NMR Determination of Protein pK_a Values in the Solid State

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Supporting Information

1. Preparation of pH titrated samples

Three pH controlled precipitation methods were tested. In the first method, GB1 was precipitated according to the procedure in Franks *et al*.[1] The protein pellet was isolated from the mother liquor using centrifugation, and the protein pellet was washed with a small quantity of the phosphate/citrate buffer cocktail. Upon the addition of a small amount of buffer (7.5 μL) the protein redissolved. In the second method, the protein solution and precipitate solution were pH controlled with a phosphate/citrate buffer cocktail. Upon the addition of the buffer to the precipitant solution, a significant quantity of solid material appeared; we attribute this to the buffer salts precipitating from the solution. Thus, the first two methods proved not to be viable for further study.

The third method, as described below, was used to create seven pH-controlled nanocrystalline uniformly-^{13}C,^{15}N labeled GB1 samples. Samples 3 through 7 (Table S.1) produced nanocrystalline protein pellets, similar in appearance as those produced by Franks *et al*.[1] and Frericks Schmidt *et al*.[2] Samples 1 and 2 produced a phase-separated solution upon adding the precipitate solution. Centrifuging further separated the phases, yielding a large white pellet that was analyzed by SSNMR.

GB1 was expressed, purified and concentrated to 40 mg of uniformly-$^{13}C^{15}N$ labeled GB1/mL according to procedures given in Franks *et al*.[1] The previous published precipitation protocol was modified to control the pH of the protein and mother liquor solution during crystallization. Specifically, both the pH of the protein/buffer solution and the pH of the precipitate solution were adjusted to defined values, prior to adding the precipitate solution to the protein/buffer solution to induce precipitation. The concentrated protein was dialyzed in a 0.2 mL dialysis button using 3,500 MWCO Snakeskin dialysis tubing (Pierce, Rockford, IL) against 40 mL of the 100 mM sodium phosphate/ 100 mM sodium citrate buffer cocktail at the pH indicated in Table S.1 for 16 h at 4 ºC. After dialysis the protein was diluted to 25 mg/mL and the pH was measured (Table S.1).

GB1 was precipitated with a 60% MPD, 30% IPA and 10% phosphoric acid solution adjusted to the pH indicated in Table S.1. Each sample was precipitated with a total of 3.5 volumes of the pH-adjusted MPD/IPA solution. After each aliquot was added, the sample was fully mixed by vortexing and allowed to sit at 25 ºC for ~10 min. The final sample composition (by volume) was 25% buffer controlled protein solution and 75% pH-adjusted MPD/IPA precipitate solution. After four days at 4 ºC, the nanocrystalline material of samples 3, 4, 5, 6 and 7 from Table S.1 were isolated by centrifuging at 3,000 rpm for 5 minutes and packed in a 3.2 mm SSNMR rotor (Varian, Inc., Palo Alto, CA); it is likely based on previous studies that significantly less than four days would have been sufficient to yield a similar quantity of nanocrystalline precipitate. Samples 1 and 2 were centrifuged at 3,000 rpm for 3 h. The white pellets were packed into 3.2 mm SSNMR rotors. Samples were positioned in the middle 80% of the coil volume by spacing with silicone rubber disks cut from 1.5 mm thick sheet (McMaster-Carr PN 8632K921) and cylindrical Kel-F spacers. The rubber disks prevented the sample from dehydrating during data acquisition. Crystalline material, after isolated by centrifugation as described above, was packed into SSNMR rotors with residual mother liquor to allow proton exchange with free carbonyl groups.

2. Solid-state NMR spectroscopy

^{13}C-^{13}C correlation spectra were acquired for all samples on a 600 MHz InfinityPlus spectrometer (Varian, Inc.) using a HXY 3.2 mm probe, tuned to the ^{1}H, ^{13}C and ^{15}N frequencies. Spectra were acquired with 25 ms of DARR mixing[3], 25.6 ms of acquisition and 1024 rows of TPPI phase encoded t_1 evolution (dw=12.5 μs). Spectra were processed with 35 Hz (F1) and 35 Hz (F2) net Lorenzian-to-Gaussian line broadening.

Heteronuclear correlation and SPC5[4,5] mixing spectra were acquired for samples 3, 4, 5, 6 and 7 with a 500 MHz InfinityPlus spectrometer (Varian, Inc.) using a HCN Balun 3.2 mm probe. ^{13}C-^{13}C correlation spectra with 2 supercycles (1080 μs total) $SPC5_3$ mixing[4] were acquired with 20.5 ms of acquisition and 768 rows of TPPI phase encoded t_1 evolution (dw=15 μs) and processed with 20 Hz (F1) and 25 Hz (F2) net Lorenzian-to-Gaussian line broadening. Heteronuclear N-(CA)-CX and N-(CO)-CX were acquired with 45 ms of DARR[3] mixing, 20.5 ms of ^{13}C acquisition and 128 rows of TPPI phase encoded t_1 evolution (dw=180 μs) and processed with 25 Hz (F1) and 15 Hz (F2) net Lorenzian-to-Gaussian line broadening. Three-dimensional N-CA-CX and N-CO-CX spectra were acquired on sample 3 and sample 7 to perform *de novo* chemical shift assignments. These spectra were acquired with 45 ms DARR[3] mixing, 20.5 ms acquisition, 64 rows of t_1 evolution (dw= 270 μs) and 32 rows t_2 evolution (dw=270 μs). The 3D spectra were processed with 60 Hz (F1), 60 Hz (F2) and 25 Hz (F3) net Lorenzian-to-Gaussian line broadening.

