Supporting Information

BSA kinetically traps protein KH1 in unfolded state

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Experimental Procedures

Protein Expression and Purification. The His-tagged KH1 gene was cloned into the pET-21a vector. The plasmid pET21a-KH1(S193A)-His, containing the His-tagged KH1 gene, was transformed into *Escherichia coli* BL21(DE3) cells. The transformed cells were plated on LB-agar plates containing 100 μg/mL ampicillin and incubated at 37°C. A single colony was inoculated into 5 mL LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) with 100 μg/mL ampicillin, and the culture was incubated overnight at 37°C with shaking at 220 rpm. Then, 100 μL of the pre-culture was used to inoculate 100 mL of TY medium (16 g/L tryptone, 10 g/L yeast extract, 10 g/L NaCl) with 100 μg/mL ampicillin in a 250 mL flask, and the culture was incubated at 37°C with shaking at 220 rpm for 1 hour. The cells were harvested by centrifugation at 2000 g for 10 minutes and then transferred to 1 L of supplemented M9 medium containing 1 g of $^{15}NH_4Cl$ in a 3 L flask. For 3FY labeling, 70 mg of D, L-m-fluorotyrosine, 60 mg of L-phenylalanine, 60 mg of L-tryptophan, and 0.5 g of glyphosate were added to the culture when the optical density at 600 nm (OD_{600}) reached 0.4. The culture was then grown at 37°C with shaking at 220 rpm and induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) when the OD600 reached approximately 0.6. Cells were induced for 20 hours at 20°C and harvested by centrifugation at 4000 g for 10 minutes. The cell pellet was stored at -80°C until purification.

KH1 was purified using a HisTrap Ni-NTA affinity column followed by gel filtration on a Superdex 75 column. The cell pellet was resuspended in 100 mL of buffer A (50 mM Tris, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed using an ATS high-pressure homogenizer at 1000 bar and 4°C. The supernatant was collected after centrifugation at 20,000 rpm at 4°C for 30 minutes and loaded onto a Ni-affinity agarose column pre-equilibrated with buffer A. The column was washed with buffer A and eluted with buffer B (50 mM Tris, 300 mM NaCl, 500 mM imidazole, pH 8.0). The eluted protein was loaded onto a size-exclusion column (GE Superdex 75 column) and eluted with buffer C (50 mM Tris, 250 mM NaCl, pH 8.0). The purified KH1 protein was desalted using a desalting column, lyophilized for 24 hours, and stored at -80°C for future use. The purification process yielded approximately 20 mg of KH1 protein from 1 L of culture.

Sample preparation for NMR. Lysozyme and bovine serum albumin (BSA) were purchased from Beijing InnoChem Science & Technology Co., Ltd. and Shanghai Ruji Biotechnology Development Co., Ltd., respectively. Protein concentrations, including those of KH1, lysozyme, and BSA, were measured using a Nanodrop 2000 spectrophotometer. The NMR buffers consisted of 10 mM Tris, 50 mM NaCl (pH 7.5), and 50 mM PBS, 100 mM NaCl (pH 7.5). The KH1 concentration for NMR measurements ranged from 0.2 to 1.0 mM. BSA was purified using gel-exclusion chromatography on a Superdex 75 column.

NMR Spectroscopy. ^{19}F and ^{1}H-^{15}N TROSY-HSQC spectra were recorded at temperatures between 25°C and 40°C on Bruker 600 MHz and 700 MHz spectrometers equipped with 5-mm H/F (C, N) triple resonance cryoprobes. One-dimensional ^{19}F spectra were obtained with a sweep width of 20 ppm, comprising between 640 and 2048 transients, depending on the sample concentration. The relaxation delay for ^{19}F spectra was set to 2 s, with an acquisition time of 0.73 s. ^{1}H-^{15}N TROSY-HSQC spectra were acquired with a ^{1}H spectral width of 12 ppm, a ^{15}N spectral width of 32 ppm, and 2048 and 128 complex points in the direct and indirect dimensions, respectively.

Data analysis. Data analysis was performed using Topspin 3.2, MestRe-c, and OriginPro 2024. A

line-broadening of 20 Hz was applied to all ^{19}F spectra. Free induction decays were processed in MestRe-c and exported as ASCII text files. Peak fitting and integration were conducted in OriginPro 2024 using Lorentzian functions. The relative populations of unfolded and native states were calculated from the integrated peak areas, and the percentage of unfolded state was determined accordingly. The percentages of unfolded state over time were fitted to an exponential model to obtain the unfolding rate constant of KH1 S193A induced by BSA. The fitting errors were used to estimate the uncertainties in the unfolding rate constants.

