SUPPLEMENTARY DATA for

The Trp-cage: Optimizing the Stability of a Globular Miniprotein

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Colour versions of figures in the article text.

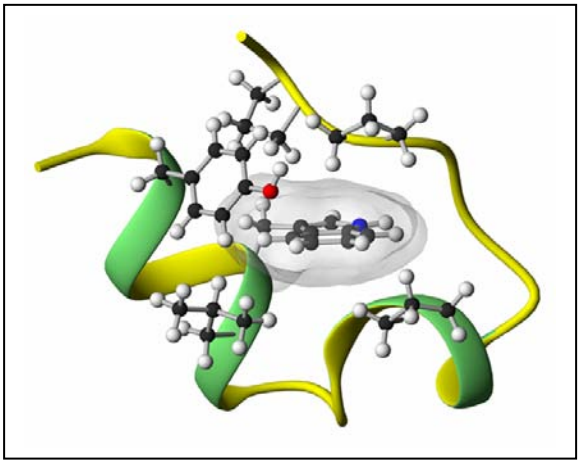

Fig. 1. *The Trp-cage fold showing the secondary structure features and the buried Trp sidechain as well as the residues that shield the Trp indole ring from solvent exposure.*

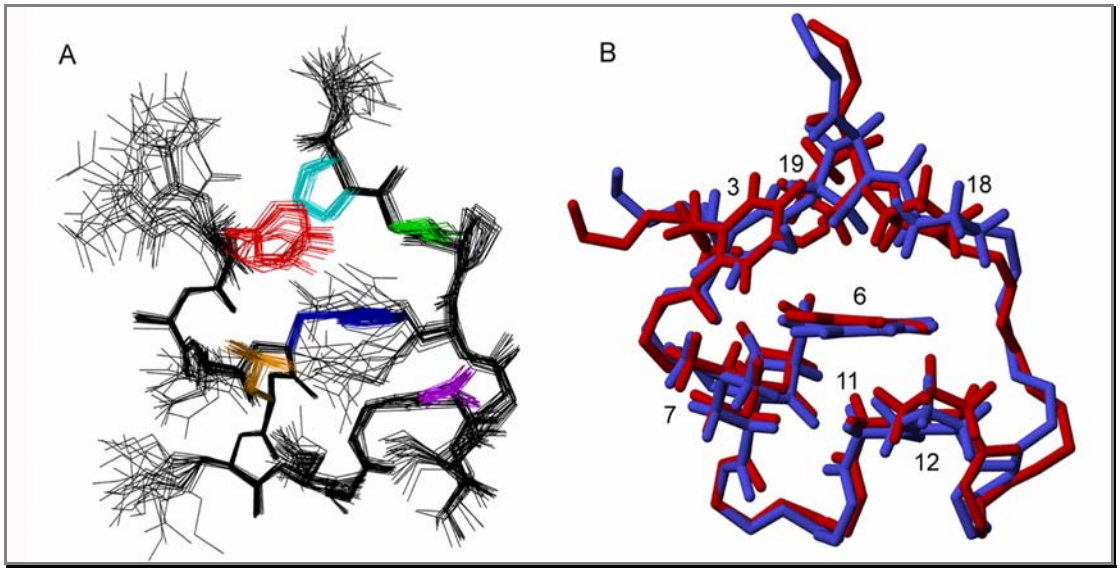

Fig. 4. *Trp-cage NMR structure ensembles: (**a**) The TC10b structure ensemble (28 structures): heavy atoms are displayed for all residue sidechains and the backbone, the hydrophobic cluster residues are shown in color -- Tyr^3 (red), Trp^6 (blue), Leu^7 (yellow), Pro^{12} (magenta), Pro^{18} (green), and Pro^{19} (cyan). (**b**) An overlay of one member each of the TC10b (blue) and TC5b (red) structure ensembles. All atoms are displayed for Tyr^3, Trp^6, Leu^7, Pro^{12}, Pro^{18}, and Pro^{19} with only N, CA, C' shown for the remaining residues.*

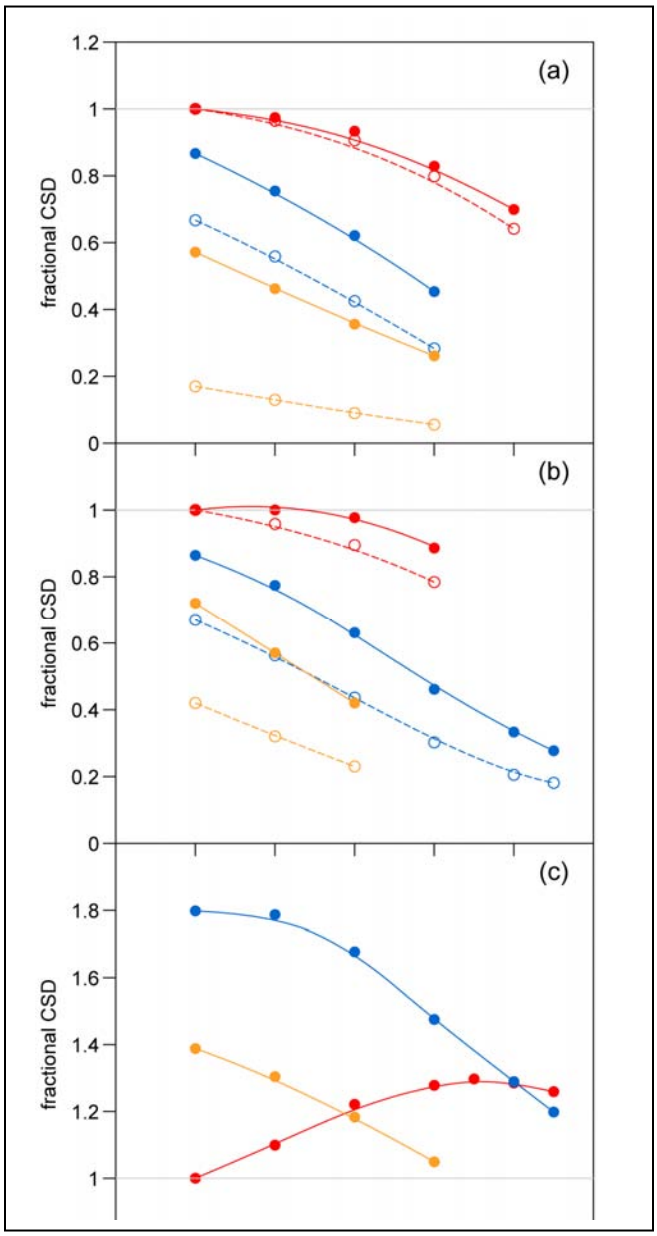

Fig. 6. *Differential melting effects upon destabilization of the Trp-cage: fractional CSDs versus T (ºC) for TC10b (red) and its S14A- (blue) and P19A-mutants (orange) -- panel (**a**) plots the "cage" (dashed lines) and "helix" (solid line) measures of folding, panel (**b**) plots the G11α2 (dashed lines) and G11α3 (solid line) CSDs, panel (**c**) shows the P12δ3 CSDs, TC10b at 7 ºC is the calibration standard for CSD = 1.0 in all panels. The P12δ3 CSDs (upfield) for the less stable systems are larger than those observed for TC10b. In the case of the P19A mutant, the only cage measure available for panel (**a**) are the P18α and β3 CSDs.*

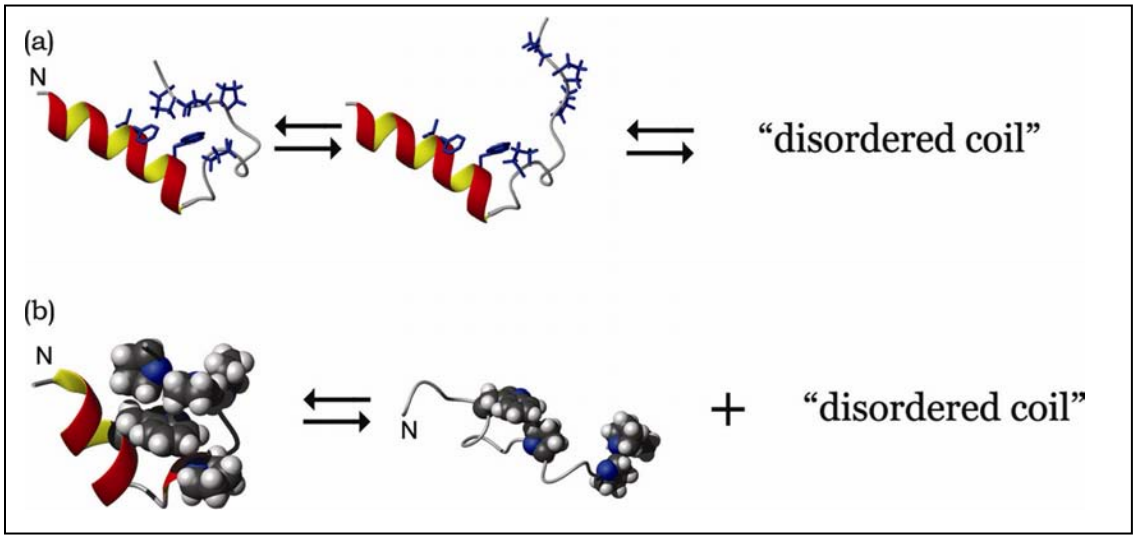

Fig. 7. *Unfolding (melting) scenarios for Trp-cages with different degrees of intrinsic helix stability: (**a**) Trp-cage formation as Pro[17,18,19] docking onto a stable helix, (**b**) Trp-cage melting producing an unfolded ensemble retaining a measurable population of a residual hydrophobic cluster, a "half-cage" structure.*

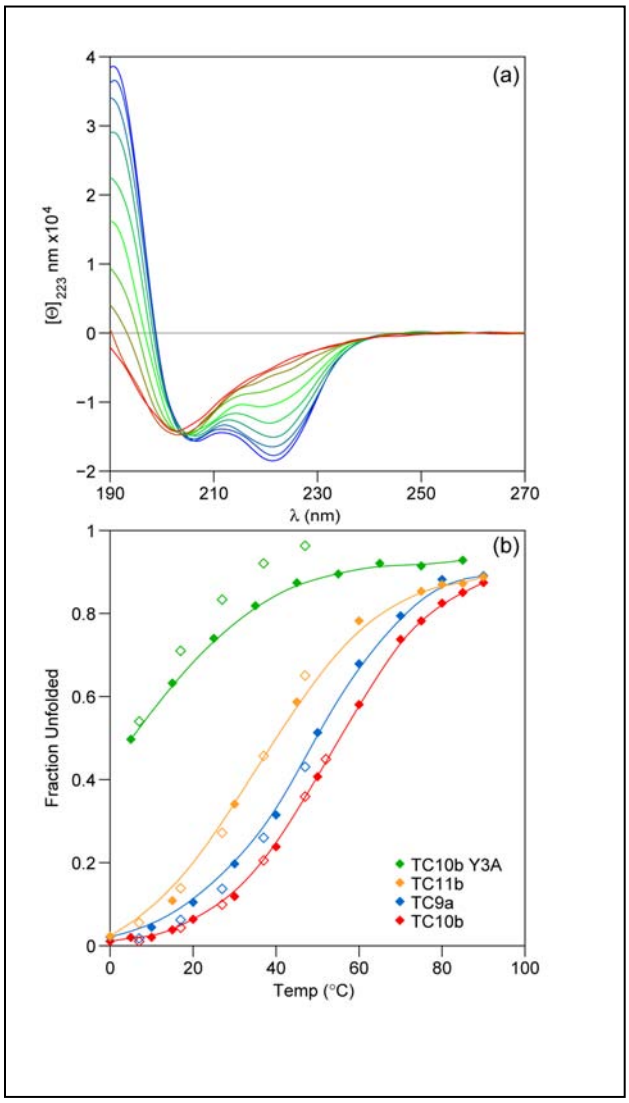

Fig. 8. *(**a**) The temperature dependence of the CD spectrum of TC10b at pH 7, traces are shown for every 10° increment from 0 to 90 °C. (**b**) Melting curves (χ_U versus T) for TC10b and some of its single site mutants. To illustrate the agreement between chemical shift and CD melts we have superimposed χ_U values from "cage CSDs" as open symbols on the plots. When the cage is substantially destabilized, illustrated here by Y3A-TC10b, some residual helicity remains after the cage structure melts.*

SUPPLEMENTARY DATA AND DISCUSSION.