3. Data fitting

Observed ^{13}C sidechain carbonyl chemical shifts for each sample were plotted as a function of sample buffer pH. pK_a values were determined by fitting a modified Henderson-Hasselbalch equation including the Hill parameter (n_H)[6] (equation 1) to the experimental data:

$$\delta_{obs} = \frac{\delta_{HA} + \delta_{A^-} * 10^{n_H(pH-pk_a)}}{1+10^{n_H(pH-pK_a)}}, \qquad (1)$$

where δ_{obs}, δ_{HA}, δ_{A^-} are the observed, protonated and deprotonated chemical shifts, respectively, for each sidechain carbonyl. Iterative curve fitting by varying the Hill parameter (n_H) and the pK_a values was performed in Igor Pro (Version 5.05A, Lake Oswego, OR) until the minimum chi-squared values were achieved. The pI of GB1 was calculated from the protein sequence using pI wrapper.[7]

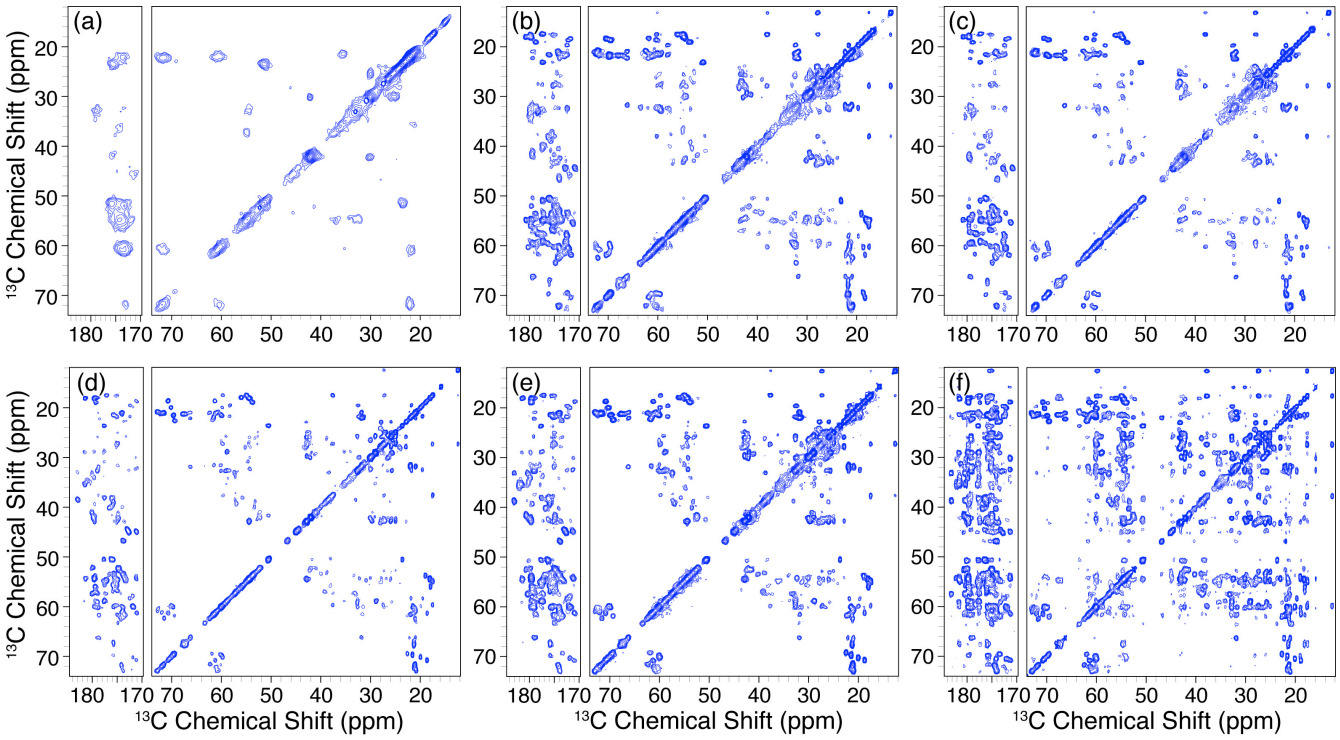

Figure S1. ^{13}C-^{13}C 2D spectra of uniformly-^{13}C,^{15}N labeled GB1 at (a) pH = 2.85 (b) pH = 3.63, (c) pH = 3.95, (d) pH = 4.55, and (e) pH = 5.22 acquired with 25 ms of DARR mixing, and (f) pH = 5.64 acquired with 90 ms DARR mixing on an Infinity Plus 600 MHz (^{1}H frequency) spectrometer.

