Supplementary Information

Robust Automated Backbone Triple Resonance NMR Assignments of Proteins Using Bayesian-Based Simulated Annealing

Anthony C. Bishop[1], Sravya Kotaru[1], Glorisé Torres-Montalvo[1], Kyle Mimun[1]
and A. Joshua Wand[1,2,3*]

Departments of Biochemistry & Biophysics,[1] Chemistry[2] and Molecular & Cellular Medicine[3], Texas A&M University, College Station, Texas USA 77843

Supplementary Table 1 – Acquisition parameters for NMR assignment spectra						
Spectrum			**IL-1β**	**IL1Ra**	**IGPS**	**MBP‡**
HSQC[1]	^{1}H (direct)	SW (ppm)	13.58	14.87	16.04	15.62
		pts	1024	1024	1024	2048
	^{15}N	SW (ppm)	30.0	27	30.0	36.0
		pts	128	82	128	128
	Field (MHz)		800	800	750	800
	NS		8	8	8	32
HNCA[2]	^{1}H (direct)	SW (ppm)	20.82	14.87	16.04	15.62
		pts	1024	1024	1024	2048
	^{15}N	SW (ppm)	30.0	29	30.0	36.0
		pts	50†	50†	52†	64†
	^{13}C	SW (ppm)	33	30	28	29.0
		pts	72†	128†	74†	98†
	Field (MHz)		800	800	750	800
	NS		8	16	32	32
HN(CO)CA[2]	^{1}H (direct)	SW (ppm)	20.82		16.04	15.62
		pts	1024		1024	2048
	^{15}N	SW (ppm)	30.0		30.0	36.0
		pts	50†		52†	64†
	^{13}C	SW (ppm)	33		28	29.0
		pts	72†		74†	98†
	Field (MHz)		800		750	800
	NS		8		32	16
HNCACB[3*]	^{1}H (direct)	SW (ppm)	20.82	14.87	16.04	15.62
		pts	1024	1024	1024	2048
	^{15}N	SW (ppm)	30	29	30.0	36.0
		pts	54†	56†	44†	64†
	^{13}C	SW (ppm)	70.0	65	65	63.0
		pts	296†	64†	60†	214†
	Field (MHz)		800	800	750	800
	NS		16	16	88	32
CBCA(CO)NH[3*]	^{1}H (direct)	SW (ppm)	20.82	14.87	16.04	15.62
		pts	1024	1024	1024	2048
	^{15}N	SW (ppm)	30	29	30.0	36.0
		pts	54†	56†	44†	64†
	^{13}C	SW (ppm)	70.0	65	65	63.0
		pts	296†	64†	60†	214†
	Field (MHz)		800	800	750	800
	NS		48	16	88	32
HNCO[4†]	^{1}H (direct)	SW (ppm)	20.82	14.87	16.04	15.62
		pts	1024	1024	1024	2048
	^{15}N	SW (ppm)	30.0	29	30.0	36.0
		pts	50†	64	52†	64†
	^{13}C	SW (ppm)	12.0	16	12.0	13.0
		pts	72†	192	32†	44†
	Field (MHz)		800	500	750	800
	NS		8	16	32	16
HN(CA)CO[5]	^{1}H (direct)	SW (ppm)	20.82	14.87	16.04	15.62
		pts	1024	1024	1024	2048
	^{15}N	SW (ppm)	30.0	29	30.0	36.0
		pts	50†	64	52†	64†
	^{13}C	SW (ppm)	12.0	16	12.0	13.0
		pts	72†	192	32†	44†
	Field (MHz)		800	500	750	800
	NS		32	32	420	64

SW = Sweep Width; pts = complex points; NS = scans/FID; ‡Spectra were acquired using TROSY[6,7] variants of the spectra; †Indirect dimensions acquired using NUS[8] with Poisson gap distribution sampling and reconstructed via hmsIST[8]; *HNCACB, HNCOCACB spectra acquired for IL-1β. TROSY-HNCOCACB and TROSY-HNCOCACB spectra were acquired for MBP; Field strengths are given as the proton Larmor frequency

Supplementary Table 2 – Execution time for Bayllagio on the model protein set‡			
System	**Average annealing run time (min)**	**Standard Deviation (min)**	**Total time (min)**
IL-1Ra	0.112	0.056	2
IL-1β	0.186	0.062	2
IGPS	0.307	0.116	3
MBP	0.489	0.180	5
CY1	0.871	0.317	8
ecTS	0.427	0.114	4
‡Performed on a 2019 6 core MacBook Pro (Intel processor) running 100 annealing runs with up to 12 simultaneous annealing runs at any given time.			