The Trp-cage displays the same chemical shift deviations over a wide range of mutants.

At this point mutations at all positions in the original sequence, except the three glycines, have been examined. Of those retaining the Trp residue, all but five adopt the Trp-cage fold as the predominant conformation in water. There is a remarkable consistency in the chemical shifts (Table S1) observed for these species under conditions that favor folding (pH 6 – 7, 280K). All of the large structuring shifts, due to ring-current effects in the hydrophobic core, that are used to measure the extent of folding in the Trp-cage appear in Table S1. The shifts are given as chemical shift deviations (CSD = δ_{obs} - δ_{rc}, where δ_{obs} is the observed chemical shift and δ_{rc} is a reference random coil chemical shif; the random coil shifts employed are also listed, with the exception of Gly^{11} these are the values produced by our current automated CSD computation algorithm (Fesinmeyer *et al.*, 2004). From Table S1, it is apparent that we had nearly achieved the fully folded shift values at the stage of TC5b. Only minor increases in structuring shifts at 280K are observed as the fold stability is increased by mutation or by the addition of TFE. Consistent with this, a recent report from another lab (Naduthambi and Zondlo, 2006) indicates CSDs of -3.58 and +0.51 for G11Hα2 and R16Hα, respectively, in a stabilized Trp-cage. If we account for the unfolded fraction of TC5b present (estimated from the NH protection data), the sum of the structuring shifts for the folded state of TC5b is greater than 95% of the sum of maximum CSDs at each position observed in higher melting constructs in Table S1. We view this as evidence that the less stable TC5b structure does not show dramatically enhanced structural fluxionality in the folded state.

As additional evidence for a common structure we document that the destabilization of a Trp-cage fold by adjusting the pH from 7 to 2.5, and thus eliminating or greatly weakening the H-bonded salt bridge between Asp^9 and Arg^{16} results in changes in the chemical shift deviation melts (Figure S1) that are entirely comparable to those observed by mutational destabilization (Figure 2).

Table S1. Chemical shift deviations for representative Trp-cage sequences at 280 K. [a]

Sequence	CSDs						
	7α	11α2[b]	12β3	16α	18α	18β3	19δ3, 19δ2
pH 6 – 7 buffer [c] -							
Random coil reference chemical shift	4.226	4.02	2.27	4.622	4.69	2.29	3.74, 3.59
TC5b NLYIQWLKD GGPSSGRPPPS[d]	-0.78	-3.29	0.25	0.41	-2.18	-1.95	-0.56, -0.63
NLY**A**QWLKD GGPSSGRPPPS	-0.81	-3.27	0.26	0.44	-2.20	-1.98	-0.59, -0.66
DLY**A**QWLKD GGPSSGRPPPS	-0.82	-3.35	0.26	0.46	-2.25	-2.03	-0.62, -0.66
AcAYAQWLKD GGPSSGRPPPS	-0.83	-3.41	0.27	0.47	-2.30	-2.11	-0.57, -0.67
N**A**YAQWLKD GGPSSGRPPPS	-0.83	-3.35	0.26	0.45	-2.28	-2.06	-0.57, -0.66
TC10b D**A**YAQWLKD GGPSSGRPPPS	-0.84	-3.37	0.27	0.46	-2.29	-2.07	-0.59, -0.66
DAY**U**QWLKD GGPSSGRPPPS	-0.79	-3.31	0.27	0.46	-2.24	-2.01	-0.58, -0.59
NA**U**Y**U**QWLKD GGPSSGRPPPS	-0.78	-3.42	0.27	0.48	-2.33	-2.12	-0.55, -0.63
NAYA**A**WLKD GGPSSGRPPPS[e]	-0.80	**-3.04**	0.24	**0.39**	-2.11	**-1.86**	-0.56, -0.61
NAYAQWL**A**D GGPSSGRPPPS	-0.86	-3.35	0.27	0.46	-2.30	-2.08	-0.57, -0.66
NAYAQWLKD GGPS**T**GRPPPS[e]	-0.84	-3.21	0.24	**0.29**	-2.20	**-1.76**	-0.58, -0.67
DAYAQWLKD GGPSSGR**A**PPS	-0.84	-3.27	0.26	0.48[f]	-2.10	-2.08	**-0.51**, -0.62
DAYAQWLKD GGPSSGRP**A**PS	-0.84	-3.41	0.26	0.46	*-2.60*	n. a.	-0.60, *-0.75*
DAYAQWLKD GGPSSGRPPP**NH_2**	-0.84	-3.32	0.27	0.44	-2.23	-2.06	-0.58, -0.63
GAAAAAAAYAQWLKD GGPSSGRPPPS	-0.84	-3.42	0.27	0.47	-2.32	-2.08	-0.61, -0.67
30% TFE [g] -							
Random coil reference chemical shift	4.261	4.055	2.27	4.657	4.723	2.29	3.74, 3.59
TC5b NLYIQWLKD GGPSSGRPPPS	-0.78	-3.20	0.24	0.44	-2.12	-1.95	-0.59, -0.67
NLY**A**QWLKD GGPSSGRPPPS	-0.75	-3.35	0.26	0.47	-2.20	-2.04	-0.64, -0.72
D**A**YAQWLKD GGPSSGRPPPS	-0.77	-3.46	0.26	0.50	-2.30	-2.11	-0.61, -0.70
AcAYAQWLKD GGPSSGRPPPS	-0.78	-3.46	0.26	0.49	-2.32	-2.12	-0.59, -0.71
NAYAQWLKD GGPS**T**GRPPPS	-0.80	-3.31	0.23	**0.35**	-2.21	**-1.89**	-0.56, -0.64
DAYAQWLKD GGPSSGR**A**PPS	-0.79	-3.44	0.27	*0.53*[f]	-2.16	-2.14	-0.55, -0.68

[a] The reference shift values employed are shown in the first row. Bold values in the body of the table highlight CSDs that are significantly smaller than the largest values observed; these imply fraction folded values (χ_F) less than 0.98. Unusually large shift are italicized.

[b] The CSD of the other Gly Hα (11Hα3) is significantly smaller, see text: the range is –0.87 to –1.02 for the systems listed in this Table.

[c] In most cases the pH was between 6.6 and 7 and spectral data was collected at 280K with 5% D_2O included as the lock signal. In purely aqueous buffers, the fold stability decreases as the pH is lowered to or beyond 4.9.

[d] The data for TC5b is pH* = 6 in 98+ % D_2O. The ring current shifts are slightly larger (by 3 – 9 %) in D_2O versus H_2O.

[e] Other data for these two analogs also suggest that χ_F for these two analogs is less than 0.96 under the strictly aqueous conditions at the lowest temperature examined.

[f] An adjustment in the R16α reference shift (-0.28 ppm), reflecting the Pro to Ala mutation in the following residue, has been included in the CSD calculation.

[g] These solutions were prepared by adding TFE to aqueous solutions at pH 6.6. The effective pH is estimated to be about 5.8.

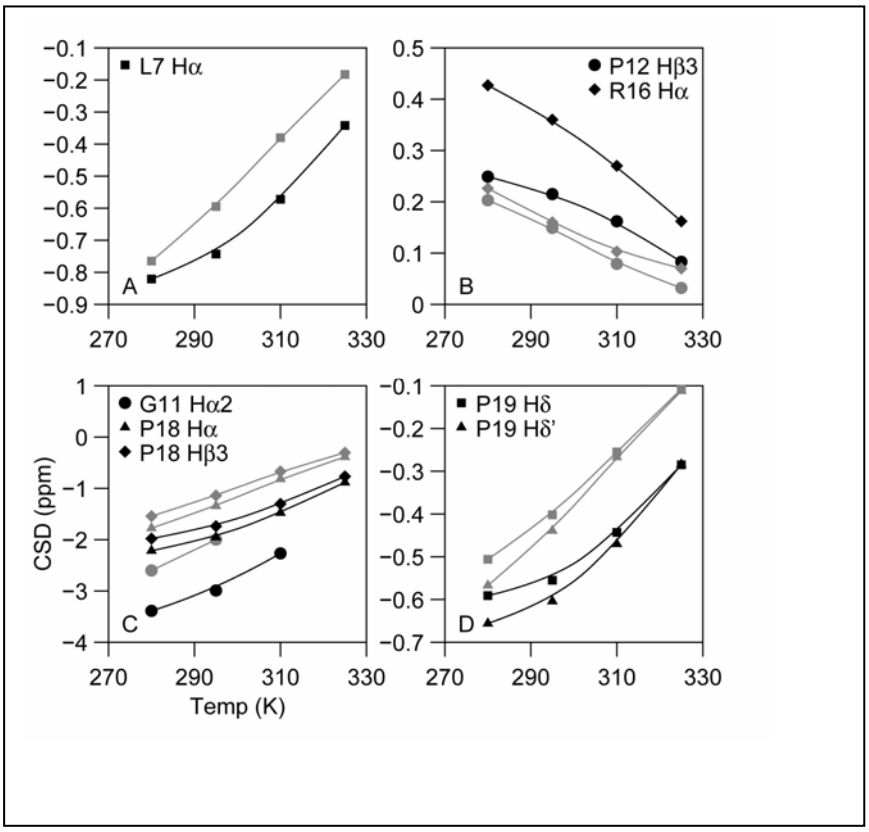

Figure S1. Plots of chemical shift deviations (CSDs, the ^{1}H sites are labeled on the panels) versus temperature for TC8a at pH 7 (optimum stability, black) and at pH 2.5 (grey lines and symbols). The lines through the data are polynomial fits with no theoretical significance. The pH effect on stability is due almost exclusively to the loss of the salt-bridge upon protonation of Asp^{9}). The format is the same as in textual Figure 2.

Structural Characterization of TC10b

The NMR structure ensemble for TC10b was generated from 186 NOE distances, including 28 long-range (i/ i+n, n>4) constraints. The NOE intensities were obtained directly from the NOESY spectra and these were converted to distances by an automated program, DIS (Fesinmeyer, 2005), which also calculates the allowed ranges, from d – d_{-} to d + d_{+}, using the relation d = A* i^{B}, where d is the distance in Å, i is the NOE intensity, and A, B are constants that were obtained by fitting a power function to a plot of distance *versus* intensity for a set of intensities for which the distances are 'known'. The following 'known' distances for a helical span (residues 3 – 8, in the present case) were used for the calibration: NN (i, i+1) = 2.8, αN (i, i) = 2.8, αN (i, i+1) = 3.5 Å in the helical span, geminal ββ' and Pro δδ' = 1.9 Å *(the geminal methylene distance is larger than the actual distance, 1.76 Å, to account for the fact that geminal NOEs are not in the linear growth regime at the mixing times employed)*. This contrasts with the procedure employed for TC5b, where the NOEs were grouped by size category and the distances were adjusted further during the refinement procedure rather than relying on an automated program for generating distance constraints. Twenty eight structures (from 40 random starts) met

the acceptance criteria. The structural and convergence statistics for the ensemble appear in Table S2.