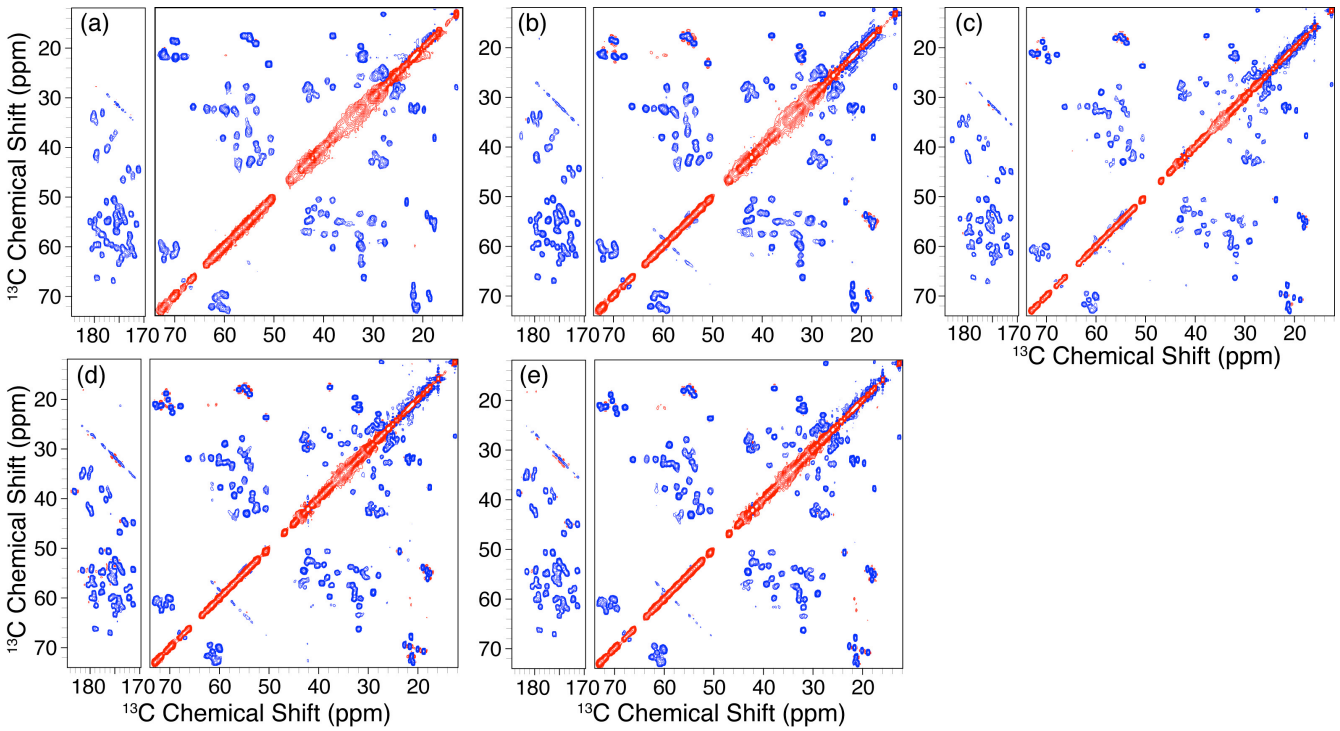

Figure S2. ^{13}C-^{13}C 2D spectra of uniformly-^{13}C,^{15}N labeled GB1 at (a) pH = 3.63, (b) pH = 3.95, (c) pH = 4.55, (d) pH = 5.22, and (e) pH = 5.64 acquired with $SPC5_3$ mixing on an Infinity Plus 500 MHz (^{1}H frequency) spectrometer.