Supplementary Table 3 – Performance of Bayllagio using SHIFTX+ predicted shifts from PDB structures									
	Bayllagio			MARS			I-PINE		
Protein	**Matching**	**Missing**	**Mismatching**	**Matching**	**Missing**	**Mismatching**	**Matching**	**Missing**	**Mismatching**
IL-1β	1.000	0.000	0.000	0.881	0.086	0.033	N/A	N/A	N/A
IL-1Ra	0.958	0.028	0.014	0.764	0.187	0.049	0.950	0.000	0.050
IGPS	0.992	0.004	0.004	0.738	0.242	0.029	0.882	0.000	0.118
MBP	1.000	0.000	0.000	0.941	0.041	0.012	N/A	N/A	N/A
CY1	0.995	0.005	0.000	0.111	0.889	0.000	N/A	N/A	N/A
ecTS	1.000	0.000	0.000	0.184	0.000	0.000	N/A	N/A	N/A

Supplementary Table 4 – Bayllagio results from randomly depleted MBP resonances					
Data Set properties			**Bayllagio results**		
fraction Cα retained	**fraction Cβ retained**	**fraction CO retained**	**Fraction Matching* (± std)**	**Fraction Missing* (± std)**	**Fraction Mismatching* (± std)**
1.00	1.00	1.00	1.000	0.000	0.000
1.00	1.00	0.75	1.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
1.00	1.00	0.50	1.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
1.00	1.00	0.25	1.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
1.00	1.00	0.00	1.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
1.00	0.75	1.00	1.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
1.00	0.75	0.75	0.997 ± 0.004	0.003 ± 0.003	0.001 ± 0.001
1.00	0.75	0.50	0.998 ± 0.002	0.002 ± 0.002	0.000 ± 0.000
1.00	0.75	0.25	0.996 ± 0.001	0.004 ± 0.001	0.000 ± 0.000
1.00	0.75	0.00	0.999 ± 0.001	0.001 ± 0.001	0.000 ± 0.000
1.00	0.5	1.00	1.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
1.00	0.5	0.75	0.996 ± 0.002	0.004 ± 0.002	0.000 ± 0.000
1.00	0.5	0.50	0.993 ± 0.006	0.006 ± 0.005	0.001 ± 0.002
1.00	0.5	0.25	0.993 ± 0.005	0.006 ± 0.004	0.001 ± 0.002
1.00	0.5	0.00	0.995 ± 0.003	0.005 ± 0.002	0.001 ± 0.001
1.00	0.25	1.00	1.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
1.00	0.25	0.75	0.994 ± 0.004	0.006 ± 0.003	0.000 ± 0.001
1.00	0.25	0.50	0.99 ± 0.004	0.009 ± 0.003	0.002 ± 0.002
1.00	0.25	0.25	0.991 ± 0.005	0.008 ± 0.003	0.001 ± 0.002
1.00	0.25	0.00	0.988 ± 0.007	0.009 ± 0.005	0.003 ± 0.003
0.88	1.000	1.00	1.000	1.000	0.000
0.88	1.000	0.75	0.997 ± 0.003	0.002 ± 0.002	0.001 ± 0.001
0.88	1.000	0.50	0.996 ± 0.004	0.003 ± 0.003	0.001 ± 0.002
0.88	1.000	0.25	0.998 ± 0.003	0.002 ± 0.002	0.001 ± 0.001
0.88	1.000	0.00	0.997 ± 0.003	0.003 ± 0.003	0.001 ± 0.001
0.88	0.75	1.00	0.998 ± 0.002	0.002 ± 0.002	0.000 ± 0.001
0.88	0.75	0.75	0.984 ± 0.007	0.014 ± 0.007	0.001 ± 0.003
0.88	0.75	0.50	0.979 ± 0.008	0.019 ± 0.007	0.001 ± 0.002
0.88	0.75	0.25	0.972 ± 0.003	0.026 ± 0.003	0.001 ± 0.002
0.88	0.75	0.0	0.97 ± 0.012	0.026 ± 0.009	0.004 ± 0.005
0.88	0.5	1.00	0.996 ± 0.003	0.003 ± 0.002	0.001 ± 0.001
0.88	0.5	0.75	0.981 ± 0.008	0.017 ± 0.006	0.001 ± 0.002
0.88	0.5	0.50	0.966 ± 0.006	0.032 ± 0.006	0.002 ± 0.003
0.88	0.5	0.25	0.948 ± 0.014	0.049 ± 0.014	0.004 ± 0.003
0.88	0.5	0.00	0.935 ± 0.015	0.056 ± 0.012	0.009 ± 0.005
0.88	0.25	1.00	0.996 ± 0.004	0.004 ± 0.004	0.000 ± 0.000