The NOE derived constraints employed for the CNS derivation of an NMR ensemble for TC10b appear in Table S3. Table S3 uses CNS nomenclature for prochiral CH_2 and CMe_2 groups; while the resonance assignment lists (*vide infra*) are given using the most recently formalized IUPAC nomenclature (Markley *et al.*, 1998). The dihedral values derived for TC10b are compared to those in the prior TC5b ensemble in Table S4.

Table S2. NMR structure statistics for the TC10b ensemble[a]

Distance constraints and r.m.s. deviations in the CNS ensemble:		
Type of constraint	Number	r.m.s. deviation[b]
Intraresidue	85	0.0506 ±0.0036
Sequential	52	0.0303 ±0.0031
i/ i+n, n = 2-4	21	0.0098 ±0.0036
i/ i+n, n ≥ 5	28	0.0158 ±0.0059

Structure statistics[b]:	
E_{TOTAL} (kcal/mol)	−30.5 ±4.2
E_{NOE} (kcal/mol)	20.7 ±1.9
E_{vdW} (kcal/mol)	−74.2 ±3.3
Bond violations (Å)	0.0037 ±0.0001
Angle violations (°)	0.478 ±0.008
Improper torsion violations (°)	0.181 ±0.012

Convergence within final ensemble, atomic r.m.s. deviations (Å)[c]:	
Pairwise over the ensemble (±s.e.)	
Backbone	0.41 ±0.14
Heavy atom	1.02 ±0.17

[a.] All statistics are over the 28 accepted structures of 40 structures.
[b.] Values are mean ±standard deviation.
[c.] All convergence measures are over residues 3-19.

Footnote – The TC5b structure ensemble (38/50 structures) was generated from 169 constraints (of which 28 were long-range) with similar acceptance criteria (NOE < 0.15 Å). Subsequent studies (to be reported elsewhere) have revealed that as few as 18 long-range constraints, with the addition of 36 medium- and short-range constraints can generate Trp-cage structure ensembles within a 0.63 ± 0.17 Å backbone RMSD of the published TC5b ensemble, which had a 0.39 ±0.12 Å pairwise backbone RMSD.

Table S3 NOE constraint list for TC10b

!! i,i				d	d−	d+	δ values
assign (residue 1 and name ha)	(residue 1 and name hb#)	2.65	0.30	0.26	4.25 2.98
assign (residue 2 and name ha)	(residue 2 and name hb#)	3.00	0.50	0.30	4.27 1.52
assign (residue 3 and name ha)	(residue 3 and name hb#)	2.53	0.27	0.24	4.03 3.14
assign (residue 3 and name ha)	(residue 3 and name hd#)	2.84	0.36	0.71	4.03 7.09
assign (residue 3 and name ha)	(residue 3 and name hn)	2.94	0.41	0.33	4.03 8.90
assign (residue 3 and name hb#)	(residue 3 and name hd#)	2.51	0.24	0.66	3.13 7.10

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assign (residue   3 and name hb#   ) (residue   3 and name hn    ) 2.47  0.25  0.23          3.13 8.90
assign (residue   4 and name ha    ) (residue   4 and name hn    ) 2.74  0.35  0.28          4.12 8.35
assign (residue   4 and name hb#   ) (residue   4 and name hn    ) 2.70  0.23  0.24          1.58 8.34
assign (residue   5 and name ha    ) (residue   5 and name hb1   ) 3.19  0.49  0.40          3.90 2.15
assign (residue   5 and name ha    ) (residue   5 and name hb2   ) 3.23  0.51  0.41          3.90 2.23
assign (residue   5 and name ha    ) (residue   5 and name hn    ) 2.61  0.31  0.26          3.90 8.12
assign (residue   5 and name hb1   ) (residue   5 and name hn    ) 2.65  0.32  0.27          2.15 8.13
assign (residue   5 and name hb2   ) (residue   5 and name hn    ) 2.64  0.32  0.26          2.24 8.13
assign (residue   6 and name ha    ) (residue   6 and name hb1   ) 3.00  0.20  0.20          4.24 3.56
assign (residue   6 and name ha    ) (residue   6 and name hb2   ) 2.40  0.40  0.40          4.24 3.14
assign (residue   6 and name ha    ) (residue   6 and name hd1   ) 2.98  0.43  0.54          4.23 6.98
assign (residue   6 and name ha    ) (residue   6 and name hn    ) 2.94  0.41  0.33          4.24 8.15
assign (residue   6 and name hb1   ) (residue   6 and name hd1   ) 3.23  0.51  0.61          3.55 6.97
assign (residue   6 and name hb1   ) (residue   6 and name he3   ) 3.07  0.45  0.56          3.55 6.97
assign (residue   6 and name hb1   ) (residue   6 and name hn    ) 2.43  0.25  0.23          3.56 8.15
assign (residue   6 and name hb2   ) (residue   6 and name hd1   ) 2.83  0.38  0.51          3.14 6.98
assign (residue   6 and name hb2   ) (residue   6 and name he3   ) 3.26  0.52  0.62          3.14 6.98
assign (residue   6 and name hb2   ) (residue   6 and name hn    ) 2.41  0.25  0.22          3.14 8.15
assign (residue   6 and name hz2   ) (residue   6 and name he1   ) 2.66  0.32  0.67          7.19 9.71
assign (residue   7 and name ha    ) (residue   7 and name hb1   ) 2.50  0.20  0.25          3.39 1.36
assign (residue   7 and name ha    ) (residue   7 and name hb2   ) 3.05  0.10  0.10          3.39 1.90
assign (residue   7 and name ha    ) (residue   7 and name hd1#  ) 4.25  0.73  0.62          3.40 0.99
assign (residue   7 and name ha    ) (residue   7 and name hd2#  ) 2.86  0.28  0.26          3.39 0.86
assign (residue   7 and name ha    ) (residue   7 and name hn    ) 2.88  0.40  0.32          3.38 8.37
assign (residue   7 and name hb1   ) (residue   7 and name hd1#  ) 3.56  0.51  0.40          1.34 0.99
assign (residue   7 and name hb1   ) (residue   7 and name hd2#  ) 3.41  0.46  0.37          1.35 0.87
assign (residue   7 and name hb1   ) (residue   7 and name hn    ) 3.49  0.46  0.46          1.36 8.37
assign (residue   7 and name hb2   ) (residue   7 and name hd1#  ) 3.43  0.47  0.37          1.90 0.99
assign (residue   7 and name hb2   ) (residue   7 and name hd2#  ) 4.33  0.75  0.65          1.90 0.86
assign (residue   7 and name hb2   ) (residue   7 and name hn    ) 2.66  0.33  0.27          1.90 8.37
assign (residue   7 and name hd1#  ) (residue   7 and name hn    ) 4.33  0.75  0.64          1.00 8.37
assign (residue   7 and name hd2#  ) (residue   7 and name hn    ) 4.74  0.88  0.83          0.87 8.37
assign (residue   7 and name hg    ) (residue   7 and name hn    ) 2.68  0.33  0.27          1.63 8.37
assign (residue   8 and name ha    ) (residue   8 and name hb#   ) 2.97  0.42  0.54          3.93 1.90
assign (residue   8 and name ha    ) (residue   8 and name hn    ) 2.52  0.28  0.24          3.92 8.12
assign (residue   8 and name hb#   ) (residue   8 and name hn    ) 2.29  0.21  0.40          1.90 8.11
assign (residue   9 and name ha    ) (residue   9 and name hb1   ) 2.51  0.28  0.24          4.51 2.94
assign (residue   9 and name ha    ) (residue   9 and name hb2   ) 3.01  0.20  0.20          4.51 2.71
assign (residue   9 and name ha    ) (residue   9 and name hn    ) 2.78  0.36  0.29          4.51 8.00
assign (residue   9 and name hb1   ) (residue   9 and name hn    ) 3.25  0.48  0.39          2.91 8.00
assign (residue   9 and name hb2   ) (residue   9 and name hn    ) 2.50  0.31  0.26          2.72 8.00
assign (residue  10 and name ha#   ) (residue  10 and name hn    ) 2.82  0.38  0.50          3.45 7.51
assign (residue  11 and name ha1   ) (residue  11 and name hn    ) 2.92  0.38  0.30          3.13 8.51
assign (residue  11 and name ha2   ) (residue  11 and name hn    ) 2.53  0.32  0.16          0.64 8.51
assign (residue  12 and name ha    ) (residue  12 and name hb1   ) 2.73  0.35  0.28          4.65 2.54
assign (residue  12 and name ha    ) (residue  12 and name hg1   ) 3.29  0.52  0.53          4.65 2.18
assign (residue  12 and name hd1   ) (residue  12 and name hg1   ) 2.85  0.39  0.31          3.48 2.17
assign (residue  12 and name hd2   ) (residue  12 and name hb2   ) 3.85  0.70  0.64          3.84 2.07
assign (residue  12 and name hd2   ) (residue  12 and name hg2   ) 2.94  0.51  0.33          3.83 2.17
assign (residue  13 and name ha    ) (residue  13 and name hb#   ) 2.69  0.31  0.26          4.49 3.94
assign (residue  13 and name ha    ) (residue  13 and name hn    ) 3.35  0.54  0.45          4.49 7.72
assign (residue  13 and name hb#   ) (residue  13 and name hn    ) 3.13  0.45  0.36          3.93 7.73
assign (residue  14 and name ha    ) (residue  14 and name hb1   ) 2.50  0.20  0.20          4.13 3.85
assign (residue  14 and name ha    ) (residue  14 and name hb2   ) 3.00  0.20  0.20          4.13 3.46
assign (residue  14 and name ha    ) (residue  14 and name hn    ) 2.90  0.40  0.32          4.13 8.25
assign (residue  14 and name hb1   ) (residue  14 and name hn    ) 3.51  0.40  0.40          3.83 8.25
assign (residue  14 and name hb2   ) (residue  14 and name hn    ) 2.76  0.36  0.29          3.46 8.25
assign (residue  15 and name ha1   ) (residue  15 and name hn    ) 2.95  0.42  0.33          4.29 7.97
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assign (residue  15 and name ha2   ) (residue  15 and name hn    )  2.41  0.25  0.22         3.80 7.98
assign (residue  16 and name ha    ) (residue  16 and name hb#   )  2.74  0.35  0.48         5.08 1.81
assign (residue  16 and name hb#   ) (residue  16 and name hn    )  2.96  0.42  0.54         1.81 8.16
assign (residue  16 and name hd#   ) (residue  16 and name he    )  3.15  0.48  0.59         3.22 7.75
assign (residue  16 and name hd#   ) (residue  16 and name hg#   )  3.10  0.46  0.77         3.31 1.81
assign (residue  17 and name ha    ) (residue  17 and name hb1   )  2.52  0.28  0.24         4.77 2.35
assign (residue  17 and name ha    ) (residue  17 and name hg1   )  3.57  0.61  0.52         4.77 2.00
assign (residue  17 and name hd1   ) (residue  17 and name hb2   )  3.70  0.65  0.57         3.86 1.80
assign (residue  17 and name hd2   ) (residue  17 and name hb2   )  3.22  0.50  0.50         3.68 1.80
assign (residue  17 and name hd2   ) (residue  17 and name hg2   )  2.75  0.39  0.31         3.68 1.99
assign (residue  18 and name ha    ) (residue  18 and name hb1   )  2.40  0.40  0.40         2.40 0.21
assign (residue  18 and name ha    ) (residue  18 and name hb2   )  3.00  0.20  0.20         2.40 1.31
assign (residue  18 and name hd#   ) (residue  18 and name hb1   )  3.90  0.70  0.62         3.52 0.21
assign (residue  19 and name ha    ) (residue  19 and name hb1   )  2.70  0.38  0.21         4.34 2.21
assign (residue  19 and name ha    ) (residue  19 and name hb2   )  3.30  0.53  0.43         4.34 1.99
assign (residue  19 and name hd1   ) (residue  19 and name hg1   )  2.70  0.44  0.35         3.14 1.79
assign (residue  19 and name hd1   ) (residue  19 and name hg2   )  3.27  0.52  0.42         3.14 1.84
assign (residue  19 and name hd2   ) (residue  19 and name hg1   )  3.38  0.55  0.46         2.94 1.79
assign (residue  19 and name hd2   ) (residue  19 and name hg2   )  3.00  0.54  0.20         2.94 1.84
assign (residue  20 and name ha    ) (residue  20 and name hb#   )  2.60  0.29  0.25         4.16 3.77
assign (residue  20 and name ha    ) (residue  20 and name hn    )  3.02  0.44  0.35         4.16 7.97