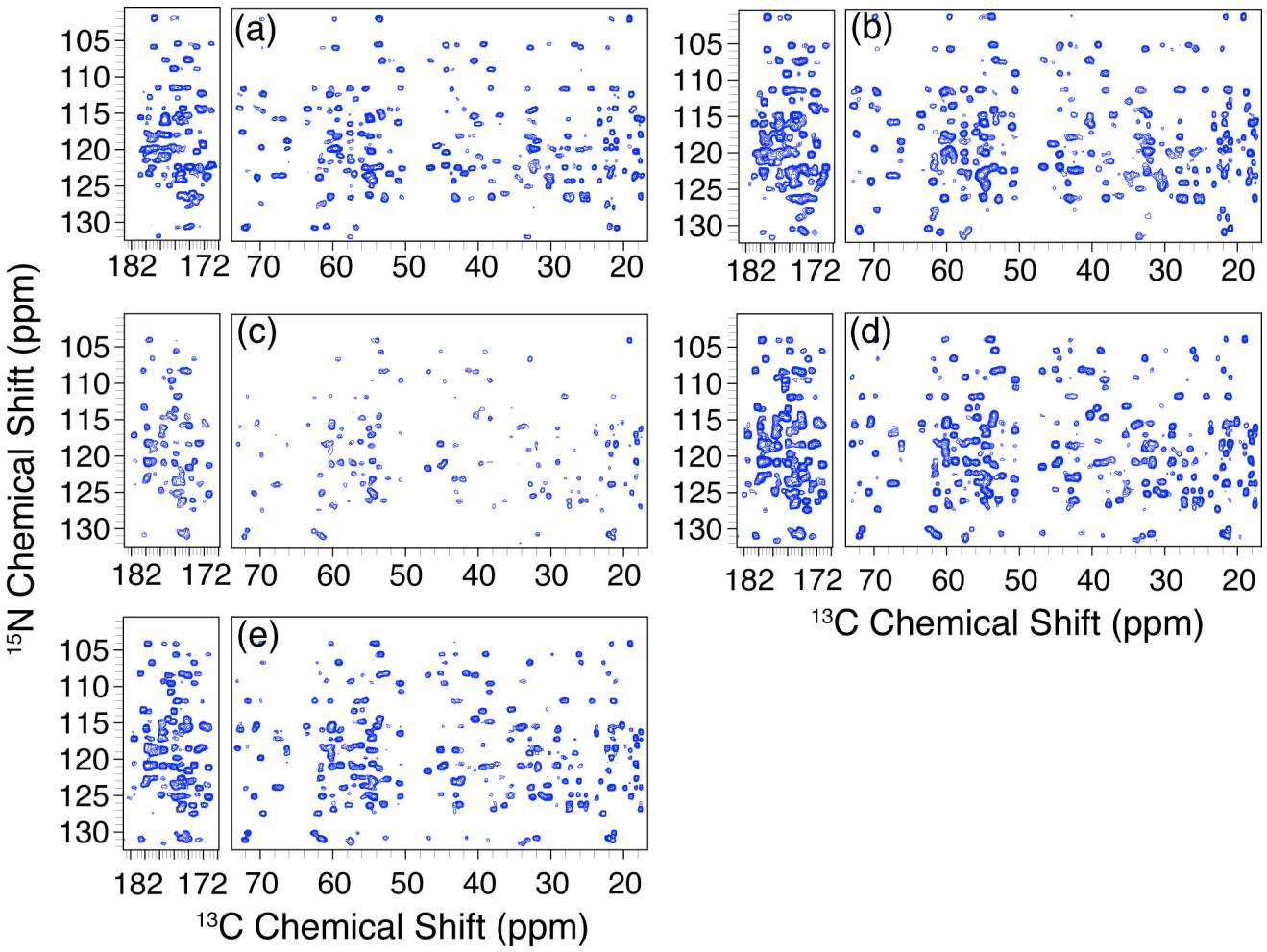

Figure S3. ^{15}N-(^{13}CO)-^{13}CX 2D spectra of uniformly-^{13}C,^{15}N labeled GB1 at (a) pH = 3.63, (b) pH = 3.95, (c) pH = 4.55, (d) pH = 5.22, and (e) pH = 5.64 acquired with 45 ms of DARR mixing on an Infinity Plus 500 MHz (^{1}H frequency) spectrometer.