0.88	0.25	0.75	0.972 ± 0.008	0.025 ± 0.007	0.004 ± 0.002
0.88	0.25	0.50	0.941 ± 0.015	0.052 ± 0.012	0.007 ± 0.005
0.88	0.25	0.25	0.911 ± 0.018	0.071 ± 0.013	0.017 ± 0.011
0.88	0.25	0.00	0.889 ± 0.018	0.09 ± 0.015	0.021 ± 0.008
0.75	1.000	1.00	0.999 ± 0.001	0.001 ± 0.001	0.000 ± 0.000
0.75	1.000	0.75	0.997 ± 0.003	0.003 ± 0.003	0.0 ± 0.001
0.75	1.000	0.50	0.993 ± 0.005	0.006 ± 0.004	0.001 ± 0.002
0.75	1.000	0.25	0.991 ± 0.008	0.007 ± 0.004	0.002 ± 0.005
0.75	1.000	0.00	0.99 ± 0.006	0.009 ± 0.005	0.001 ± 0.001
0.75	0.75	1.00	0.995 ± 0.004	0.004 ± 0.003	0.001 ± 0.001
0.75	0.75	0.75	0.976 ± 0.006	0.023 ± 0.005	0.002 ± 0.002
0.75	0.75	0.50	0.951 ± 0.016	0.044 ± 0.015	0.004 ± 0.004
0.75	0.75	0.25	0.942 ± 0.011	0.054 ± 0.012	0.004 ± 0.003
0.75	0.75	0.00	0.925 ± 0.011	0.068 ± 0.008	0.007 ± 0.006
0.75	0.5	1.00	0.993 ± 0.005	0.006 ± 0.004	0.001 ± 0.001
0.75	0.5	0.75	0.945 ± 0.013	0.05 ± 0.013	0.005 ± 0.002
0.75	0.5	0.50	0.906 ± 0.013	0.079 ± 0.011	0.015 ± 0.007
0.75	0.5	0.25	0.846 ± 0.017	0.127 ± 0.016	0.026 ± 0.009
0.75	0.5	0.00	0.825 ± 0.02	0.143 ± 0.019	0.031 ± 0.008
0.75	0.25	1.00	0.99 ± 0.005	0.008 ± 0.005	0.002 ± 0.001
0.75	0.25	0.75	0.921 ± 0.015	0.072 ± 0.013	0.007 ± 0.005
0.75	0.25	0.50	0.834 ± 0.031	0.131 ± 0.017	0.035 ± 0.02
0.75	0.25	0.25	0.749 ± 0.036	0.184 ± 0.024	0.066 ± 0.02
0.75	0.25	0.00	0.701 ± 0.026	0.224 ± 0.024	0.075 ± 0.02
0.5	1.000	1.00	0.998 ± 0.001	0.002 ± 0.001	0.000 ± 0.000
0.5	1.000	0.75	0.989 ± 0.008	0.008 ± 0.006	0.003 ± 0.002
0.5	1.000	0.50	0.984 ± 0.008	0.014 ± 0.007	0.002 ± 0.002
0.5	1.000	0.25	0.985 ± 0.004	0.014 ± 0.004	0.001 ± 0.002
0.5	1.000	0.00	0.982 ± 0.007	0.016 ± 0.006	0.002 ± 0.001
0.5	0.75	1.00	0.99 ± 0.003	0.009 ± 0.003	0.002 ± 0.001
0.5	0.75	0.75	0.94 ± 0.018	0.051 ± 0.014	0.009 ± 0.006
0.5	0.75	0.50	0.887 ± 0.023	0.096 ± 0.019	0.017 ± 0.006
0.5	0.75	0.25	0.833 ± 0.026	0.142 ± 0.015	0.025 ± 0.014
0.5	0.75	0.00	0.809 ± 0.019	0.158 ± 0.012	0.033 ± 0.013
0.5	0.5	1.00	0.982 ± 0.009	0.015 ± 0.008	0.003 ± 0.002
0.5	0.5	0.75	0.879 ± 0.027	0.102 ± 0.025	0.019 ± 0.008
0.5	0.5	0.50	0.736 ± 0.012	0.217 ± 0.014	0.047 ± 0.008
0.5	0.5	0.25	0.622 ± 0.024	0.311 ± 0.025	0.067 ± 0.01
0.5	0.5	0.00	0.559 ± 0.029	0.353 ± 0.025	0.088 ± 0.023
0.5	0.25	1.00	0.974 ± 0.004	0.021 ± 0.005	0.006 ± 0.002