!! i,i+1
assign (residue   2 and name ha    ) (residue   3 and name hn    )   3.46  0.58  0.48         4.27 8.90
assign (residue   2 and name hb#   ) (residue   3 and name hn    )   3.80  0.60  0.57         1.52 8.90
assign (residue   3 and name ha    ) (residue   4 and name hn    )   3.91  0.72  0.67         4.03 8.35
assign (residue   3 and name hb#   ) (residue   4 and name hn    )   2.76  0.34  0.28         3.14 8.35
assign (residue   3 and name hd#   ) (residue   4 and name ha    )   3.47  0.56  0.87         4.10 7.09
assign (residue   3 and name hn    ) (residue   4 and name hn    )   2.83  0.38  0.30         8.35 8.90
assign (residue   4 and name ha    ) (residue   5 and name hn    )   3.71  0.66  0.58         4.11 8.12
assign (residue   4 and name hb#   ) (residue   5 and name hn    )   3.28  0.42  0.34         1.58 8.12
assign (residue   4 and name hn    ) (residue   5 and name hn    )   2.80  0.30  0.30         8.35 8.13
assign (residue   5 and name hb1   ) (residue   6 and name hn    )   2.92  0.41  0.33         2.15 8.14
assign (residue   5 and name hb2   ) (residue   6 and name hn    )   3.32  0.54  0.44         2.24 8.14
assign (residue   6 and name ha    ) (residue   7 and name hn    )   4.05  0.76  0.74         4.24 8.36
assign (residue   6 and name hb1   ) (residue   7 and name hn    )   2.94  0.41  0.33         3.56 8.37
assign (residue   6 and name he3   ) (residue   7 and name ha    )   3.66  0.64  0.76         3.39 6.98
assign (residue   6 and name he3   ) (residue   7 and name hd2#  )   3.55  0.50  0.64         0.86 6.97
assign (residue   6 and name he3   ) (residue   7 and name hg    )   2.73  0.45  0.48         1.63 6.97
assign (residue   6 and name he3   ) (residue   7 and name hn    )   3.16  0.48  0.59         6.98 8.37
assign (residue   6 and name hn    ) (residue   7 and name hn    )   2.57  0.27  0.23         8.36 8.15
assign (residue   6 and name hz3   ) (residue   7 and name hd2#  )   3.35  0.44  0.60         0.86 7.13
assign (residue   7 and name ha    ) (residue   8 and name hn    )   3.41  0.56  0.46         3.39 8.11
assign (residue   7 and name hb1   ) (residue   8 and name hn    )   3.47  0.58  0.49         1.36 8.11
assign (residue   7 and name hn    ) (residue   8 and name hn    )   2.51  0.21  0.27         8.37 8.12
assign (residue   8 and name ha    ) (residue   9 and name hn    )   3.41  0.56  0.47         3.93 8.00
assign (residue   8 and name hb#   ) (residue   9 and name hn    )   3.40  0.56  0.66         1.98 8.00
assign (residue   8 and name hn    ) (residue   9 and name hn    )   2.47  0.27  0.33         8.01 8.11
assign (residue   9 and name ha    ) (residue  10 and name hn    )   3.63  0.73  0.55         4.52 7.51
assign (residue   9 and name hn    ) (residue  10 and name hn    )   2.96  0.42  0.33         7.51 8.00
assign (residue  10 and name ha#   ) (residue  11 and name hn    )   3.10  0.47  0.57         3.46 8.50
assign (residue  10 and name hn    ) (residue  11 and name hn    )   2.42  0.25  0.23         7.51 8.50
assign (residue  11 and name ha1   ) (residue  12 and name hd1   )  2.41  0.35  0.22         3.48 3.11
assign (residue  11 and name ha1   ) (residue  12 and name hd2   )  2.47  0.27  0.23         3.84 3.11
assign (residue  11 and name hn    ) (residue  12 and name hd2   )  3.90  0.72  0.77         3.83 8.50
assign (residue  12 and name hb2   ) (residue  13 and name hn    )  4.04  0.76  0.74         2.07 7.72
assign (residue  12 and name hd1   ) (residue  13 and name hn    )  4.00  0.70  1.00
assign (residue  12 and name hd2   ) (residue  13 and name hn    )  3.71  0.66  0.58         3.84 7.73
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```
assign (residue  13 and name ha    ) (residue  14 and name hn    )  3.50  0.59  0.50        4.48 8.25
assign (residue  13 and name hn    ) (residue  14 and name hn    )  2.72  0.34  0.28        7.73 8.25
assign (residue  14 and name ha    ) (residue  15 and name hn    )  3.80  0.73  0.68        4.13 7.98
assign (residue  14 and name hn    ) (residue  15 and name hn    )  3.09  0.46  0.37        7.99 8.25
assign (residue  15 and name ha1   ) (residue  16 and name hn    )  3.98  0.74  0.70        4.30 8.16
assign (residue  15 and name ha2   ) (residue  16 and name hn    )  3.79  0.68  0.62        3.80 8.16
assign (residue  15 and name hn    ) (residue  16 and name hn    )  2.83  0.38  0.30        7.99 8.16
assign (residue  16 and name ha    ) (residue  17 and name hd1   )  2.51  0.38  0.24        5.08 3.87
assign (residue  16 and name ha    ) (residue  17 and name hd2   )  3.01  0.44  0.35        5.09 3.68
assign (residue  16 and name hb#   ) (residue  17 and name hd2   )  3.29  0.53  0.63        3.68 1.92
assign (residue  17 and name ha    ) (residue  18 and name hd#   )  2.14  0.14  0.18        4.77 3.51
assign (residue  17 and name hb1   ) (residue  18 and name hd#   )  3.31  0.51  0.41        3.51 2.35
assign (residue  18 and name ha    ) (residue  19 and name hd1   )  2.55  0.29  0.20        3.15 2.40
assign (residue  18 and name ha    ) (residue  19 and name hd2   )  2.84  0.25  0.29        2.93 2.40
assign (residue  18 and name hb2   ) (residue  19 and name hd2   )  3.89  0.72  0.66        2.94 1.30
assign (residue  19 and name ha    ) (residue  20 and name hn    )  2.42  0.25  0.23        4.34 7.97
assign (residue  19 and name hb2   ) (residue  20 and name hn    )  3.91  0.72  0.67        2.00 7.96

!! i,i+2
assign (residue   8 and name ha    ) (residue  10 and name hn    )  3.93  0.73  0.68        3.93 7.51
assign (residue   8 and name hn    ) (residue  10 and name hn    )  3.86  0.71  0.75        8.11 7.52
assign (residue   9 and name hn    ) (residue  11 and name hn    )  3.87  0.71  0.65        8.01 8.50
assign (residue  13 and name ha    ) (residue  15 and name hn    )  3.81  0.69  0.62        4.49 7.98

!! i,i+3
assign (residue   2 and name ha    ) (residue   5 and name hb1   )  3.63  0.63  0.55        4.27 2.15
assign (residue   2 and name ha    ) (residue   5 and name hb2   )  3.67  0.65  0.56        4.27 2.25
assign (residue   2 and name ha    ) (residue   5 and name hn    )  3.36  0.55  0.45        4.27 8.13
assign (residue   2 and name hb#   ) (residue   5 and name hn    )  4.54  0.82  0.83        1.52 8.12
assign (residue   3 and name ha    ) (residue   6 and name hb1   )  2.56  0.30  0.25        4.03 3.56
assign (residue   3 and name ha    ) (residue   6 and name he3   )  4.00  0.80  0.60
assign (residue   3 and name ha    ) (residue   6 and name hn    )  3.79  0.68  0.61        4.03 8.15
assign (residue   3 and name he#   ) (residue   6 and name hz3   )  3.11  0.45  0.98        6.83 7.13
assign (residue   4 and name ha    ) (residue   7 and name hb2   )  3.41  0.56  0.47        4.12 1.90
assign (residue   4 and name ha    ) (residue   7 and name hd1#  )  3.62  0.53  0.42        4.12 0.99
assign (residue   6 and name ha    ) (residue   9 and name hb1   )  4.00  0.20  1.00
assign (residue   6 and name ha    ) (residue   9 and name hb2   )  3.20  0.50  0.40        4.23 2.71
assign (residue   6 and name ha    ) (residue   9 and name hn    )  3.81  0.69  0.62        4.22 8.00

!! i,i+4
assign (residue   3 and name hd#   ) (residue   7 and name hd1#  )  4.12  0.66  1.04        0.99 7.10
assign (residue   3 and name he#   ) (residue   7 and name hd1#  )  3.46  0.45  0.86        0.99 6.83
assign (residue   3 and name he#   ) (residue   7 and name hd2#  )  4.11  0.66  1.03        0.86 6.83
assign (residue   7 and name ha    ) (residue  11 and name hn    )  2.74  0.45  0.29        3.39 8.50

!! i,i+n
assign (residue   3 and name ha    ) (residue  19 and name hb1   )  4.00  0.50  1.50
assign (residue   3 and name ha    ) (residue  19 and name hb2   )  4.00  0.50  1.50
assign (residue   3 and name ha    ) (residue  19 and name hd2   )  3.67  0.64  0.56        4.04 2.93
assign (residue   3 and name ha    ) (residue  19 and name hg1   )  3.39  0.56  0.46        4.04 1.80
assign (residue   3 and name ha    ) (residue  19 and name hg2   )  3.44  0.57  0.48        4.03 1.85
assign (residue   3 and name hd#   ) (residue  19 and name hd2   )  3.29  0.51  0.82        2.93 7.10
assign (residue   3 and name he#   ) (residue  18 and name hb1   )  3.87  0.59  1.12        0.20 6.83
assign (residue   3 and name he#   ) (residue  18 and name hb2   )  3.41  0.54  0.85        1.31 6.83
assign (residue   6 and name hd1   ) (residue  16 and name hb#   )  2.94  0.41  0.73        1.81 6.98
assign (residue   6 and name hd1   ) (residue  16 and name he    )  3.51  0.60  0.70        6.99 7.75
assign (residue   6 and name hd1   ) (residue  16 and name hg#   )  2.52  0.28  0.64        1.65 6.98
assign (residue   6 and name hd1   ) (residue  18 and name ha    )  3.33  0.54  0.64        2.40 6.98
```