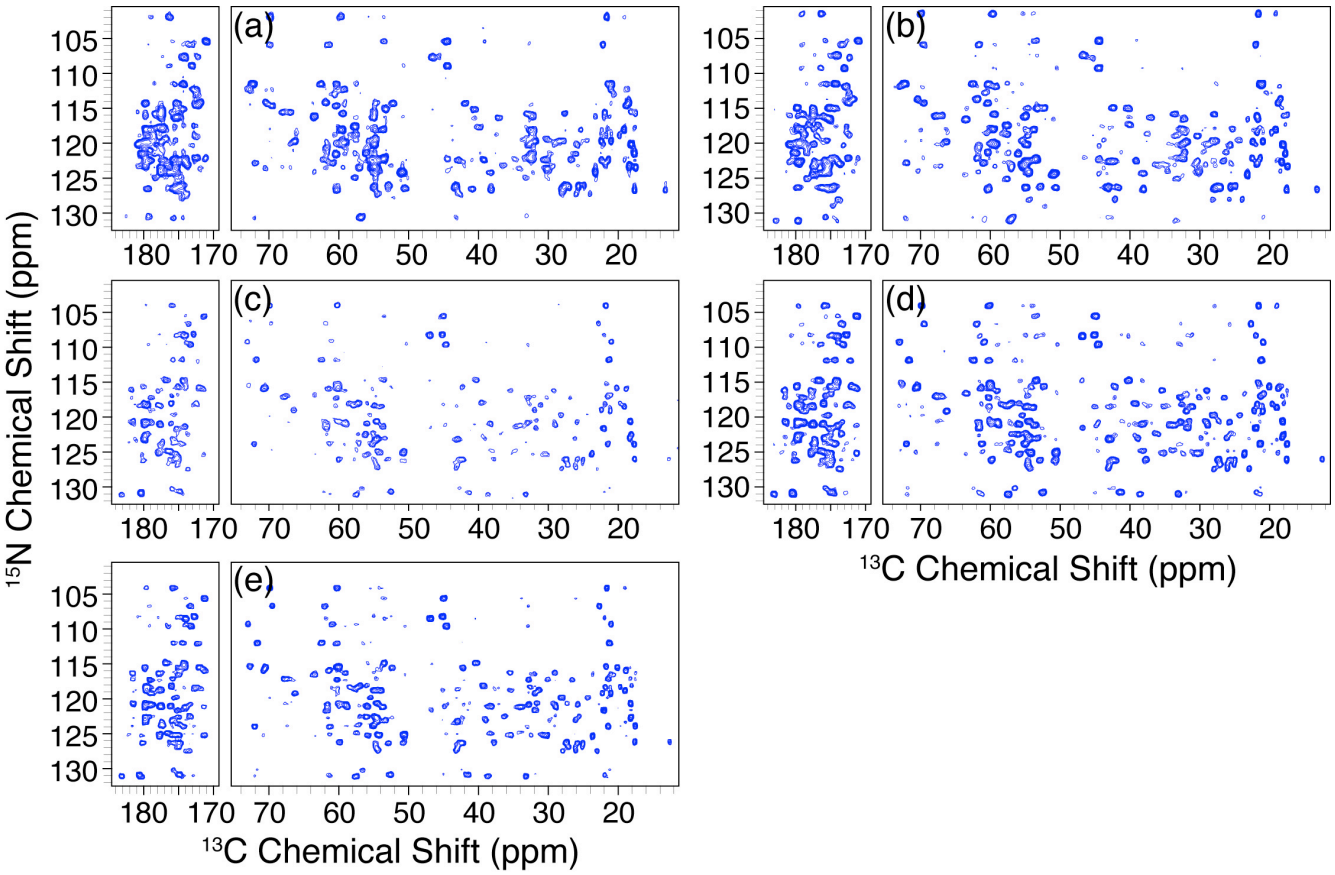

Figure S4. ^{15}N-(^{13}CA)-^{13}CX 2D spectra of uniformly-^{13}C,^{15}N labeled GB1 at (a) pH = 3.63, (b) pH = 3.95, (c) pH = 4.55, (d) pH = 5.22, and (e) pH = 5.64 acquired with 45 ms of DARR mixing on an Infinity Plus 500 MHz (^{1}H frequency) spectrometer.

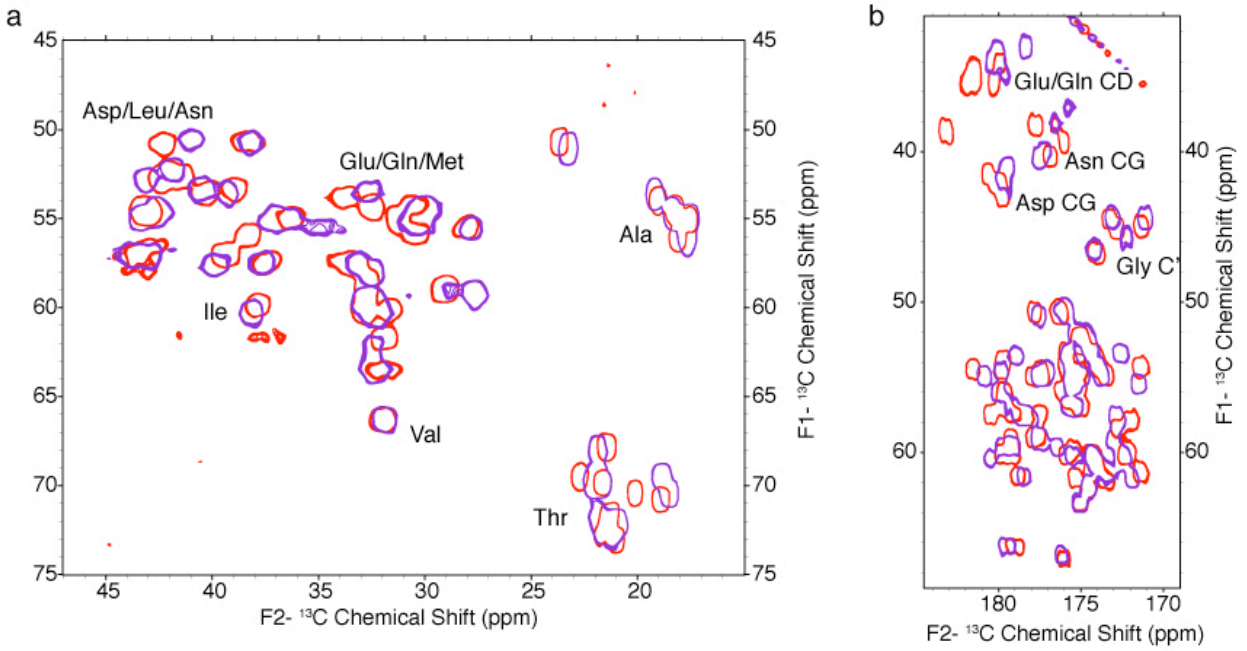

Figure S5. ^{13}C-^{13}C spectra of GB1 at pH 5.64 (red) and pH=3.63 (purple). (a) Expansions of the CA-CB and Thr CB-CG and (b) CA-CO regions of the ^{13}C-^{13}C 2D spectra acquired with $SPC5_3$ mixing .