0.5	0.25	0.75	0.773 ± 0.029	0.184 ± 0.022	0.043 ± 0.015
0.5	0.25	0.50	0.562 ± 0.03	0.369 ± 0.024	0.069 ± 0.022
0.5	0.25	0.25	0.412 ± 0.034	0.483 ± 0.032	0.104 ± 0.014
0.5	0.25	0.00	0.356 ± 0.021	0.544 ± 0.025	0.099 ± 0.019
0.25	1.00	1.00	0.997 ± 0.000	0.003 ± 0.000	0.000± 0.000
0.25	1.00	0.75	0.987 ± 0.004	0.012 ± 0.003	0.001 ± 0.001
0.25	1.00	0.50	0.973 ± 0.011	0.022 ± 0.008	0.005 ± 0.005
0.25	1.00	0.25	0.969 ± 0.006	0.026 ± 0.003	0.005 ± 0.007
0.25	1.00	0.00	0.971 ± 0.006	0.026 ± 0.005	0.003 ± 0.003
0.25	0.75	1.00	0.987 ± 0.006	0.012 ± 0.005	0.001 ± 0.001
0.25	0.75	0.75	0.924 ± 0.013	0.065 ± 0.012	0.011 ± 0.007
0.25	0.75	0.50	0.808 ± 0.028	0.159 ± 0.025	0.033 ± 0.007
0.25	0.75	0.25	0.743 ± 0.025	0.207 ± 0.023	0.05 ± 0.014
0.25	0.75	0.00	0.685 ± 0.024	0.252 ± 0.017	0.063 ± 0.016
0.25	0.5	1.00	0.974 ± 0.008	0.021 ± 0.006	0.005 ± 0.004
0.25	0.5	0.75	0.776 ± 0.03	0.179 ± 0.028	0.045 ± 0.011
0.25	0.5	0.50	0.564 ± 0.036	0.352 ± 0.038	0.084 ± 0.023
0.25	0.5	0.25	0.443 ± 0.016	0.462 ± 0.025	0.095 ± 0.014
0.25	0.5	0.00	0.364 ± 0.02	0.548 ± 0.028	0.088 ± 0.017
0.25	0.25	1.00	0.965 ± 0.005	0.028 ± 0.005	0.007 ± 0.003
0.25	0.25	0.75	0.569 ± 0.042	0.361 ± 0.034	0.069 ± 0.013
0.25	0.25	0.50	0.363 ± 0.036	0.534 ± 0.03	0.103 ± 0.02
0.25	0.25	0.25	0.214 ± 0.025	0.671 ± 0.034	0.115 ± 0.021
0.25	0.25	0.00	0.168 ± 0.01	0.765 ± 0.019	0.067 ± 0.015

*Entries with standard deviations were generated from the average of 10 independent runs on distinct, randomly-generated data sets