```
assign (residue   6 and name hd1   ) (residue  19 and name hd2   )  3.58  0.62  0.73        2.93 6.99
assign (residue   6 and name he1   ) (residue  16 and name hb#   )  3.50  0.59  0.90        1.82 9.71
assign (residue   6 and name he1   ) (residue  16 and name hg#   )  3.68  0.65  0.97        1.66 9.71
assign (residue   6 and name he1   ) (residue  16 and name hn    )  3.83  0.70  0.83        8.17 9.71
assign (residue   6 and name he1   ) (residue  18 and name ha    )  3.50  0.59  0.70        2.40 9.71
assign (residue   6 and name hh2   ) (residue  12 and name ha    )  3.51  0.59  0.80        4.64 7.22
assign (residue   6 and name hh2   ) (residue  12 and name hd1   )  2.92  0.41  0.53        3.49 7.22
assign (residue   6 and name hh2   ) (residue  12 and name hg#   )  3.24  0.51  0.81        2.17 7.22
assign (residue   6 and name hz2   ) (residue  12 and name ha    )  2.92  0.51  0.53        4.64 7.18
assign (residue   6 and name hz2   ) (residue  12 and name hg#   )  3.44  0.57  0.87        2.17 7.19
assign (residue   6 and name hz2   ) (residue  18 and name hd1   )  2.76  0.34  0.49        3.51 7.18
assign (residue   6 and name hz2   ) (residue  18 and name hg1   )  3.65  0.64  0.75        1.65 7.18
assign (residue   9 and name hb1   ) (residue  14 and name hb1   )  3.00  0.43  0.34        3.84 2.94
assign (residue   9 and name hb1   ) (residue  14 and name hb2   )  3.57  0.61  0.52        3.47 2.94
assign (residue   9 and name hb2   ) (residue  14 and name hb1   )  4.30  0.40  2.00
assign (residue   9 and name hb2   ) (residue  14 and name hb2   )  4.30  0.40  2.00
```

Table S4. Comparison of dihedral angles (±s.d.) between the TC10b (28 structures) and TC5b (24 structures) NMR ensembles.

Residue	ϕ	ψ	χ1	Residue	ϕ	ψ	χ1

TC5b				**TC10b**			
N1		-48.2(69.5)	-100.3(62.9)	D1		-111.2(93.2)	-117.1(72.3)
L2	-28.0(68.4)	-22.2(19.7)	-87.6 (58.2)	A2	-103.5(61.8)	-0.7 (32.0)	
Y3	-58.2 (3.1)	-35.6 (3.0)	-146.3 (8.4)	Y3	-69.5 (11.7)	-38.3 (2.0)	-152.1 (7.9)
I4	-61.3 (2.5)	-48.1 (2.8)	-52.3 (4.3)	A4	-57.8 (1.1)	-41.5 (1.4)	
Q5	-62.3 (3.3)	-41.6 (5.5)	-109.5(30.4)	Q5	-73.4 (2.9)	-36.1 (2.3)	-149.5 (2.6)
W6	-63.1 (4.1)	-44.6 (2.9)	-180.0 (2.2)	W6	-55.4 (1.5)	-44.6 (1.2)	-171.8 (1.9)
L7	-62.7 (2.1)	-38.2 (3.1)	-74.7 (2.3)	L7	-70.2 (2.2)	-37.6 (2.2)	-68.4 (2.9)
K8	-56.1 (2.3)	-33.6 (4.6)	-124.5(61.0)	K8	-58.0 (1.1)	-32.7 (4.4)	-124.6(31.8)
D9	-72.6 (4.6)	-18.0(10.6)	-125.7(37.9)	D9	-87.4 (8.7)	20.4(22.0)	-73.0 (9.5)
G10	119.5(13.3)	-10.3 (6.2)		G10	83.2(22.4)	21.7 (13.8)	
G11	55.9 (3.3)	-126.3(7.6)		G11	59.0 (3.6)	-119.9(3.4)	
P12	-56.6 (1.9)	-27.1 (3.7)	-20.5 (1.7)	P12	-77.1(14.5)	8.2 (18.1)	14.7 (24.9)
S13	-76.9 (10.1)	-0.5 (18.2)	-31.2 (84.0)	S13	-79.5(10.4)	-34.3(12.2)	-104.4(68.7)
S14	-94.6 (15.8)	-1.8 (7.5)	-55.7 (33.2)	S14	-71.7(12.6)	-28.9(18.7)	-32.1 (24.8)
G15	73.1 (3.8)	-1.7 (28.7)		G15	86.3(12.1)	13.6(15.8)	
R16	-112.3(38.7)	136.3(4.6)	109.6 (68.0)	R16	-96.4(19.5)	134.5 (7.0)	125.5(73.0)
P17	-68.6 (2.8)	161.2 (2.4)	28.6 (1.7)	P17	-63.5(12.0)	129.4 (9.4)	-4.5 (23.8)
P18	-69.2 (2.4)	148.7 (8.0)	26.7 (1.5)	P18	-62.2 (3.4)	135.9 (5.2)	19.7 (2.3)
P19	-79.1 (3.0)	97.2 (73.5)	31.5 (1.7)	P19	-81.8 (1.6)	112.2(20.6)	30.8 (0.8)
S20	-119.0(82.0)		-102.7(96.0)	S20	-121.3(26.3)		-79.9 (76.2)

Incorporating other NMR data into TC10b structural models

Method details –

The NMR ensemble of TC10b, which was calculated without any H-bonding restraints, was sequentially modified by including a distance restraint (D9Cγ/R16Cζ = 3.9 – 5.1 Å) intended to maintain the ends of the Asp[9] and Arg[16] sidechains within salt-bridging distance and the consensus H-bonds ($6H_N$-2O', 6Hε1-16O', $7H_N$-3O', $8H_N$-4O') from the initial TC10b ensemble were restrained with H_N-O' distances corresponding to those observed over the entire NMR ensemble. This produced a more converged ensemble (residue 3-19 backbone RMSD = 0.41 ± 0.15 Å) with a decrease in the E_{NOE} parameter (11.6 versus 20.7 kcal/mol), and remained structurally consistent with the ensemble generated without H-bond restraints as reflected in the inter-ensemble residue 3-19 backbone rmsd (0.43 ± 0.15 Å). A control MD annealing run applying the original NOE constraints to the same folded structure ensemble did not improve convergence and resulted in a smaller decrease in the E_{NOE} parameter compared to runs with added non-NOE constraints.

The effects of adding, serially in alternative modes, additional H-bond restraints (16Hε-14Oγ, $9H_N$-5O' versus $9H_N$-6O', $11H_N$-6O' versus $11H_N$-7O', and 14Hγ-9O' versus 14Hγ-10O' versus 14Hγ-11O'), and then narrowing the restraint for α-helical H_N / O' H-bonds to 2.0 + 0.2 Å was examined by additional CNS annealing and Powell minimization protocols. Most sets of additional H-bond restraints did not increase the E_{NOE} term or the violations of the 49 medium- and long-range NOE constraints that define the Trp-cage motif. The only pattern that could be eliminated based on a significantly less good fit to the NOE-derived constraints, higher E_{TOT} measures and/or inability to fit expected H-bonding distances were models incorporating the 14Hγ-10O' H-bond as a restraint. The discrimination between alternative models increased when the more restrictive H_N/O' distances were employed. The CNS runs with varying added restraints indicated that a $9H_N$-5O' was superior to a $9H_N$-6O' H-bond but no clear choice could be made between 14Hγ-9O' and 14Hγ-11O' H-bonding. Defining the H-bond acceptor for the fully exchange protected Gly[11]-H_N was less problematic. Based on E_{TOT} and E_{NOE} criteria, as well as H-bond geometries, the $11H_N$-7O' restraint was better independent of both the 14Hγ and $9H_N$ acceptor specifications. For example with the 14Hγ-9O' and $9H_N$-5O' bonding pattern, an $11H_N$-6O' restraint still produces $11H_N$-7O' H-bonds *and* the $11H_N$-7O' bonds displayed geometries that were in closer accord with expectations for a strong H-bond. As a result, the Gly[11] amide NH is protected in this model with the buried Trp[6]-C=O also sequestered to a significant extent.

The observation that the Trp-cage can accommodate Thr (and allo-Thr) in place of Ser[14] provides additional tests of the validity of the proposed hydrogen bonding networks. The ensembles accepted based on energies and NOE distance RMSDs position S14Hγ pointed toward the carbonyl of D9 or G11. In the best two ensembles, the χ^1 dihedral angles are identical (S14Hγ-D9O' with Ser[14] χ^1 = -16.8° ± 16.5°; S14Hγ-11O' with χ^1 = -15.5° ± 16.4°) thus the S14Hβ protons are positioned uniquely. Given that the Ser to allo-Thr and Ser to Thr mutational effects on stability are small, < 0.7 and circa 2 kJ/mol, respectively(textual Table 3), we can assume that the 14Hγ H-bonding is retained in both mutants. As a result, the replacement of S14 with Thr and allo-Thr in these ensembles can be done without changing the χ^1 dihedral angle and provides models for comparison with CSD and NOE data. In the NOESY spectra of S14T, the Thr methyl (Hγ2) displayed only a single non-intraresidue NOE, a very large peak with the shift coincident $C_\beta H_2$ of D9; this was much diminished in the allo-Thr analog (in which the diastereotopic shift difference for D9-$C_\beta H_2$ is restored. The (S14T)-TC10b ensembles based on structures generated with $9H_N$-5O'/$11H_N$-7O'/14Hγ-9O' and $9H_N$-5O'/$11H_N$-6O'/14Hγ-11O' H-bond restraints displayed the

