Supporting Information

Anion binding to Ubiquitin and its Relevance to the Hofmeister Effects

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1. Materials, instrumentation and sample preparation procedures

1.1. Materials and Instrumentation

All reagents were purchased from Sigma-Aldrich or Fisher and were used without purification. Ubiquitin was of purity ≥ 98%, salt-free, and were used as received. Ubiquitin contains the following ionizable residues: four arginines (pKa of conjugate acid ≈ 12.5), seven lysines (pKa of conjugate acid ≈ 11.1), one tyrosine residue (pKa ≈ 9.8), one free methionine N-terminus (pKa ≈ 9.2), one histidine (pKa of conjugate acid ≈ 6.8), and six glutamic and five aspartic acids (pKa ≈ 3.1 to 4.5). Using these approximate values, the Protein Calculator (http://protcalc.sourceforge.net/) gives the following charges (in parenthesis) at the following pH values. For the SLS experiments: 2.3 (+12.8), 2.7 (+12.5), 3.0 (+12.1), 3.3 (+11.6), 4.0 (+9.0), 4.3 (+7.2), 4.6 (+5.3), 5.0 (+3.2). For the DSC experiments: 2.3 (+12.8), 2.6 (+12.6), 3.0 (+12.1), 4.0 (+9.0), 4.3 (+7.2), 4.5 (+5.9), 5.0 (+3.2). For the NMR experiments: 2.3 (+12.8), 2.8 (+12.4), 3.8 (+10.0), 4.8 (+4.1), 5.8 (+1.3), 7.3 (0.0).

All sodium salts were of purity ≥ 99 % and were used as received. All solvents were purchased from Fisher Scientific and were used as received. All solutions were prepared in 18.2 MΩ·cm Milli-Q H_2O. All circular dichroism (CD) study were collected on an Olis DSM 1000 CD instrument. All differential scanning calorimetry (DSC) measurements were collected on a Malvern MicroCal PEAQ-DSC instrument. All static light scattering (SLS) measurements were collected on a Fluence Analytics ARGEN SMSLS v5 instrument. All dynamic light scattering (DLS) measurements were performed on a Nicomp ZLS Z3000 particle size analyzer (Particle Sizing Systems – Port Richey, FL) with a 50 mW laser diode (660 nm wavelength) and an avalanche photodiode (APD) detector. Measurements of scattered light were made at 90 °C, with data collected at 25 °C and processed using a non-negative least squares Nicomp analysis. All ^{1}H-^{15}N HSQC NMR spectra were collected on a Bruker 500 MHz spectrometer at 25 °C. All data were analyzed by OriginPro 2019, Matlab 2018a or Microsoft Excel.

1.2. Static Light Scattering

All ubiquitin samples were prepared at 2 mg/mL stock solution and diluted to the desired concentration by 10 mM phosphate buffer. All protein/salt mixed solutions were loaded into the 1 cm pathlength sample cell (~ 2 mL). The buffer solution was used as a baseline correction. The temperature ramp experiments were carried out from 25 to 90 °C in 7 hours. The fixed temperature experiments were measured over 3-8 hours.

1.3. Circular Dichroism

All ubiquitin samples were prepared at 2 mg/mL stock solution and diluted to the desired concentration by 10 mM phosphate buffer. The sample was loaded in a 1 mm optical path length quartz cuvette, and high purity N_2 gas purged into the spectropolarimeter and the sample chamber during the measurements. The spectra of each sample were the average of five scans collected at a scan speed of 50 nm/min at the far-UV range (190 – 250 nm). The salt solutions were subtracted as baseline in corresponding protein/salt mixed solution. Data were processed using Origin 2019.

1.4. Differential Scanning Calorimetry

All ubiquitin samples were prepared at 2 mg/mL stock solution and diluted to the desired concentration by 10 mM phosphate buffer. All protein/salt mixed solution were loaded into the sample cell (~ 250 µL) with the salt solution in the reference cell. (The salt solutions were used as baseline corrections for corresponding protein/salt mixed solution.) All experiments were collected between 20-110 °C or 40-130 °C, at a 90 °C /hour temperature ramping rate under 50-60 psi pressure. Each experimental result was the average of two or three measurements and processed with the instrument packaged software and processed using OriginPro 2019.