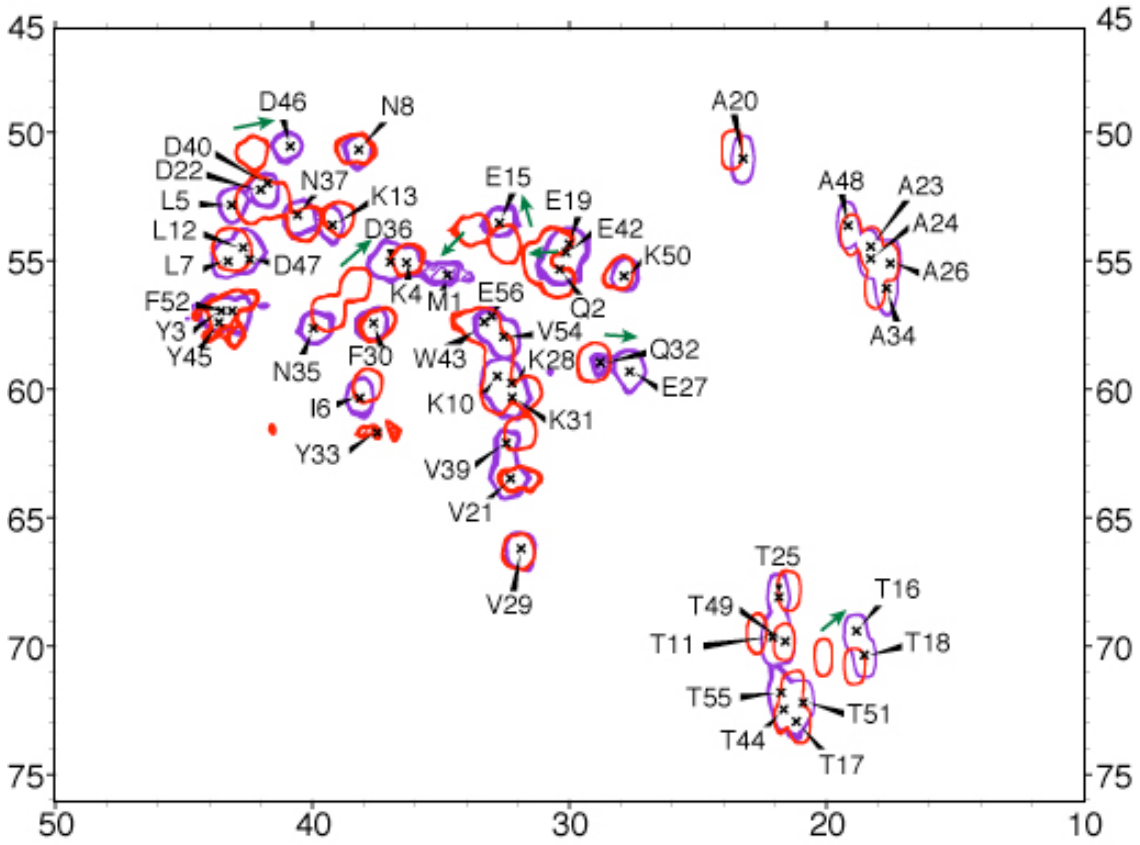

Figure S6. Assigned CA-CB correlations of GB1 at pH 5.64 (red) and pH=3.63 (purple). Expansions of the CA-CB and Thr CB-CG regions of the ^{13}C-^{13}C 2D spectra acquired with $SPC5_3$ mixing. Chemical shift assignments for GB1 at pH=3.63 are indicated.