shortest 14Hγ2/9$C_\beta H_2$ distances and rationalized the changes observed in the chemical shifts of 9$C_\beta H_2$. The allo-Thr analog displayed a new NOE between Hγ2 and W6Hδ1 which is fully consistent with the location of this methyl implied by the low energy TC10b structure generated with 9H_N-5O'/11H_N-7O'/14Hγ-9O' or 9H_N-5O'/11H_N-6O'/14Hγ-11O' H-bond restraints. Distinguishing between a 14Hγ-9O' and 14Hγ-11O' H-bond is not possible; as this corresponds to nothing more than a change in χ^2 of S14.

Results and Discussion –

The addition of H-bond restraints did not produce significant changes in the ϕ/ψ values from residue 3 – 18; there were small but significant changes in a few χ^1 dihedrals: notable a narrowing of D9 and R16 χ-values reflecting the salt bridge and Ser^{14} with $\chi^1 \cong$ -16° reflecting H-bonding interactions. The ensemble generated using 9HN-5O', 11HN-7O', 14Hγ-9O', and 16Hε-14Oγ restraints is viewed as that most consistent with both the NOEs and exchange protection data. Views of a representative structure from this ensemble, illustrating the H-bonding network in the vicinity of the Ser^{14} sidechain the D9/R16 salt bridge appear in the left hand panel of Figure S2; the right hand panel is a view of this region of the computer simulated fold of TC5b derived by Simmerling and Roitberg. The Cartesian coordinates for our model appear in Table S5 and can be requested from the authors as a PDB file.

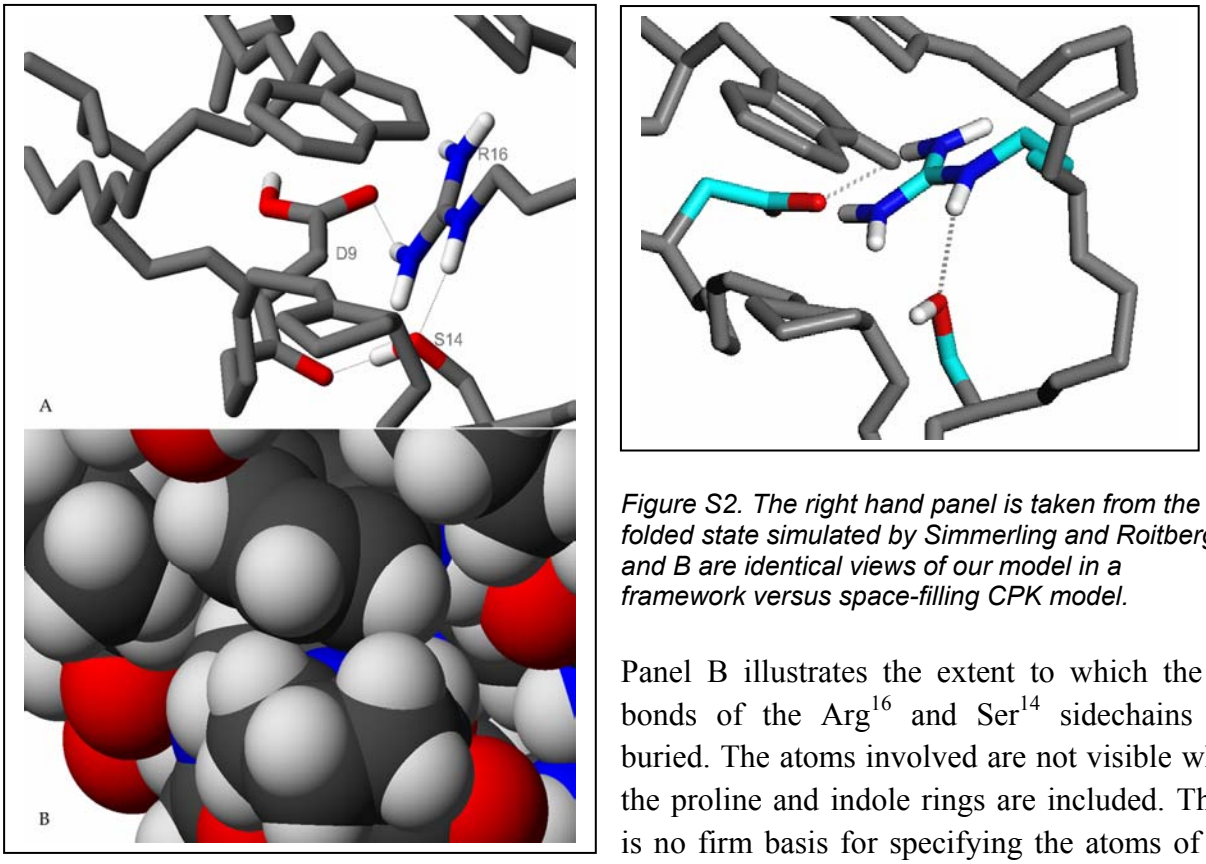

Figure S2. The right hand panel is taken from the folded state simulated by Simmerling and Roitberg. A and B are identical views of our model in a framework versus space-filling CPK model.

Panel B illustrates the extent to which the H-bonds of the Arg^{16} and Ser^{14} sidechains are buried. The atoms involved are not visible when the proline and indole rings are included. There is no firm basis for specifying the atoms of the D9 and R16 that are involved in H-bond that stabilizes the salt-bridge. In panel A, D9 is shown with a protonated carboxyl.

The present model represents our best model for the structure of TC10b (and other stable Trp-cage species). Hopefully, collaborators will eventually obtain an X-ray crystal structure for one

of our Trp-cage constructs. Until that time we recommend the use of the model specified by the atomic coordinates in Table S5 as the starting point for computer simulations of Trp-cage unfolding.

Table S5 – Atomic coordinates for a representative low-energy structure of TC10b with added non-NOE salt bridge and hydrogen bond information (D9Cγ-R16Cζ, 6H_N-2O', 6Hε1-16O', 7H_N-3O', 8H_N-4O', 9HN-5O', 11HN-7O', 14Hγ-9O', and 16Hε-14Oγ).

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ATOM   1    N  ASP   1   -2.119 13.313 -4.420 / ATOM   2  HT1  ASP   1   -1.247 13.403 -4.923
ATOM   3  HT2  ASP   1   -2.772 12.774 -4.974 / ATOM   4  HT3  ASP   1   -2.505 14.232 -4.245
ATOM   5   CA  ASP   1   -1.899 12.628 -3.133 / ATOM   6   HA  ASP   1   -1.167 13.200 -2.559
ATOM   7   CB  ASP   1   -3.193 12.578 -2.313 / ATOM   8  HB2  ASP   1   -4.000 13.102 -2.828
ATOM   9  HB1  ASP   1   -3.508 11.548 -2.136 / ATOM  10   CG  ASP   1   -2.889 13.252 -0.991
ATOM  11  OD1  ASP   1   -3.059 14.487 -0.958 / ATOM  12  OD2  ASP   1   -2.349 12.532 -0.125
ATOM  13    C  ASP   1   -1.339 11.226 -3.333 / ATOM  14    O  ASP   1   -1.965 10.421 -4.017
ATOM  15    N  ALA   2   -0.171 10.932 -2.753 / ATOM  16   HN  ALA   2    0.307 11.628 -2.197
ATOM  17   CA  ALA   2    0.429  9.597 -2.816 / ATOM  18   HA  ALA   2    0.414  9.254 -3.852
ATOM  19   CB  ALA   2    1.892  9.701 -2.371 / ATOM  20  HB1  ALA   2    2.430 10.386 -3.027
ATOM  21  HB2  ALA   2    1.952 10.066 -1.345 / ATOM  22  HB3  ALA   2    2.363  8.719 -2.431
ATOM  23    C  ALA   2   -0.342  8.560 -1.975 / ATOM  24    O  ALA   2   -0.412  7.388 -2.353
ATOM  25    N  TYR   3   -0.978  8.987 -0.872 / ATOM  26   HN  TYR   3   -0.904  9.963 -0.615
ATOM  27   CA  TYR   3   -1.760  8.108  0.006 / ATOM  28   HA  TYR   3   -1.096  7.324  0.371
ATOM  29   CB  TYR   3   -2.273  8.907  1.216 / ATOM  30  HB2  TYR   3   -1.605  9.752  1.395
ATOM  31  HB1  TYR   3   -3.257  9.324  0.997 / ATOM  32   CG  TYR   3   -2.345  8.084  2.490
ATOM  33  CD1  TYR   3   -1.444  8.349  3.538 / ATOM  34  HD1  TYR   3   -0.745  9.167  3.453
ATOM  35  CE1  TYR   3   -1.449  7.558  4.700 / ATOM  36  HE1  TYR   3   -0.757  7.758  5.503
ATOM  37   CZ  TYR   3   -2.365  6.493  4.820 / ATOM  38   OH  TYR   3   -2.355  5.722  5.941
ATOM  39   HH  TYR   3   -3.007  5.022  5.903 / ATOM  40  CE2  TYR   3   -3.288  6.237  3.781
ATOM  41  HE2  TYR   3   -3.997  5.430  3.872 / ATOM  42  CD2  TYR   3   -3.279  7.037  2.623
ATOM  43  HD2  TYR   3   -3.988  6.842  1.833 / ATOM  44    C  TYR   3   -2.924  7.438 -0.743
ATOM  45    O  TYR   3   -3.235  6.266 -0.519 / ATOM  46    N  ALA   4   -3.528  8.167 -1.690
ATOM  47   HN  ALA   4   -3.210  9.115 -1.832 / ATOM  48   CA  ALA   4   -4.574  7.649 -2.560
ATOM  49   HA  ALA   4   -5.394  7.290 -1.935 / ATOM  50   CB  ALA   4   -5.097  8.789 -3.441
ATOM  51  HB1  ALA   4   -5.422  9.622 -2.817 / ATOM  52  HB2  ALA   4   -4.314  9.130 -4.120
ATOM  53  HB3  ALA   4   -5.948  8.440 -4.026 / ATOM  54    C  ALA   4   -4.092  6.471 -3.418
ATOM  55    O  ALA   4   -4.901  5.590 -3.712 / ATOM  56    N  GLN   5   -2.814  6.443 -3.826
ATOM  57   HN  GLN   5   -2.192  7.184 -3.522 / ATOM  58   CA  GLN   5   -2.216  5.310 -4.535
ATOM  59   HA  GLN   5   -2.960  4.918 -5.225 / ATOM  60   CB  GLN   5   -0.990  5.762 -5.347
ATOM  61  HB2  GLN   5   -1.182  6.741 -5.787 / ATOM  62  HB1  GLN   5   -0.123  5.850 -4.691
ATOM  63   CG  GLN   5   -0.672  4.769 -6.479 / ATOM  64  HG2  GLN   5    0.239  5.091 -6.983
ATOM  65  HG1  GLN   5   -0.497  3.775 -6.066 / ATOM  66   CD  GLN   5   -1.798  4.713 -7.513
ATOM  67  OE1  GLN   5   -2.122  5.699 -8.158 / ATOM  68  NE2  GLN   5   -2.473  3.594 -7.678
ATOM  69 HE21  GLN   5   -3.143  3.613 -8.422 / ATOM  70 HE22  GLN   5   -2.252  2.709 -7.177
ATOM  71    C  GLN   5   -1.844  4.183 -3.567 / ATOM  72    O  GLN   5   -2.134  3.024 -3.851
ATOM  73    N  TRP   6   -1.269  4.528 -2.405 / ATOM  74   HN  TRP   6   -1.046  5.510 -2.265
ATOM  75   CA  TRP   6   -0.961  3.579 -1.329 / ATOM  76   HA  TRP   6   -0.177  2.907 -1.681
ATOM  77   CB  TRP   6   -0.421  4.340 -0.107 / ATOM  78  HB2  TRP   6    0.351  5.034 -0.442
ATOM  79  HB1  TRP   6   -1.222  4.935  0.328 / ATOM  80   CG  TRP   6    0.165  3.495  0.983
ATOM  81  CD1  TRP   6    1.453  3.088  1.051 / ATOM  82  HD1  TRP   6    2.215  3.325  0.324
ATOM  83  NE1  TRP   6    1.648  2.336  2.193 / ATOM  84  HE1  TRP   6    2.551  1.927  2.450