Supplementary Table 5 – Bayllagio results from CY1 data sets with fractions of the Cβ resonances

Data Set properties			Bayllagio results		
Fraction Cα Retained	**Fraction Cβ Retained**	**Faction CO Retained**	**Fraction Matching* (± std)**	**Fraction Missing* (± std)**	**Fraction Mismatching* (± std)**
1.0	0.1	1.0	0.953 ± 0.007	0.038 ± 0.004	0.010 ± 0.005
1.0	0.2	1.0	0.959 ± 0.009	0.035 ± 0.007	0.007 ± 0.003
1.0	0.3	1.0	0.960 ± 0.012	0.034 ± 0.008	0.007 ± 0.006
1.0	0.4	1.0	0.978 ± 0.007	0.020 ± 0.005	0.002 ± 0.003
1.0	0.5	1.0	0.977 ± 0.003	0.020 ± 0.002	0.002 ± 0.002
1.0	0.6	1.0	0.981 ± 0.007	0.018 ± 0.007	0.001 ± 0.001
1.0	0.7	1.0	0.984 ± 0.005	0.015 ± 0.005	0.000 ± 0.001
1.0	0.8	1.0	0.983 ± 0.003	0.015 ± 0.002	0.002 ± 0.003
1.0	0.9	1.0	0.986 ± 0.004	0.012 ± 0.003	0.002 ± 0.003

*Entries with standard deviations were generated from the average of 10 independent runs on distinct, randomly-generated data sets.

Supplementary Table 6 – Performance of Bayllagio using SHIFTX+ predicted shifts from AlphaFold2 models			
	Matching	**Missing**	**Mismatching**
IL-1β	1.000	0.000	0.000
IL-1Ra	0.972	0.029	0.000
IGPS	1.000	0.000	0.000
MBP	0.997	0.000	0.003
CY1	0.988	0.012	0.000
ecTS	0.992	0.004	0.004

Materials and Methods

Interleukin 1- β (IL-1 β)

A vector encoding IL1β was transformed into *E. coli* BL21DE3 cells and expressed in 1 L of 95% D_2O M9 media containing $^{15}NH_4Cl$ and $^{2}H,^{13}C$ glucose as the sole nitrogen and carbon sources, respectively. Cells were grown at 37°C to an OD_{600} of 0.9 and induced with 1 mM IPTG. Induction continued for 4 hrs at 37°C until harvesting via centrifugation at 3500xg and frozen overnight. The cell pellet was then thawed, resuspended in 10 mM potassium phosphate pH 8.0, 0.2 mM EDTA, 5 mM DTT and 1 mM PMSF. The cells were then lysed by sonication and centrifuged at 32,000xg for 30 min at 4°C. Lysate was then brought to 80% saturation with NH_4SO_4 and allowed to stir for 1 hr at 4°C. The suspension was then centrifuged for 30 min at 32,000xg 4°C and the pellet was resuspended in 25 mM ammonium acetate pH 4.5, 1 mM BME and dialyzed overnight (8 kDa MWCO) in the same buffer at 4°C. The dialyzed protein was then loaded onto a HiTrap Capto S column (Cytiva Life Sciences) equilibrated in 25 mM ammonium acetate pH 4.5, 1 mM BME and eluted with a linear gradient up to 500 mM ammonium acetate pH 4.5, 1 mM BME. Protein was then frozen and lyophilized. The lyophilized protein was dissolved in 20 mM Tris pH 8.0, 7M urea, 20 mM DTT and added drop wise to 20x volume of 20 mM tris, 100 mM NaCl, 5 mM DTT pH 8.0 under constant stirring. The refolded protein was then dialyzed against 50 mM sodium acetate pH 5.0, 5 mM DTT and concentrated to 0.67 mM. To this sample 0.02% NaN_3, 100 μM DSS and 5% D_2O was added. Triple resonance spectra were acquired at 23°C on an 800 MHz Bruker Neo spectrometer with a CryoProbe (Supplementary Table 1). Spectra were processed using the NMRPipe software package on the NMRbox platform. Spin systems were built by manual peaking picking using NMRFAM-SPARKY and referenced using DSS.

