              | 0.2                                   | -0.5  | $\pm 0.08$                               |
| M120    | -0.9                                 | -0.20                             | -0.3                                  | $\pm 0.4$                                   | $\pm 0.04$                               |
| L121    | -1.0                                 | -0.49                             | $\pm 0.3$                             | --  | $\pm 0.09$                               |
| Q122    | -0.3                                 | 0.47                              | 0.4                                   | 0.5   | $\pm 0.08$                               |
| Q123    | $\pm 0.4$                            | $\pm 0.07$                        | $\pm 0.3$                             | $\pm 0.3$                                   | $\pm 0.07$                               |
| K124    | 0.2                                  | -0.10                             | $\pm 0.1$                             | --  | --                                       |
| R125    | -0.5                                 | $\pm 0.04$                        | $\pm 0.1$                             | 0.6   | $\pm 0.04$                               |
| W126    | 0.5                                  | 0.13                              | $\pm 0.2$                             | --  | --                                       |
| D127    | $\pm 0.4$                            | $\pm 0.02$                        | $\pm 0.1$                             | $\pm 0.3$                                   | $\pm 0.00$                               |
| E128    | $\pm 0.0$                            | $\pm 0.07$                        | $\pm 0.2$                             | $\pm 0.4$                                   | $\pm 0.02$                               |
| A129    | $\pm 0.5$                            | -0.02                             | $\pm 0.2$                             | $\pm 0.4$                                   | $\pm 0.02$                               |
| A130    | 0.7                                  | 0.11                              | -0.4                                  | $\pm 0.0$                                   | $\pm 0.13$                               |
| V131    | -0.6                                 | 0.09                              | $\pm 0.3$                             | $\pm 0.3$                                   | $\pm 0.01$                               |
| N132    | $\pm 0.4$                            | $\pm 0.14$                        | $\pm 0.2$                             | $\pm 0.5$                                   | $\pm 0.02$                               |
| L133    | -0.7                                 | -0.10                             | 0.9                                   | --  | --                                       |
| A134    | 2.2                                  | 0.40                              | 0.3                                   | $\pm 0.7$                                   | $\pm 0.15$                               |
| K135    | -3.2                                 | 0.11                              | 0.7                                   | 0.6   | $\pm 0.04$                               |
| S136    | 2.5                                  | $\pm 0.03$                        | -0.3                                  | 1.0   | -0.23                                    |
| R137    | -1.1                                 | -0.40                             | $\pm 0.3$                             | -0.8  | $\pm 0.03$                               |
| W138    | -0.9                                 | 0.18                              | -0.4                                  | 2.5   | -0.37                                    |
| Y139    | 0.9                                  | 0.23                              | 0.5                                   | -0.4  | $\pm 0.10$                               |
| N140    | $\pm 0.9$                            | --                                | -0.5                                  | $\pm 0.5$                                   | $\pm 0.12$                               |
| Q141    | -1.0                                 | -0.54                             | -0.9                                  | $\pm 1.1$                                   | --                                       |
| T142    | 4.5                                  | -0.58                             | --                                    | --  | --                                       |
| P143    | --                                   | --                                | $\pm 0.2$                             | $\pm 0.5$                                   | 0.16                                     |
| N144    | 0.3                                  | 0.21                              | $\pm 0.0$                             | -0.4  | $\pm 0.08$                               |