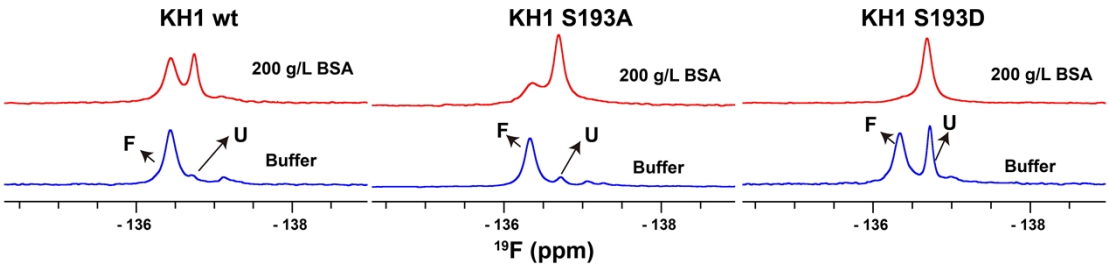

Figure S1 The ^{19}F NMR spectra of KH1 and its mutants in both buffer and 200 g/L BSA solutions at pH 7.5 and 300 K.

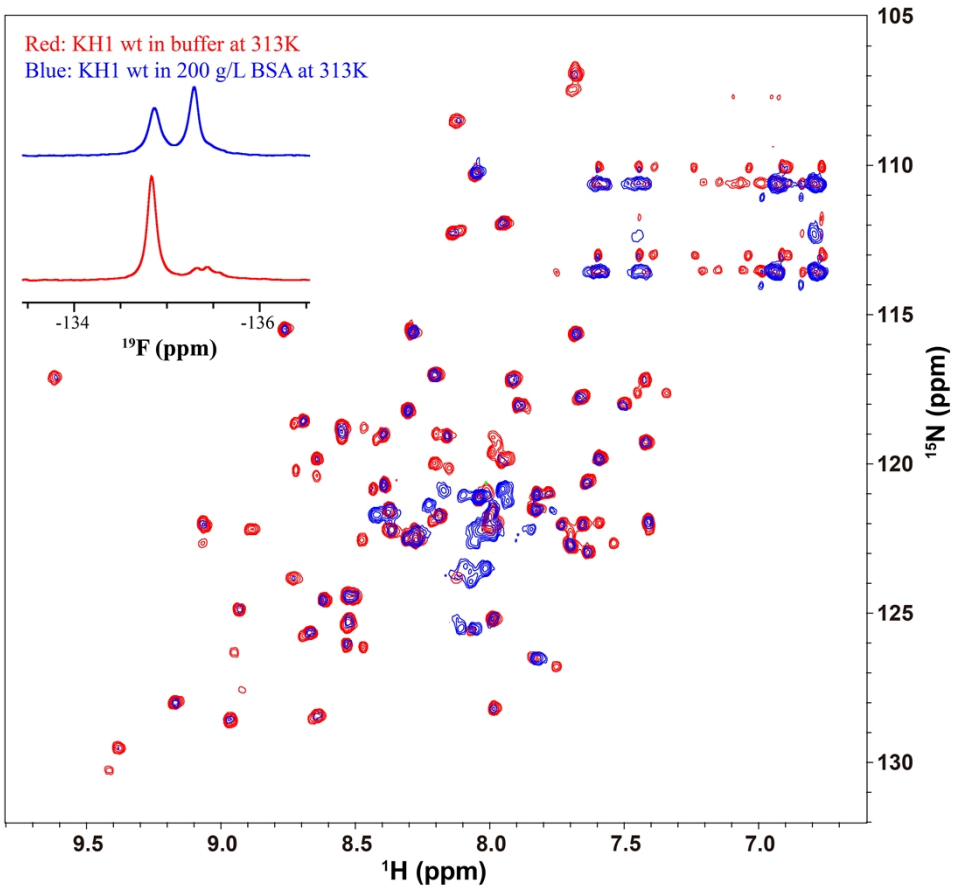

Figure S2 Overlay of ^{1}H-^{15}N TROSY-HSQC spectra of KH1 wt in buffer (Red) and 200 g/L BSA (Blue) at 313K and pH 7.5. Inset showed the corresponding ^{19}F spectra of KH1 wt.