Interleukin-1 Receptor Antagonist (IL-1Ra) C66A/C121A

A vector encoding human interleukin-1 receptor antagonist (IL-1Ra) containing C66A/C121A amino acid substitutions was expressed using *E. coli* BL21(DE3) cells in M9 minimal media. The M9 minimal media contained $^{15}NH_4Cl$ and ^{13}C-glucose as the sole nitrogen and carbon sources respectively. The culture was centrifuged at 5000 rpm, and the cell pellet was resuspended in 20 mM Tris, 500 mM NaCl, 20 mM imidazole, pH 7.9 for sonication. Sonicated cells were centrifuged at 15000 rpm, and supernatant was loaded onto a His60 column (Takara Bio USA). The column was washed with 20 mM Tris, 500 mM NaCl, 40 mM imidazole, pH 7.9; and protein was eluted with 20 mM Tris, 500 mM NaCl, 500 mM imidazole, pH 7.9. The collected protein fraction was buffer exchanged to 12.5 mM HEPES, 50 mM NaCl, 5 mM CaCl2, pH 6.5 for His-tag removal by FXa protease (New England Biolabs) and further purified both by affinity (His60 resin) and size exclusion chromatography (S-75 Sephadex, Cytiva Life Sciences). The NMR sample was prepared by buffer exchanging the protein into 100 mM NaCl, 25 mM MES, pH 6.0 and concentrated to 1 mM, with the addition of 100 mM DSS, 5% D2O, and 0.02% NaN3 (Supplementary Table 1). Triple resonance assignment experiments were acquired at 35 °C on either a 500 MHz Bruker Avance spectrometer or an 800 MHZ Bruker Neo spectrometer both equipped with a Cryoprobe. NUS spectra were reconstructed using hmsIST and all spectra were processed with NMRpipe on NMRBox. Peaks were picked using NMRFAM-SPARKY and referenced using DSS.

Indole-3-glycerol phosphate synthase R43S

A R43S variant of the gene for indole-3-glycerol phosphate synthase from *S. solfataricus* (IGPS) was cloned in a modified pGS-21a vector downstream of an N-terminal His-tag and TEV protease site. This expression plasmid was a gift from the lab of Professor Robert Matthews, University of Massachusetts Medical School, Worcester. IGPS R43S protein was expressed in BL21(DE3) competent cells with

ampicillin antibiotic selection. Cells were grown at 37°C until they reached an OD600nm of 0.6 and 1mM IPTG was added to induce expression for 16-20h at 25°C. To isotopically label the protein for NMR spectroscopy, cells were grown in M9 minimal medium with $^{15}NH_4Cl$ and ^{13}C-glucose as the nitrogen and carbon sources, respectively. Cells were lysed in 100mM potassium phosphate, pH 7.5, 50mM KCl, 5mM imidazole by sonication. The lysate was loaded onto a Ni^{2+}-NTA column pre-equilibrated with the lysis buffer. Impurities weakly bound to the column were washed away with 100mM potassium phosphate, pH 7.5, 150mM KCl, 75mM imidazole, followed by equilibration into the low salt buffer 100mM potassium phosphate, pH 7.5, 50mM KCl, 75mM imidazole. Protein was eluted with 100mM potassium phosphate, pH 7.5, 50mM KCl, 500mM imidazole and dialyzed into lysis buffer. Purified His-tagged protein was concentrated to 5mL, and tag was cleaved with TEV protease added at 1:30 mass ratio and mixing at RT overnight. Untagged protein was separated TEV protease and uncleaved protein by Ni^{2+}-affinity chromatography. Protein aliquots were flash frozen and stored at -80°C. NMR samples of $^{15}N^{13}C$-labelled IGPS were prepared at 250µM concentration in 60 mM potassium phosphate, pH 7.2, 50mM KCl, 5% D_2O, 100 uM DSS. All data were collected on a 750MHz Bruker AVANCE spectrometer equipped with a CryoProbe at 50°C (Supplementary Table 1). All data were processed using the NMRPipe software package and peaks were picked manually using NMRFAM-SPARKY.