| Residue | $\Delta\varpi_{^{15}\text{N}}$ (ppm) | $\Delta\varpi_{^1\text{H}}$ (ppm) | $\Delta\varpi_{^{13}\text{C}'}$ (ppm) | $\Delta\varpi_{^{13}\text{C}^\alpha}$ (ppm) | $\Delta\varpi_{^1\text{H}^\alpha}$ (ppm) |
|---------|--------------------------------------|-----------------------------------|---------------------------------------|---|--|
| R145    | 1.6                                  | 0.07                              | $\pm 0.1$                             | -1.2  | $\pm 0.08$                               |
| A146    | 0.5                                  | 0.44                              | $\pm 0.3$                             | -0.1  | 0.28                                     |
| K147    | 1.0                                  | -0.23                             | $\pm 0.2$                             | $\pm 0.4$                                   | $\pm 0.15$                               |
| R148    | $\pm 0.0$                            | 0.20                              | 0.5                                   | $\pm 0.5$                                   | $\pm 0.04$                               |
| V149    | 0.2                                  | 0.27                              | $\pm 0.3$                             | $\pm 0.5$                                   | $\pm 0.06$                               |
| I150    | 0.8                                  | -0.27                             | $\pm 0.3$                             | --  | $\pm 0.09$                               |
| T151    | 0.5                                  | -0.18                             | $\pm 0.2$                             | 0.1   | $\pm 0.11$                               |
| T152    | -0.7                                 | 0.08                              | -0.6                                  | 0.2   | 0.14                                     |
| F153    | 0.4                                  | 0.12                              | -0.4                                  | $\pm 1.1$                                   | --                                       |
| R154    | -0.8                                 | -0.31                             | 0.4                                   | -0.5  | $\pm 0.12$                               |
| T155    | 1.0                                  | 0.07                              | $\pm 0.2$                             | $\pm 0.4$                                   | $\pm 0.02$                               |
| G156    | $\pm 0.3$                            | $\pm 0.00$                        | $\pm 0.2$                             | $\pm 0.5$                                   | $\pm 0.11/\pm 0.10$                      |
| T157    | 0.3                                  | $\pm 0.04$                        | $\pm 0.1$                             | $\pm 0.3$                                   | $\pm 0.01$                               |
| W158    | $\pm 0.0$                            | -0.09                             | $\pm 0.2$                             | $\pm 0.3$                                   | $\pm 0.02$                               |
| D159    | $\pm 0.1$                            | $\pm 0.02$                        | $\pm 0.0$                             | $\pm 0.4$                                   | $\pm 0.00$                               |
| A160    | $\pm 0.1$                            | $\pm 0.06$                        | $\pm 0.2$                             | $\pm 0.3$                                   | $\pm 0.02$                               |
| Y161    | $\pm 0.2$                            | -0.00                             | $\pm 0.1$                             | --  | --                                       |
| K162    | $\pm 0.0$                            | 0.09                              | $\pm 0.1$                             | --  | $\pm 0.10$                               |
| N163    | $\pm 0.0$                            | 0.01                              | $\pm 0.1$                             | $\pm 0.4$                                   | $\pm 0.03$                               |
| L164    | 0.2                                  | 0.08                              | --                                    | --  | --                                       |

**Supplementary Table 6 | Comparison of backbone (N,C $\alpha$ ,C') RMSDs between various sets of structures.**

RMSD values were calculated for only those regions that were allowed to vary during computations (100-120, 132-146) after the molecules were first superimposed using regions that were held fixed (residues 1-99,121-131,147-164).

| No | Structures Being Compared                       |   | RMSD (Å) |
|----|---|---|----------|
| 1  | Calculated Ground State Structures (10)         | Calculated Ground State Structures (10)         | 0.6±0.2  |
| 2  | Calculated Ground State Structures (10)         | Ground State Crystal Structure (3DMV)           | 0.6±0.2  |
| 3  | Calculated Excited State Structures (10)        | Calculated Excited State Structures (10)        | 0.7±0.2  |
| 4  | Calculated Excited State Structures (10)        | Ground State Crystal Structure (3DMV)           | 2.5±0.2  |
| 5  | Calculated Excited State Structures (10)        | Calculated Ground State Structures (10)         | 2.3±0.2  |
| 6  | Calculated Structures L99A,G113A,R119P T4L (10) | Calculated Structures L99A,G113A,R119P T4L (10) | 0.9±0.3  |
| 7  | Calculated Structures L99A,G113A,R119P T4L (10) | Ground State Crystal Structure (3DMV)           | 2.3±0.3  |
| 8  | Calculated Structures L99A,G113A,R119P T4L (10) | Calculated Excited State Structures (10)        | 0.8±0.3  |

**Supplementary Table 7 | Rosetta structure based design calculations identifying substitutions stabilizing the excited state relative to the ground state (negative  $\Delta\Delta G_E - \Delta\Delta G_G$ ).**

$\Delta\Delta G$  values are in ‘Rosetta energy units’.

| Mutation | $\Delta\Delta G_E$ | $\Delta\Delta G_G$ | $\Delta\Delta G_E - \Delta\Delta G_G$ |
|----------|--------------------|--------------------|---------------------------------------|
| G113A    | -2.048             | -1.296             | -0.752                                |
| R119P    | 2.680              | 10.014             | -7.334                                |

**Supplementary Table 8 | Exchange parameters obtained from quantitative magnetization exchange experiments recorded on a Met- $^{13}\text{C}^{\text{e}}$ -L99A,G113A T4L sample, 1°C, in the absence (two state exchange) or presence (three state exchange) of benzene.**

Data from Met 102, where separate peaks were observed for ground, excited and benzene bound states.  $k_{exij} = k_{ij} + k_{ji}$ .