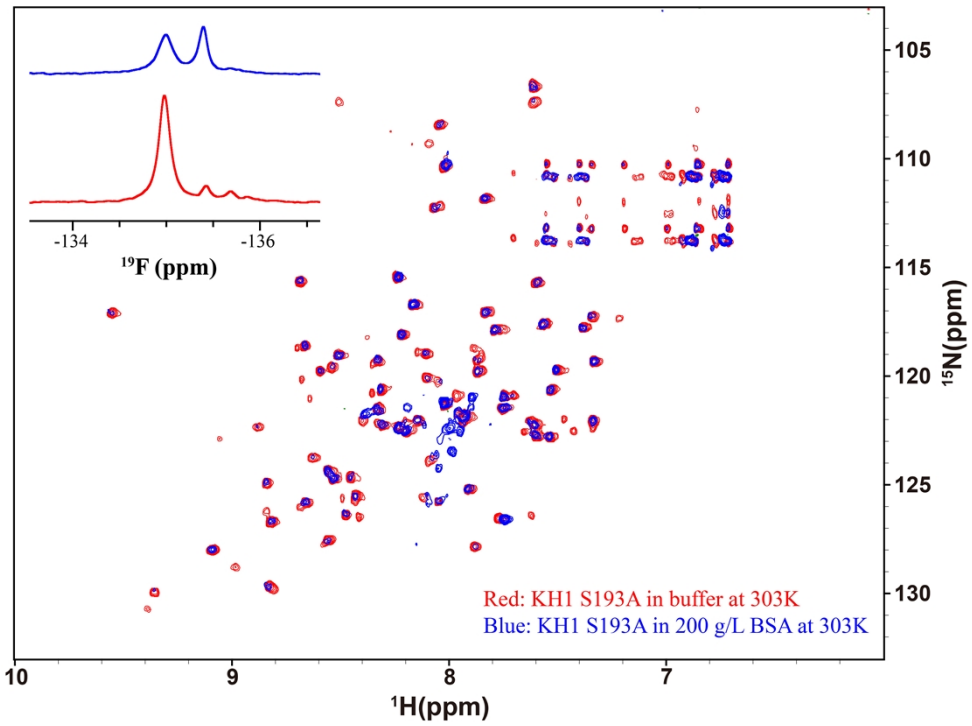

Figure S3 Overlay of ^{1}H-^{15}N TROSY-HSQC spectra of KH1 S193A in buffer (Red) and 200 g/L BSA (Blue) at 303K and pH 7.5. Inset showed the corresponding ^{19}F spectra of KH1 S193A.

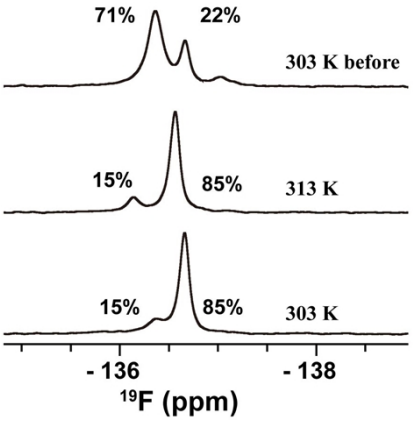

Figure S4 ^{19}F spectra of KH1 in 200 g/L BSA at pH 7.5, 303 K, 313 K, and 303 K after heated at 313 K for 260 min.