Maltose Binding Protein (MBP)

A vector encoding MBP was transformed into BL21DE3 cells and expressed in 1 L of 95% D_2O M9 media containing $^{15}NH_4Cl$ and $^{2}H,^{13}C$ glucose as the sole nitrogen and carbon sources respectively. Cells were grown at 37°C to an OD_{600} of 0.9 and induced with 1 mM IPTG. Induction continued for 4 hrs at 37°C until harvesting via centrifugation at 3500xg. The cell pellet was frozen overnight and resuspended in 20 mM Tris-HCl, 20 mM NaCl pH 8.0, 1 mM DTT. 6 mg of Lysozyme was added and was incubated under stirring for 30 min at room temperature. Cells were further lysed by sonication and centrifuged at 32000xg for 30 min at 4°C. Clarified lysate was filtered (0.45 um pore size) and loaded onto a 25 ml DEAE column equilibrated in 20 mM Tris, 20 mM NaCl, pH 8.0, 1 mM DTT. The protein was eluted using a gradient to 20 mM Tris, 500 mM NaCl. Protein was concentrated to 1-2 ml and run on a 112 ml Superdex 75 column equilibrated in 20 mM Tris, 20 mM NaCl, 2 mM DTT pH 8.0. The protein was pooled and unfolded by dialysis in 4 M GuCHl, 20 mM Tris-HCl, 1 mM DTT pH 7.5. Protein was refolded by repeated 10x dilution with 20 mM sodium phosphate pH 7.1, 1 mM EDTA, 2 mM β-cyclodextrin followed by concentration (4 times). From this a 0.5 mM sample of MBP was created. Spectra were acquired at 37°C on an 800 MHz Bruker Neo spectrometer with a CryoProbe using non-uniform Poission gap sampling in the indirect dimensions (Supplementary Table 1). Spectra were reconstructed using HMSIst[8] and processed using the NMRPipe[9] software package on the NMRBox[10] platform. Spin systems were built by manual peaking picking using NMRFAM-SPARKY[11].

References

1. Schleucher, J. *et al.* A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients. *J. Biomol. NMR* **4**, 301–306 (1994).
2. Bax, A. & Ikura, M. An efficient 3D NMR technique for correlating the proton and15N backbone amide resonances with the α-carbon of the preceding residue in uniformly15N/13C enriched proteins. *J. Biomol. NMR* **1**, 99–104 (1991).
3. Wittekind, M. & Mueller, L. HNCACB, a High-Sensitivity 3D NMR Experiment to Correlate Amide-Proton and Nitrogen Resonances with the Alpha- and Beta-Carbon Resonances in Proteins. *J. Magn. Reson. Ser. B* **101**, 201–205 (1993).
4. Clubb, R. T., Thanabal, V. & Wagner, G. A constant-time three-dimensional triple-resonance pulse scheme to correlate intraresidue 1HN, 15N, and 13C′ chemical shifts in 15N13C-labelled proteins. *J. Magn. Reson.* **97**, 213–217 (1992).
5. Grzesiek, S. & Bax, A. Improved 3D triple-resonance NMR techniques applied to a 31 kDa protein. *J. Magn. Reson.* **96**, 432–440 (1992).
6. Pervushin, K., Riek, R., Wider, G. & Wüthrich, K. Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 12366–12371 (1997).
7. Salzmann, M., Wider, G., Pervushin, K., Senn, H. & Wu, K. TROSY-type Triple-Resonance Experiments for Sequential NMR Assignments of Large Proteins. *J. Am. Chem. Soc.* **121**, 844–848 (1999).
8. Hyberts, S. G., Milbradt, A. G., Wagner, A. B., Arthanari, H. & Wagner, G. Application of iterative soft thresholding for fast reconstruction of NMR data non-uniformly sampled with multidimensional Poisson Gap scheduling. *J Biomol NMR* **52**, 315–327 (2012).
9. Delaglio, F. *et al.* NMRPipe: A multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **6**, 277–293 (1995).
10. Maciejewski, M. W. *et al.* NMRbox: A Resource for Biomolecular NMR Computation. *Biophys. J.* **112**, 1529–1534 (2017).
11. Lee, W., Tonelli, M. & Markley, J. L. NMRFAM-SPARKY: Enhanced software for biomolecular NMR spectroscopy. *Bioinformatics* **31**, 1325–1327 (2015).