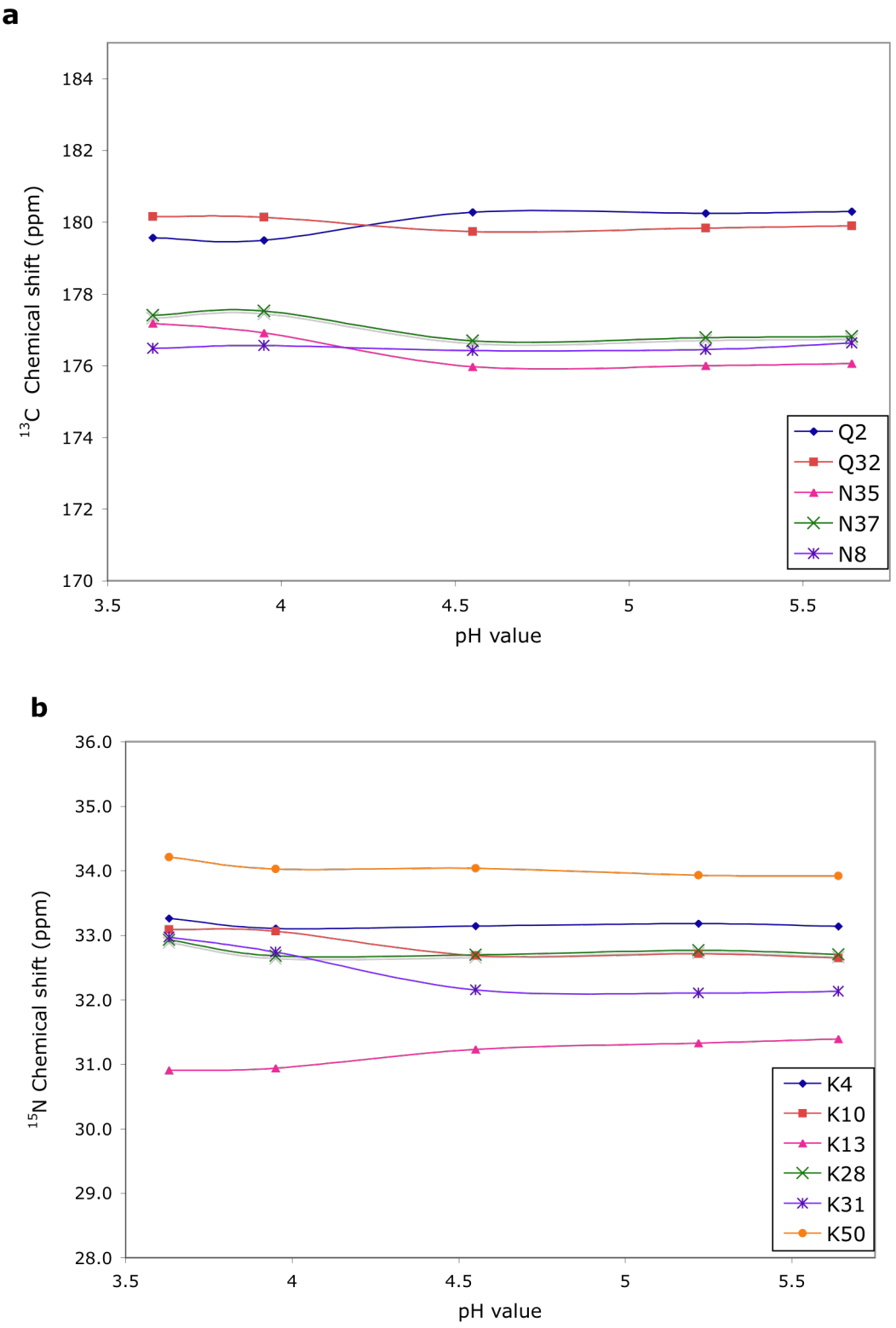

Figure S7. (a) Asparagine and Glutamine sidechain carbonyl chemical shifts over the pH titrated range. (b) Lysine sidechain amide chemical shifts over the pH titrated range.

Table S.1. pH value of titrated GB1 samples.

Sample	Buffer pH (before dialysis)	Buffer pH (after dialysis)	Precipitant pH	Final pH of mother liquor
1	2.42	2.53	2.41	2.42
2	2.74	2.85	2.75	2.92
3	3.55	3.63	3.55	4.44
4	3.89	3.95	3.88	5.19
5	4.53	4.55	4.52	5.98
6	5.27	5.22	5.28	6.30
7	5.68	5.64	5.70	6.70

Table S.2. Chemical shift assignments of GB1 charged residues at pH=3.63, 3.95, 4.55, 5.22 and 5.64.

Group	Atom	Chemical shift at pH=3.63 (ppm)	Chemical shift at pH=3.95 (ppm)	Chemical shift at pH=4.55 (ppm)	Chemical shift at pH=5.22 (ppm)	Chemical shift at pH=5.64 (ppm)
D22	C	175.25	175.17	175.05	174.93	175.09
D22	CA	52.19	52.27	52.29	52.27	52.49
D22	CB	41.92	42.33	42.09	42.07	42.24
D22	CG	179.68	179.78	179.90	179.81	179.87
D22	N	114.31	114.94	115.66	115.61	115.53
D36	C	175.44		176.07	176.08	176.17
D36	CA	55.03	55.00	55.82	55.81	55.94
D36	CB	37.02	36.99	38.23	38.18	38.32
D36	CG	175.57	175.63	177.63	177.69	177.77
D36	N	116.29	116.27	120.94	120.97	121.10
D40	C	174.38		174.83	174.88	175.00
D40	CA	52.13	52.19	52.40	52.52	52.73
D40	CB			41.13	41.45	41.57
D40	CG			180.28	180.58	180.63
D40	N	127.86	129.02	130.76	130.79	130.94
D46	C	175.75	175.89	176.18	176.14	176.20
D46	CA	50.50	50.62	50.58	50.75	50.84
D46	CB	40.84	41.69	41.86	42.33	42.30
D46	CG	179.33	179.62	180.04	180.10	180.12
D46	N	126.60	126.35	126.19	126.21	126.33
D47	C	177.10	177.23	177.36	177.31	177.39
D47	CA	54.81	54.79	54.56	54.64	54.71