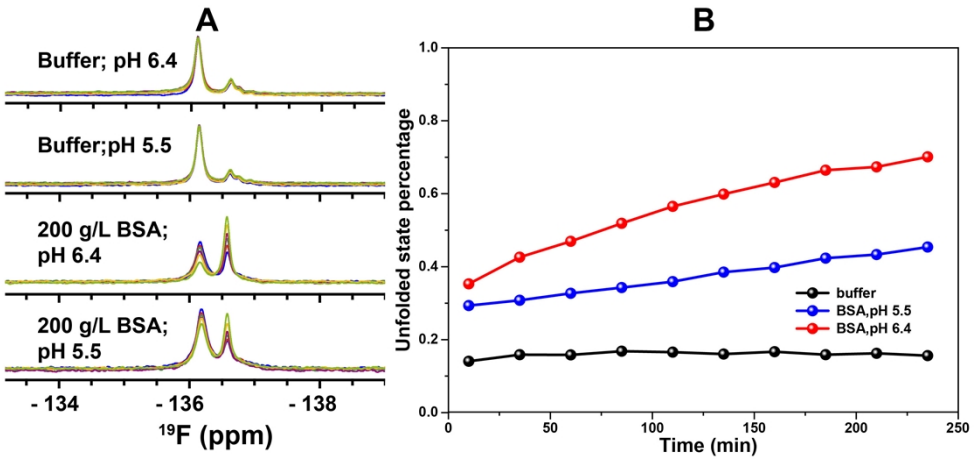

Figure S5 (A) ^{19}F spectra of KH1 in buffer and 200 g/L BSA at 310K, pH 6.5 and 5.5. (B) Percentages of unfolded state of KH1 S193A in different conditions as a function of time.

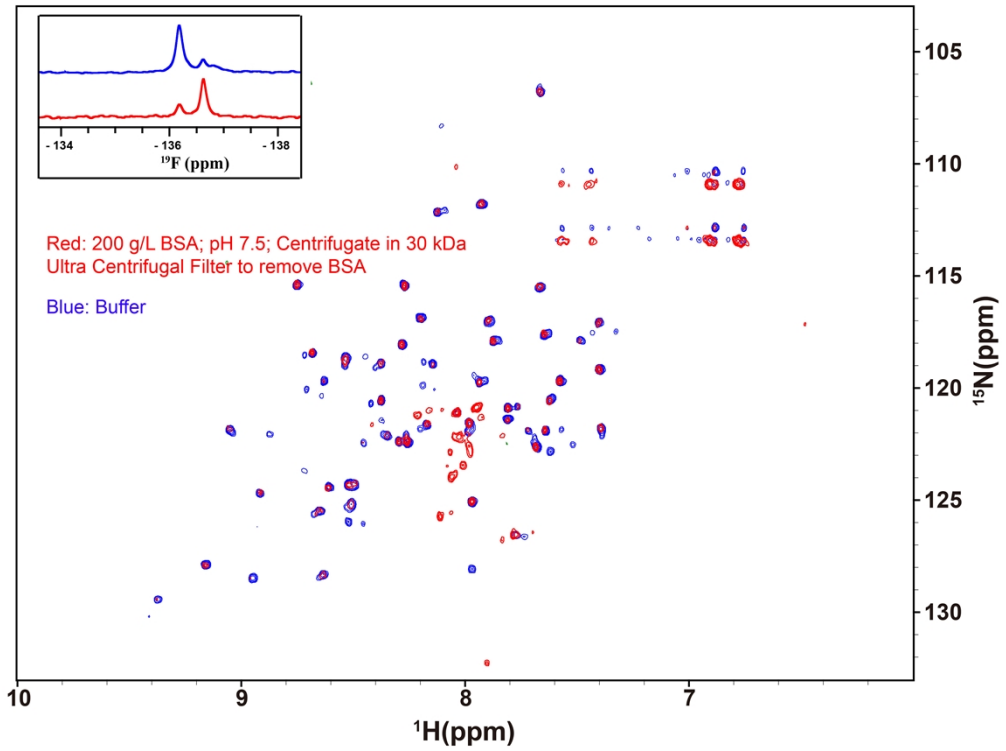

Figure S6 Overlay of ^{1}H-^{15}N TROSY-HSQC spectra of KH1 in buffer (Blue) and BSA -unfolded KH1 in solution after removing BSA by centrifuging in 30 kDa Ultra centrifugal filter (Red) at pH 7.5 and 310K. Inset showed the corresponding ^{19}F spectra.