ATOM  85  CE2  TRP   6    0.491  2.245  2.937 / ATOM  86  CZ2  TRP   6    0.192  1.645  4.169
ATOM  87  HZ2  TRP   6    0.960  1.114  4.710 / ATOM  88  CH2  TRP   6   -1.109  1.761  4.688
ATOM  89  HH2  TRP   6   -1.354  1.309  5.639 / ATOM  90  CZ3  TRP   6   -2.090  2.469  3.971
ATOM  91  HZ3  TRP   6   -3.089  2.554  4.375 / ATOM  92  CE3  TRP   6   -1.774  3.076  2.739
ATOM  93  HE3  TRP   6   -2.524  3.637  2.204 / ATOM  94  CD2  TRP   6   -0.477  2.979  2.189
ATOM  95    C  TRP   6   -2.181  2.706 -1.000 / ATOM  96    O  TRP   6   -2.092  1.484 -1.080
ATOM  97    N  LEU   7   -3.343  3.318 -0.723 / ATOM  98   HN  LEU   7   -3.341  4.335 -0.679
ATOM  99   CA  LEU   7   -4.599  2.588 -0.504 / ATOM 100   HA  LEU   7   -4.435  1.855  0.283
ATOM 101   CB  LEU   7   -5.711  3.549 -0.057 / ATOM 102  HB2  LEU   7   -5.768  4.372 -0.771
ATOM 103  HB1  LEU   7   -6.664  3.016 -0.085 / ATOM 104   CG  LEU   7   -5.519  4.113  1.361
ATOM 105   HG  LEU   7   -4.538  4.578  1.436 / ATOM 106  CD1  LEU   7   -6.582  5.182  1.620
ATOM 107 HD11  LEU   7   -6.490  5.978  0.881 / ATOM 108 HD12  LEU   7   -7.578  4.742  1.553
ATOM 109 HD13  LEU   7   -6.446  5.609  2.613 / ATOM 110  CD2  LEU   7   -5.641  3.034  2.445
ATOM 111 HD21  LEU   7   -6.596  2.515  2.352 / ATOM 112 HD22  LEU   7   -4.832  2.310  2.358
ATOM 113 HD23  LEU   7   -5.583  3.491  3.432 / ATOM 114    C  LEU   7   -5.062  1.783 -1.725
ATOM 115    O  LEU   7   -5.433  0.621 -1.556 / ATOM 116    N  LYS   8   -5.033  2.370 -2.936
ATOM 117   HN  LYS   8   -4.681  3.317 -2.988 / ATOM 118   CA  LYS   8   -5.410  1.686 -4.189
ATOM 119   HA  LYS   8   -6.470  1.437 -4.146 / ATOM 120   CB  LYS   8   -5.129  2.581 -5.416
ATOM 121  HB2  LYS   8   -4.669  3.518 -5.117 / ATOM 122  HB1  LYS   8   -4.416  2.079 -6.069
ATOM 123   CG  LYS   8   -6.376  2.861 -6.260 / ATOM 124  HG2  LYS   8   -6.053  3.242 -7.230
ATOM 125  HG1  LYS   8   -6.893  1.917 -6.440 / ATOM 126   CD  LYS   8   -7.354  3.856 -5.612
ATOM 127  HD2  LYS   8   -8.351  3.415 -5.642 / ATOM 128  HD1  LYS   8   -7.100  4.027 -4.564
ATOM 129   CE  LYS   8   -7.402  5.188 -6.374 / ATOM 130  HE2  LYS   8   -7.387  4.981 -7.448
ATOM 131  HE1  LYS   8   -8.346  5.686 -6.140 / ATOM 132   NZ  LYS   8   -6.269  6.068 -6.013
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ATOM 133  HZ1  LYS   8   -6.230  6.185 -5.001 / ATOM 134  HZ2  LYS   8   -5.390  5.633 -6.256
ATOM 135  HZ3  LYS   8   -6.340  6.967 -6.470 / ATOM 136    C  LYS   8   -4.667  0.361 -4.348
ATOM 137    O  LYS   8   -5.265 -0.659 -4.680 / ATOM 138    N  ASP   9   -3.371  0.378 -4.054
ATOM 139   HN  ASP   9   -2.967  1.285 -3.830 / ATOM 140   CA  ASP   9   -2.453 -0.735 -4.249
ATOM 141   HA  ASP   9   -2.821 -1.362 -5.063 / ATOM 142   CB  ASP   9   -1.095 -0.164 -4.675
ATOM 143  HB2  ASP   9   -0.700  0.408 -3.834 / ATOM 144  HB1  ASP   9   -0.438 -1.018 -4.849
ATOM 145   CG  ASP   9   -1.033  0.731 -5.938 / ATOM 146  OD1  ASP   9    0.117  0.964 -6.381
ATOM 147  OD2  ASP   9   -2.068  1.219 -6.449 / ATOM 148    C  ASP   9   -2.375 -1.619 -2.977
ATOM 149    O  ASP   9   -1.293 -2.052 -2.555 / ATOM 150    N  GLY  10   -3.551 -1.857 -2.368
ATOM 151   HN  GLY  10   -4.358 -1.420 -2.802 / ATOM 152   CA  GLY  10   -3.804 -2.737 -1.224
ATOM 153  HA2  GLY  10   -4.879 -2.911 -1.161 / ATOM 154  HA1  GLY  10   -3.334 -3.698 -1.401
ATOM 155    C  GLY  10   -3.355 -2.193  0.138 / ATOM 156    O  GLY  10   -3.122 -2.970  1.067
ATOM 157    N  GLY  11   -3.227 -0.869  0.270 / ATOM 158   HN  GLY  11   -3.376 -0.298 -0.555
ATOM 159   CA  GLY  11   -2.737 -0.203  1.473 / ATOM 160  HA2  GLY  11   -2.689  0.869  1.297
ATOM 161  HA1  GLY  11   -3.437 -0.378  2.287 / ATOM 162    C  GLY  11   -1.324 -0.680  1.833
ATOM 163    O  GLY  11   -0.408 -0.522  1.025 / ATOM 164    N  PRO  12   -1.114 -1.294  3.014
ATOM 165   CD  PRO  12   -2.097 -1.577  4.052 / ATOM 166  HD2  PRO  12   -2.930 -2.161  3.658
ATOM 167  HD1  PRO  12   -2.465 -0.642  4.475 / ATOM 168   CG  PRO  12   -1.363 -2.380  5.127
ATOM 169  HG2  PRO  12   -1.500 -3.447  4.946 / ATOM 170  HG1  PRO  12   -1.705 -2.114  6.128
ATOM 171   CB  PRO  12    0.104 -2.007  4.919 / ATOM 172  HB2  PRO  12    0.776 -2.794  5.264
ATOM 173  HB1  PRO  12    0.316 -1.077  5.447 / ATOM 174   CA  PRO  12    0.206 -1.768  3.409
ATOM 175   HA  PRO  12    0.947 -0.990  3.226 / ATOM 176    C  PRO  12    0.658 -3.027  2.649
ATOM 177    O  PRO  12    1.840 -3.362  2.719 / ATOM 178    N  SER  13   -0.226 -3.715  1.902
ATOM 179   HN  SER  13   -1.179 -3.375  1.806 / ATOM 180   CA  SER  13    0.123 -4.976  1.229
ATOM 181   HA  SER  13    0.476 -5.670  1.992 / ATOM 182   CB  SER  13   -1.100 -5.615  0.565
ATOM 183  HB2  SER  13   -0.875 -6.658  0.338 / ATOM 184  HB1  SER  13   -1.955 -5.575  1.241
ATOM 185   OG  SER  13   -1.405 -4.951 -0.640 / ATOM 186   HG  SER  13   -2.155 -5.383 -1.055
ATOM 187    C  SER  13    1.247 -4.833  0.195 / ATOM 188    O  SER  13    2.009 -5.779  0.006
ATOM 189    N  SER  14    1.398 -3.656 -0.435 / ATOM 190   HN  SER  14    0.720 -2.928 -0.249
ATOM 191   CA  SER  14    2.514 -3.382 -1.347 / ATOM 192   HA  SER  14    2.661 -4.281 -1.947
ATOM 193   CB  SER  14    2.209 -2.258 -2.362 / ATOM 194  HB2  SER  14    3.128 -1.700 -2.544
ATOM 195  HB1  SER  14    1.929 -2.719 -3.309 / ATOM 196   OG  SER  14    1.199 -1.319 -2.013
ATOM 197   HG  SER  14    0.297 -1.715 -2.122 / ATOM 198    C  SER  14    3.864 -3.162 -0.634
ATOM 199    O  SER  14    4.873 -2.994 -1.316 / ATOM 200    N  GLY  15    3.926 -3.184  0.708
ATOM 201   HN  GLY  15    3.070 -3.335  1.238 / ATOM 202   CA  GLY  15    5.189 -3.215  1.460
ATOM 203  HA2  GLY  15    4.974 -3.490  2.492 / ATOM 204  HA1  GLY  15    5.838 -3.984  1.038
ATOM 205    C  GLY  15    5.967 -1.894  1.482 / ATOM 206    O  GLY  15    7.164 -1.894  1.760
ATOM 207    N  ARG  16    5.296 -0.772  1.193 / ATOM 208   HN  ARG  16    4.307 -0.885  1.027
ATOM 209   CA  ARG  16    5.866  0.584  1.152 / ATOM 210   HA  ARG  16    6.949  0.482  1.167
ATOM 211   CB  ARG  16    5.510  1.315 -0.157 / ATOM 212  HB2  ARG  16    5.537  2.393  0.015
ATOM 213  HB1  ARG  16    6.269  1.078 -0.904 / ATOM 214   CG  ARG  16    4.142  0.927 -0.729
ATOM 215  HG2  ARG  16    4.207 -0.104 -1.082 / ATOM 216  HG1  ARG  16    3.371  0.989  0.039
ATOM 217   CD  ARG  16    3.750  1.811 -1.908 / ATOM 218  HD2  ARG  16    3.367  2.766 -1.549
ATOM 219  HD1  ARG  16    4.629  1.986 -2.531 / ATOM 220   NE  ARG  16    2.731  1.112 -2.691
ATOM 221   HE  ARG  16    2.227  0.354 -2.233 / ATOM 222   CZ  ARG  16    2.526  1.201 -3.990
ATOM 223  NH1  ARG  16    2.959  2.188 -4.735 / ATOM 224 HH11  ARG  16    3.473  2.939 -4.316
ATOM 225 HH12  ARG  16    2.727  2.177 -5.715 / ATOM 226  NH2  ARG  16    1.855  0.250 -4.560
ATOM 227 HH21  ARG  16    1.463 -0.467 -3.968 / ATOM 228 HH22  ARG  16    1.369  0.469 -5.445
ATOM 229    C  ARG  16    5.419  1.411  2.368 / ATOM 230    O  ARG  16    4.230  1.379  2.700
ATOM 231    N  PRO  17    6.328  2.183  3.001 / ATOM 232   CD  PRO  17    7.762  2.227  2.735
ATOM 233  HD2  PRO  17    7.944  2.799  1.824 / ATOM 234  HD1  PRO  17    8.190  1.228  2.649