Group	Atom	Chemical shift at pH=3.63 (ppm)	Chemical shift at pH=3.95 (ppm)	Chemical shift at pH=4.55 (ppm)	Chemical shift at pH=5.22 (ppm)	Chemical shift at pH=5.64 (ppm)
D47	CB	42.52	42.64	42.96	42.93	43.02
D47	CG	179.17	179.27	179.74	179.70	179.77
D47	N	122.54	122.57	123.34	123.20	123.33
E15	C	172.52	172.45	174.02	174.02	174.04
E15	CA	53.47	53.48	53.67	53.72	53.88
E15	CB	32.81	32.79	33.44	33.56	33.76
E15	CD	179.92	180.53	181.27	181.30	181.48
E15	CG	33.52	33.59	34.41	34.24	34.83
E15	N	122.12	122.18	121.28	121.19	121.19
E19	C	175.51	175.56	175.75	175.79	175.93
E19	CA	54.40	54.46	54.19	54.06	54.29
E19	CB	30.09	30.25	30.21	30.33	30.58
E19	CD	180.60	181.36	181.88	181.97	182.24
E19	CG	34.04	34.61	35.24	35.33	35.55
E19	N	123.67	124.15	125.09	125.12	125.20
E27	C	177.41	177.48	177.81	177.73	177.86
E27	CA	59.35	59.33	58.93	59.07	59.09
E27	CB	27.63	27.88	29.05	28.95	29.03
E27	CD	178.43	178.94	181.59	181.57	181.69
E27	CG	33.10	33.69	35.39	35.45	35.50
E27	N	115.72	115.87	116.19	116.21	116.28
E42	C	176.82	176.96	177.59	177.74	177.93
E42	CA	54.71	54.66	54.81	54.84	55.01
E42	CB	30.23	30.31	31.03	31.12	31.26
E42	CD	178.41	178.82	181.16	181.17	181.32

Group	Atom	Chemical shift at pH=3.63 (ppm)	Chemical shift at pH=3.95 (ppm)	Chemical shift at pH=4.55 (ppm)	Chemical shift at pH=5.22 (ppm)	Chemical shift at pH=5.64 (ppm)
E42	CG	33.11	33.40	34.57	35.17	35.47
E42	N	119.39	118.92	118.29	118.49	118.75
E56	C	179.27	179.64	180.13	180.45	180.48
E56	CA	57.08	57.50	57.36	57.47	57.58
E56	CB	32.93	32.98	33.09	33.09	33.22
E56	CD	182.50	182.81	183.05	183.10	183.20
E56	CG	35.93	38.50	38.63	38.69	38.69
E56	N	130.52	131.09	131.08	131.05	131.20
N35	C	177.92	178.20	179.45	179.27	179.68
N35	CA	57.59	57.62	57.08	57.04	57.01
N35	CB	39.85	40.02	39.44	39.27	39.31
N35	CG	177.18	176.92	175.98	176.00	176.07
N35	N	117.69	117.40	117.99	118.06	118.17
N37	C	174.38	174.39	174.13	174.13	174.27
N37	CA	53.31	53.25	53.42	53.47	53.54
N37	CB	40.54	40.26	40.19	40.16	40.32
N37	CG	177.41	177.53	176.69	176.78	176.81
N37	N	115.11	115.00	114.71	114.78	114.86
N8	C	176.07	176.24	176.06	176.18	176.45
N8	CA	50.72	50.51	50.55	50.54	50.68
N8	CB	38.13	38.11	38.21	38.23	38.32
N8	CG	176.49	176.56	176.42	176.46	176.64
N8	N	124.24	126.40	124.86	124.89	125.06
Q2	C	175.40	175.44	175.14	174.86	175.08
Q2	CA	55.42	55.21	55.83	55.76	55.85

Group	Atom	Chemical shift at pH=3.63 (ppm)	Chemical shift at pH=3.95 (ppm)	Chemical shift at pH=4.55 (ppm)	Chemical shift at pH=5.22 (ppm)	Chemical shift at pH=5.64 (ppm)
Q2	CB	30.41	30.52	30.51	30.47	30.49
Q2	CD	179.57	179.50	180.28	180.25	180.30
Q2	CG	34.81	35.02	35.40	35.22	35.31
Q2	N	122.46	123.14	125.36	125.25	125.30
Q32	C	177.45	177.52	177.66	177.40	177.57
Q32	CA	59.05	59.08	58.90	58.82	58.85
Q32	CB	28.76	28.92	28.84	28.87	28.94
Q32	CD	180.16	180.14	179.74	179.84	179.90
Q32	CG	34.36	34.32	34.04	34.06	34.15
Q32	N	119.89	120.11	121.11	121.20	121.16

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