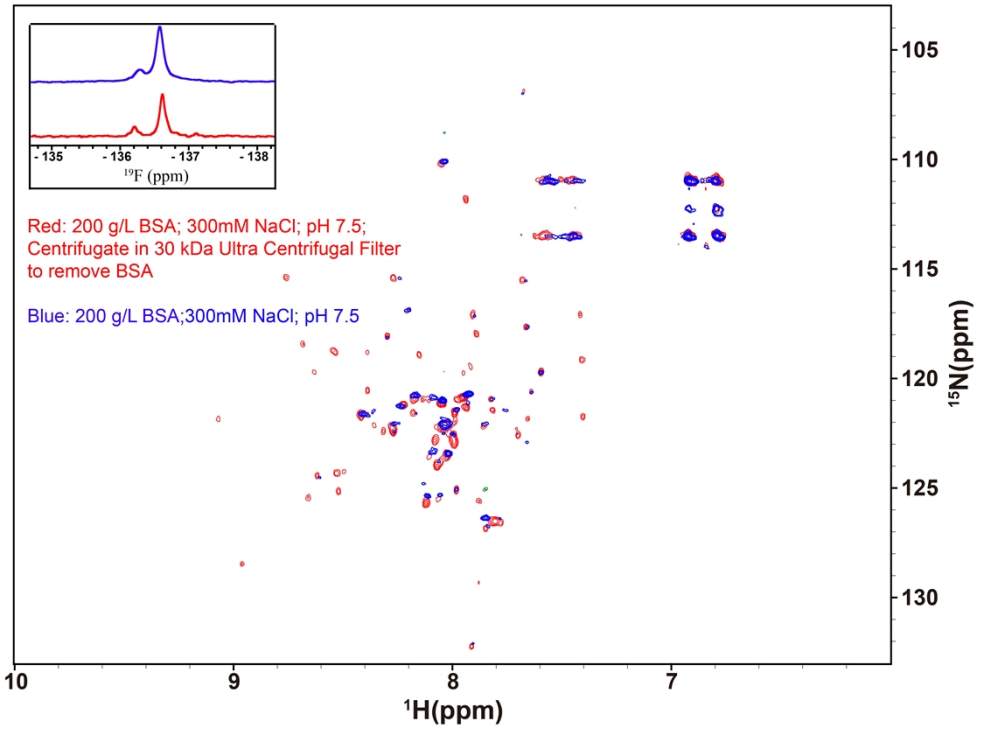

Figure S7 Overlay of ^{1}H-^{15}N TROSY-HSQC spectra of KH1 in 200 g/L BSA and 300 mM NaCl (Blue) and BSA-unfolded KH1 in solution after removing BSA by centrifuging in 30 kDa Ultra centrifugal filter (Red) at pH 7.5 and 310K. Inset showed the corresponding ^{19}F spectra.