ATOM 235   CG  PRO  17    8.389  2.939  3.929 / ATOM 236  HG2  PRO  17    9.300  3.469  3.649
ATOM 237  HG1  PRO  17    8.592  2.214  4.719 / ATOM 238   CB  PRO  17    7.283  3.892  4.372
ATOM 239  HB2  PRO  17    7.335  4.799  3.767 / ATOM 240  HB1  PRO  17    7.371  4.139  5.431
ATOM 241   CA  PRO  17    5.991  3.117  4.075 / ATOM 242   HA  PRO  17    5.737  2.523  4.950
ATOM 243    C  PRO  17    4.838  4.071  3.690 / ATOM 244    O  PRO  17    4.922  4.705  2.635
ATOM 245    N  PRO  18    3.765  4.190  4.500 / ATOM 246   CD  PRO  18    3.473  3.386  5.676
ATOM 247  HD2  PRO  18    4.335  3.305  6.337 / ATOM 248  HD1  PRO  18    3.146  2.395  5.356
ATOM 249   CG  PRO  18    2.335  4.098  6.401 / ATOM 250  HG2  PRO  18    2.754  4.814  7.109
ATOM 251  HG1  PRO  18    1.682  3.390  6.912 / ATOM 252   CB  PRO  18    1.598  4.849  5.293
ATOM 253  HB2  PRO  18    1.142  5.764  5.672 / ATOM 254  HB1  PRO  18    0.829  4.209  4.865
ATOM 255   CA  PRO  18    2.674  5.131  4.239 / ATOM 256   HA  PRO  18    2.259  4.904  3.260
ATOM 257    C  PRO  18    3.158  6.596  4.298 / ATOM 258    O  PRO  18    3.839  6.967  5.255
ATOM 259    N  PRO  19    2.822  7.439  3.301 / ATOM 260   CD  PRO  19    2.097  7.083  2.087
ATOM 261  HD2  PRO  19    1.094  6.723  2.320 / ATOM 262  HD1  PRO  19    2.656  6.320  1.542
ATOM 263   CG  PRO  19    2.015  8.352  1.240 / ATOM 264  HG2  PRO  19    1.072  8.864  1.432
ATOM 265  HG1  PRO  19    2.118  8.126  0.179 / ATOM 266   CB  PRO  19    3.184  9.195  1.754
ATOM 267  HB2  PRO  19    3.028 10.261  1.586 / ATOM 268  HB1  PRO  19    4.105  8.870  1.268
ATOM 269   CA  PRO  19    3.248  8.837  3.244 / ATOM 270   HA  PRO  19    4.280  8.932  3.586
ATOM 271    C  PRO  19    2.357  9.737  4.121 / ATOM 272    O  PRO  19    1.366 10.290  3.643
ATOM 273    N  SER  20    2.714  9.886  5.401 / ATOM 274   HN  SER  20    3.528  9.395  5.746
ATOM 275   CA  SER  20    2.018 10.714  6.402 / ATOM 276   HA  SER  20    1.288 11.345  5.899
ATOM 277   CB  SER  20    1.257  9.822  7.387 / ATOM 278  HB2  SER  20    0.815 10.450  8.164
ATOM 279  HB1  SER  20    0.455  9.310  6.854 / ATOM 280   OG  SER  20    2.116  8.859  7.974
ATOM 281   HG  SER  20    1.700  8.498  8.759 / ATOM 282    C  SER  20    2.944 11.687  7.140
ATOM 283  OT1  SER  20    4.173 11.619  6.935 / ATOM 284  OT2  SER  20    2.373 12.520  7.886
END
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Detailed assignments for four Trp-cage constructs. All assignment at pH 7, 280K: α- β- and δ-CH_2 shifts shown in bold are stereospecifically assigned.

a) TC10b

Residue	HN	Hα (α2, α3)[a]	Hβ2, Hβ3[a]	Others[b]
Asp 1	Exch[c]	4.245	3.138, 2.963	
Ala 2	Exch	4.277	1.516	
Tyr 3	8.899	4.028	3.136 (both)	δ 7.106; ε 6.827
Ala 4	8.348	4.120	1.578	
Gln 5	8.126	3.903	2.235, 2.155	γ, γ' 2.454, 2.384; δNH2 7.915, 7.068
Trp 6	8.150	4.237	**3.138, 3.558**	ε1 9.720; δ1 6.987; ε3 6.983; ζ3 7.133; ηƩ 7.223; ζ2 7.187
Leu 7	8.369	3.387	**1.901, 1.363**	γ 1.635; δ2, δ3 0.866, 0.995
Lys 8	8.117	3.934	**1.975, 1.900**	γ2, γ3 1.513; δ2, δ3 1.656; ε2, ε3 2.972
Asp 9	8.004	4.512	**2.726, 2.910**	
Gly 10	7.513	**3.454, 4.172**		
Gly 11	8.507	**0.647, 3.128**		
Pro 12	--	4.649	**2.080, 2.541**	γ2, γ3 2.176; δ2, δ3 **3.842, 3.480**
Ser 13	7.732	4.486	3.925 (both)	
Ser 14	8.256	4.134	**3.844, 3.465**	
Gly 15	7.984	**3.795, 4.296**		
Arg 16	8.177	5.086	1.918, 1.810	γ, γ' 1.810, 1.652; δ, δ' 3.312, 3.222; ε 7.748
Pro 17	--	4.771	**1.785, 2.365**	γ2, γ3 1.997; δ2, δ3 **3.678, 3.888**
Pro 18	--	2.401	**1.312, 0.213**	γ2, γ3 1.657, 1.718; δ2, δ3 3.518
Pro 19	--	4.343	**1.997, 2.213**	γ2, γ3 1.788, 1.853; δ2, δ3 **2.936, 3.151**
Ser 20	7.968	4.162	3.783 (both)	

b) (P18A)-TC10b

Asp 1	Exch	4.256	3.146, 2.965	
Ala 2	Exch	4.269	1.514	
Tyr 3	8.917	4.025	3.125 (both)	δ 7.110; ε 6.829
Ala 4	8.357	4.101	1.571	
Gln 5	8.108	3.892	2.234, 2.143	γ, γ' 2.450, 2.376; δNH2 7.908, 7.067
Trp 6	8.166	4.230	**3.101, 3.561**	ε1 9.754; δ1 6.945; ε3 6.973; ζ3 7.112; ηƩ 7.224; ζ2 7.210
Leu 7	8.366	3.389	**1.879, 1.354**	γ 1.617; δ2, δ3 0.856, 0.978
Lys 8	8.084	3.929	**1.973, 1.888**	γ2, γ3 1.497; δ2, δ3 1.661; ε2, ε3 2.967
Asp 9	7.997	4.505	**2.722, 2.927**	
Gly 10	7.511	**3.445, 4.158**		
Gly 11	8.536	**0.613, 3.139**		
Pro 12	--	4.649	**2.074, 2.554**	γ2, γ3 2.175; δ2, δ3 **3.838, 3.498**
Ser 13	7.704	4.487	3.920 (both)	
Ser 14	8.269	4.114	**3.835, 3.453**	
Gly 15	7.994	**3.773, 4.290**		
Arg 16	8.173	5.081	1.913, 1.794	γ2, γ3 1.611; δ, δ' 3.298, 3.213; ε 7.760
Pro 17	--	4.508	**1.760, 2.259**	γ2, γ3 1.955; δ2, δ3 **3.649, 3.841**
Ala 18	8.346	1.988	0.303	
Pro 19	--	4.328	**1.970, 2.213**	γ2, γ3 1.798; δ2, δ3 **2.844, 3.140**
Ser 20	7.959	4.159	3.768 (both)	

c) (R16Nva)-TC9b

Asn 1	Exch	4.135	3.136, 3.020	γNH2 7.937, 7.142
Ala 2	Exch	4.259	1.478	
Tyr 3	8.792	4.100	3.089 (both)	δ 7.080; ε 6.817
Ala 4	8.310	4.104	1.518	
Gln 5	8.110	3.939	2.424, 2.173	γ2, γ3 2.173; δNH2 7.959, 6.999
Trp 6	8.151	4.348	**3.196, 3.510**	ε1 9.663; δ1 7.150; ε3 7.089; ζ3 7.207; η2 7.258; ζ2 7.266
Leu 7	8.322	3.443	**1.781, 1.425**	γ 1.581; δ2, δ3 0.852, 0.945
Lys 8	8.014	3.961	**1.942, 1.888**	γ2, γ3 1.472; δ2, δ3 1.654; ε2, ε3 2.967
Asp 9	8.064	4.568	**2.692, 2.814**	
Gly 10	7.576	**3.541, 4.152**		
Gly 11	8.218	**1.591, 3.023**		
Pro 12	--	4.541	**2.042, 2.473**	γ2, γ3 2.103; δ2, δ3 **3.691, 3.207**
Ser 13	7.971	4.421	3.921 (both)	
Ser 14	8.153	4.310	**3.940, 3.684**	
Gly 15	8.015	**3.869, 4.208**		
Nva 16	8.012	4.745	1.835, 1.734	γ2, γ3 1.435; δ 0.970
Pro 17	--	4.622	**1.765, 2.200**	γ2, γ3 2.003; δ2, δ3 **3.668, 3.865**
Pro 18	--	2.909	**1.435, 0.790**	γ2, γ3 1.736, 1.788; δ2, δ3 3.494, 3.534
Pro 19	--	4.352	**1.946, 2.235**	γ2, γ3 1.851; δ2, δ3 **3.006, 3.215**
Ser 20	8.023	4.175	3.789 (both)	

d) (S14T)-TC9b

Asn 1	Exch	4.141	3.188, 3.045	γNH2 7.957, 7.159
Ala 2	Exch	4.261	1.504	
Tyr 3	8.949	4.028	3.089 (both)	δ 7.088; ε 6.817
Ala 4	8.360	4.101	1.552	
Gln 5	8.112	3.927	2.188 (both)	γ2, γ3 2.441; δNH2 8.007, 7.037
Trp 6	8.133	4.267	**3.169, 3.549**	ε1 9.464; δ1 7.122; ε3 7.071; ζ3 7.089; η2 7.238; ζ2 7.208
Leu 7	8.408	3.395	**1.794, 1.446**	γ 1.576; δ2, δ3 0.916, 0.963
Lys 8	8.113	3.916	**1.956, 1.893**	γ2, γ3 1.479; δ2, δ3 1.667; ε2, ε3 2.969
Asp 9	8.213	4.405	**2.707, 2.707**	
Gly 10	7.437	**3.494, 4.260**		
Gly 11	8.512	**0.801, 3.156**		
Pro 12	--	4.610	**2.055, 2.507**	γ2, γ3 2.181; δ2, δ3 **3.489, 3.814**
Ser 13	7.783	4.300	3.920 (both)	
Thr 14	8.099	4.133	4.239	γ 1.069; OHγ 5.009
Gly 15	7.972	**3.876, 4.197**		
Arg 16	8.287	4.921	1.890, 1.716	γ, γ' 1.695, 1.644; δ, δ' 3.358, 3.262; ε 7.561
Pro 17	--	4.667	**1.760, 2.280**	γ2, γ3 2.000; δ2, δ3 **3.661, 3.868**
Pro 18	--	2.496	**0.538, 1.383**	γ2, γ3 1.701, 1.803; δ2, δ3 3.483
Pro 19	--	4.346	**1.914, 2.238**	γ2, γ3 1.845; δ2, δ3 **2.926, 3.163**
Ser 20	8.011	4.171	3.782 (both)	

(a) If the entry is bold, then the atoms are diastereotopically assigned and the first number is the lower IUPAC number (i.e. α2 or β2). (b) Entries are diastereotopically assigned if noted by IUPAC numbering. (c) 'Exch' indicates unable to assign due to H_N exchange with solvent.

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