| States | Populations (%)                           |          |          | Exchange Rates ( $\text{s}^{-1}$ ) |            |            | $\frac{p_E}{p_E + p_G}$ |
|--------|---|----------|----------|------------------------------------|------------|------------|-------------------------|
|        | $p_G$                                     | $p_E$    | $p_B$    | $k_{exGE}$                         | $k_{exGB}$ | $k_{exEB}$ |                         |
| Two:   | 66.0±1.4                                  | 34.0±1.8 | -        | 47.6±1.1                           | -          | -          | 34.0±1.8                |
| Three: | 47.5±1.0                                  | 20.8±1.2 | 31.7±1.2 | 52.5±1.2                           | 29±0.3     | 0±0        | 30.5±1.6                |
|        | Forward Reverse Rates ( $\text{s}^{-1}$ ) |          |          |                                    |            |            |                         |
|        | $k_{GE}$                                  | $k_{EG}$ | $k_{GB}$ | $k_{BG}$                           | $k_{EB}$   | $k_{BE}$   |                         |
| Two:   | 16.2±0.7                                  | 31.4±1.4 | -        | -                                  | -          | -          | -                       |
| Three: | 16±0.7                                    | 36.5±1.5 | 11.6±0.3 | 17.4±0.4                           | 0±0        | 0±0        |                         |

**Supplementary Table 9 | Experimental  $^3J_{C'C\gamma}$  and  $^3J_{NC\gamma}$  coupling constants and side-chain  $\chi_1$  angles of L99A,G113A,R119P T4L and L99A T4L.**

$^3J_{C'C\gamma}$  and  $^3J_{NC\gamma}$  values were obtained using the ratio of the peak intensities measured in ‘dephased’ and reference 2D spin-echo spectra as previously described <sup>2,3</sup>. Errors in the measurements were propagated from the estimated uncertainty of peak intensities. When the ‘dephased’ and the reference peaks had similar intensities (that is within error), an upper-limit value was estimated based on the average intensity of the peaks and their uncertainty.  $\chi_1$  angles were directly extracted from the excited state CS-Rosetta structure ( $\chi_{1,E}$ ) and the ground state X-ray crystal structure ( $\chi_{1,G}$ ) of L99A T4L. No values were reported in cases of strong overlap or in the absence of peaks. Residue F114 (highlighted) is the only residue showing significantly different coupling values, as expected from a comparison of excited and ground state structures of L99A T4L.

| Residue     | L99A,G113A,R119P T4L            |                                 |                  | L99A T4L                        |                       |                  |
|-------------|---------------------------------|---------------------------------|------------------|---------------------------------|-----------------------|------------------|
|             | $^3J_{C'C\gamma}$ (Hz)          | $^3J_{NC\gamma}$ (Hz)           | $\chi_{1,E}$ (°) | $^3J_{C'C\gamma}$ (Hz)          | $^3J_{NC\gamma}$ (Hz) | $\chi_{1,G}$ (°) |
| F4          | $2.7 \pm 0.2$                   | $1.4 \pm 0.1$                   | -72              | -                               | $2.3 \pm 0.1$         | -69              |
| Y18         | < 1.1                           | $0.7 \pm 0.2$                   | 65               | $0.9 \pm 0.4$                   | $0.6 \pm 0.3$         | 66               |
| Y24         | $3.7 \pm 0.1$                   | < 0.7                           | -60              | $3.9 \pm 0.1$                   | < 0.6                 | -60              |
| Y25         | $4.2 \pm 0.1$                   | < 0.5                           | -69              | $4.2 \pm 0.1$                   | < 0.5                 | -70              |
| H31         | < 0.7                           | $3.2 \pm 0.1$                   | 178              | < 0.6                           | $3.2 \pm 0.1$         | -179             |
| F67         | < 1.3                           | $2.7 \pm 0.1$                   | -179             | < 0.9                           | $2.6 \pm 0.1$         | -178             |
| Y88         | < 0.7                           | $2.7 \pm 0.1$                   | -176             | < 0.5                           | $2.7 \pm 0.1$         | -171             |
| F104        | -                               | $2.6 \pm 0.1$                   | -173             | -                               | -                     | -170             |
| <b>F114</b> | <b><math>0.6 \pm 0.4</math></b> | <b><math>2.6 \pm 0.1</math></b> | <b>-164</b>      | <b><math>4.1 \pm 0.1</math></b> | <b>&lt; 0.5</b>       | <b>-80</b>       |
| W126        | $4.4 \pm 0.1$                   | $1.0 \pm 0.1$                   | -52              | $4.3 \pm 0.1$                   | $1.0 \pm 0.1$         | -53              |
| W138        | < 1.0                           | $2.6 \pm 0.4$                   | 176              | $1.2 \pm 0.5$                   | $3.1 \pm 0.1$         | 170              |
| Y139        | < 1.4                           | $2.6 \pm 0.1$                   | 176              | < 0.8                           | $2.6 \pm 0.2$         | 175              |
| F153        | $3.9 \pm 0.1$                   | < 0.9                           | -84              | $4.2 \pm 0.1$                   | < 0.6                 | -83              |
| W158        | $4.4 \pm 0.1$                   | < 1.1                           | -60              | $4.3 \pm 0.1$                   | < 0.7                 | -69              |
| Y161        | $3.6 \pm 0.3$                   | < 0.7                           | -67              | $3.8 \pm 0.1$                   | < 1.1                 | -70              |



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