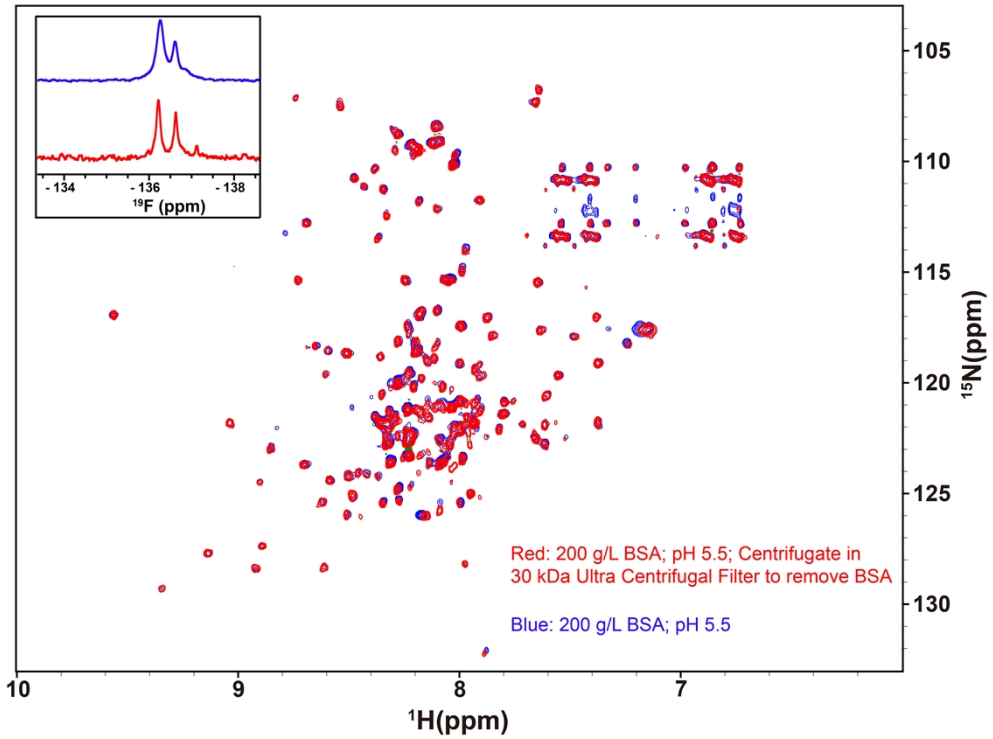

Figure S8 Overlay of ^{1}H-^{15}N TROSY-HSQC spectra of KH1 in 200 g/L BSA (Blue) and BSA-unfolded KH1 in solution after removing BSA by centrifuging in 30 kDa Ultra centrifugal filter (Red) at pH 5.5 and 310K. Inset showed the corresponding ^{19}F spectra.

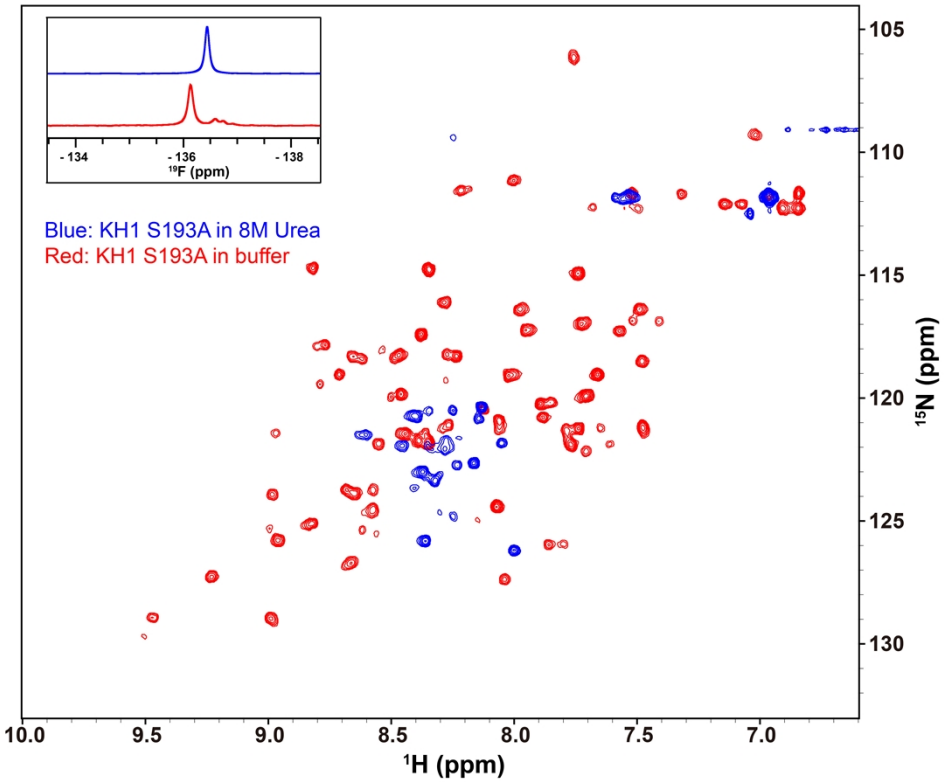

Figure S9 Overlay ^{1}H-^{15}N HSQC spectra of KH1 S193A in buffer (red) and 8M Urea (blue) at pH 7.5 and 310K. Inset showed the corresponding ^{19}F spectra.

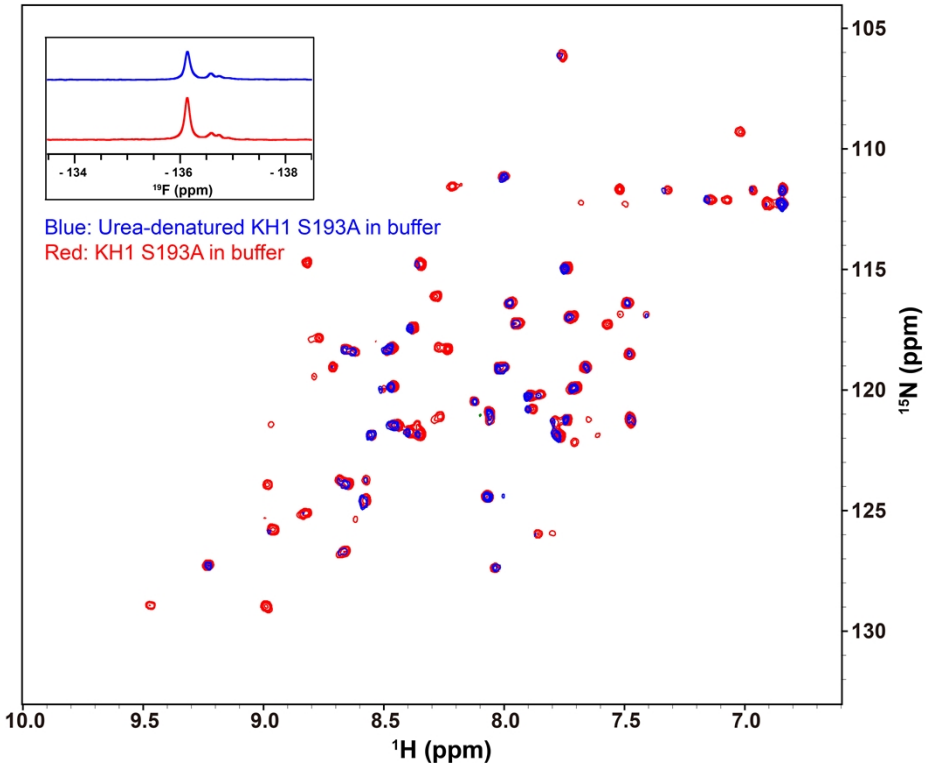

Figure S10 Overlay ^{1}H-^{15}N HSQC spectra of KH1 S193A in buffer (red) and Urea-unfolded KH1 in solution after removing BSA by centrifuging in 3 kDa MWCO filter (blue) at pH 7.5 and 310K. Inset showed the corresponding ^{19}F spectra.

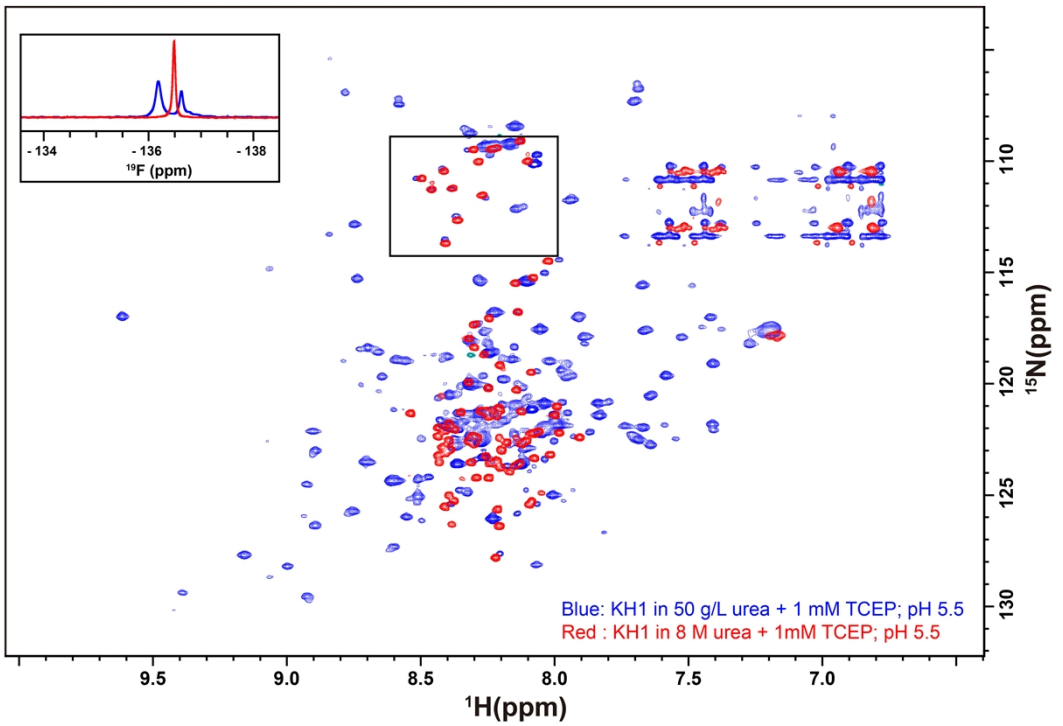

Figure S11 Overlay of ^{1}H-^{15}N TROSY-HSQC of partially unfolded and fully unfolded KH1 S193A by urea in buffer at pH 5.5 and 310 K. Inset showed the corresponding ^{19}F spectra and the signals from 13 Glycine in the unfolded states.

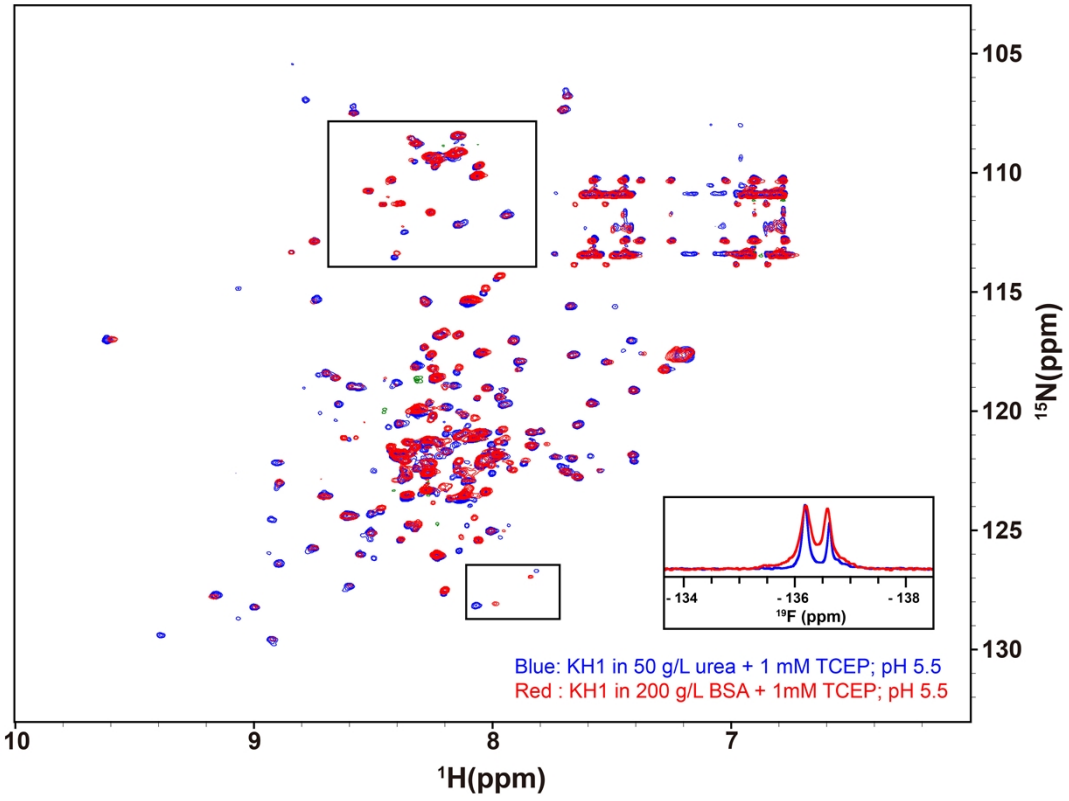

Figure S12 Overlay of ^{1}H-^{15}N TROSY-HSQC of partially unfolded KH1 S193A by urea and BSA in the presence of 1 mM TCEP at pH 5.5 and 310 K. Inset showed the corresponding ^{19}F spectra and the signals from 13 Glycine in the unfolded states.

Table S1 Unfolding rate constant (k_u) for KH1 S193A in different concentrations of BSA at 310 K and pH 7.5.

	50 g/L	100 g/L	150 g/L	200 g/L
k_u (s^{-1}) x 10^4	0.8 ± 0.1	1.7 ± 0.1	2.6 ± 0.1	3.1 ± 0.2

Table S2 Unfolding rate constant (k_u) for KH1 S193A in 200 g/L BSA at 310 K with pH 5.5, 6.4 and 7.5

	pH 5.5	pH 6.4	pH 7.5
k_u (s^{-1}) x 10^4	0.05 ± 0.11	1.0 ± 0.1	3.2 ± 0.4

Table S3 Unfolding rate constant (k_u) for KH1 S193A in 200 g/L BSA at 301 K, 303 K, 307 K and 310 K with pH 7.5.

	301 K	303 K	307 K	310 K
k_u (s^{-1}) x 10^4	1.3 ± 0.1	1.7 ± 0.1	2.3 ± 0.1	3.2 ± 0.4