1.5. ^{1}H ^{15}N HSQC NMR

^{15}N-enriched human ubiquitin (98 atom %, recombinant expressed in E. coli) was purchased from Sigma-Aldrich and used without further purification. All samples were prepared from 100 mg/mL stock solution and diluted to the desired concentration and pH by 50 mM phosphate buffer in 90% H_2O/ 10% D_2O. The salt solutions were prepared at 2 M concentration for the titration experiments. All experiments were carried out in Shigemi NMR tube. The experiments were carried out on a 500 MHz Bruker NMR fitted with a BBI probe at 25 °C. The spectra were recorded with the Phase-sensitive ^{1}H-^{15}N 2D HSQC using PEP with gradients in back-inept pulse program with a 1.5 second delay time and 512 × 128 data points. The number of scans varied from 32 to 256 depends on the concentration of the protein sample and the dilution effect by the salt solution.

2. Static Light Scattering (SLS) Experiments

2.1. Aggregation Rates

By carrying out Arrhenius plots, we measured the energy of activation (E_a), pre-exponential factor (ln A), and aggregation rate constants (k_{agg}) for Ub in the presence of eight anions, ReO_4^-, PF_6^-, TfO^-, ClO_4^-, NO_3^-, Br^-, Cl^- and $MeSO_3^-$ at pH 2.3. E_a, ln A, and the relative rates of aggregation (k_{rel}) at 298 K and shown in Table S1.

Preliminary studies indicated required concentrations of ubiquitin and salt to be respectively 1mg/mL and 1 M. All ubiquitin samples were prepared at 2 mg/mL stock solution and diluted to the desired concentration by 10 mM phosphate buffer. 1 mL of ubiquitin solution was then mixed thoroughly with 1 mL of salt solution in a 1 cm pathlength capped cuvette. The respective buffer solution was used as reference. The temperature was fixed at the desired temperature based on the preliminary study and each test run for 3 h.

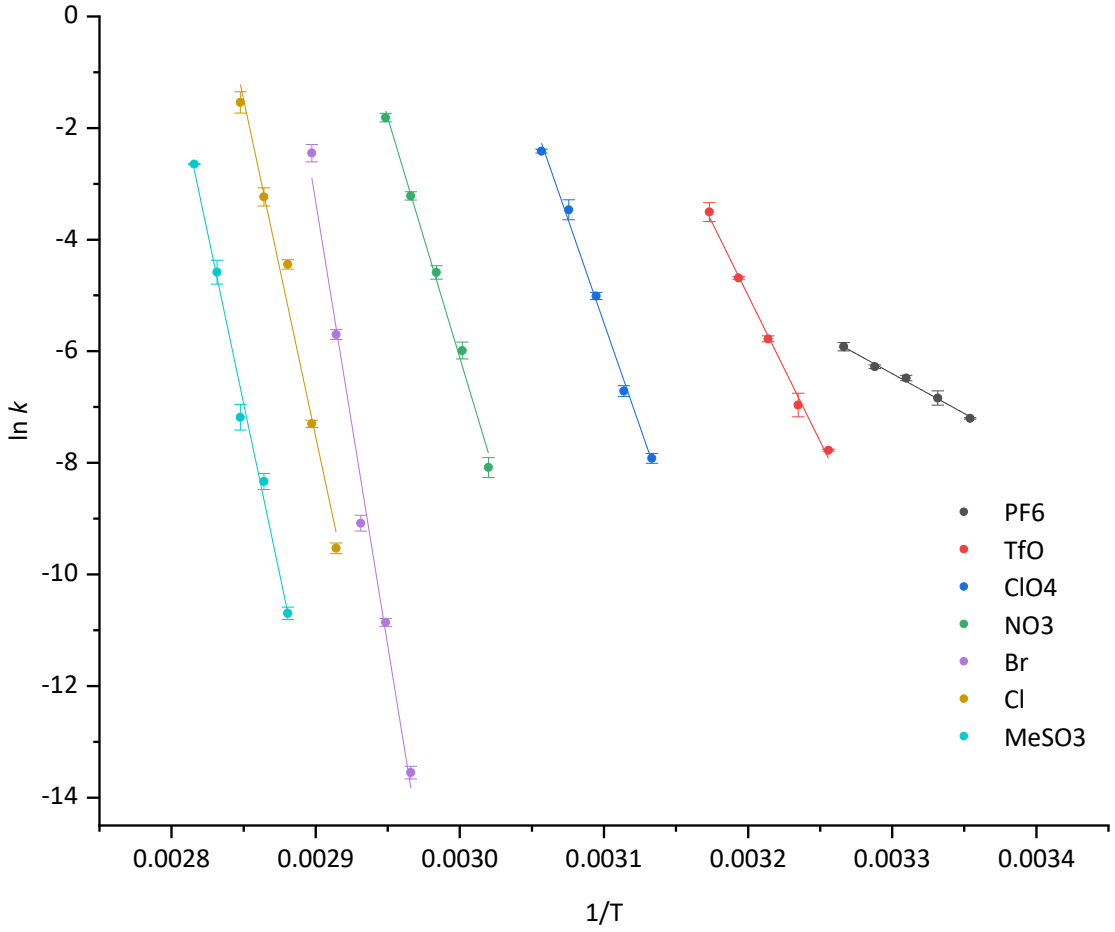
Notably, ReO_4^- was found to be the strongest precipitant, inducing the precipitation of ubiquitin at rt. This salt was not therefore studied further. For the others, five temperature values were selected for each salt covering the range of 25 - 82 ˚C. The data for the other anions is shown below. All salts fitted well to a linear Arrhenius relationship:

$$lnk = \frac{-E_a}{R}\left(\frac{1}{T}\right) + lnA \quad \text{(Eq. S1)}$$

The energy of activation (E_a), pre-exponential factor (ln A), and aggregation rate constants (k_{agg}) were calculated directly by the SLS instrument packaged software. Arrhenius plots for each salt are shown in Figure S1. The raw data for each salt is shown in Figure S2 to Figure S8. For clarity, the signal change after each neutral density (ND) filter increased was removed from the data.

Table S1: Summary of the Arrhenius fitting for seven anions at pH = 2.3

Arrhenius Fitting	**Salts**						
	PF_6^-	**TfO^-**	**ClO_4^-**	**NO_3^-**	**Br^-**	**Cl^-**	**$MeSO_3^-$**
E_a (kJ/mol)	119	436	619	715	1322	1006	1014
ln A	41	162	225	252	458	343	342
k_{rel} (298 K)	5.8×10^{29}	5.6×10^{26}	1.1×10^{22}	8.5×10^{16}	1	2.6×10^{5}	4.0×10^{3}



Equation	y = a + b*x						
Plot	PF6	TfO	ClO4	NO3	Br	Cl	MeSO3
Weight	No Weighting						
Intercept	40.7 ± 2.29	162 ± 6.58	225 ± 9.75	252 ± 12.9	458 ± 30.2	343 ± 27.4	342 ± 20.2
Slope	-1.43E+04 ±	-5.23E+04 ±	-7.45E+04 ±	-8.60E+04 ±	-1.59E+05 ± 1	-1.21E+05 ±	-1.22E+05 ±
Residual Su	0.01	0.05	0.11	0.18	0.94	0.75	0.40
Pearson's r	-1.00	-1.00	-1.00	-1.00	-0.99	-0.99	-1.00
R-Square (	0.99	1.00	0.99	0.99	0.99	0.98	0.99
Adj. R-Squ	0.99	0.99	0.99	0.99	0.98	0.98	0.99

Figure S1 The Arrhenius equation fitting of ubiquitin with seven different salts at pH 2.3. Each measurement (data point) was duplicated or triplicated and error is smaller than 5% for all data points.

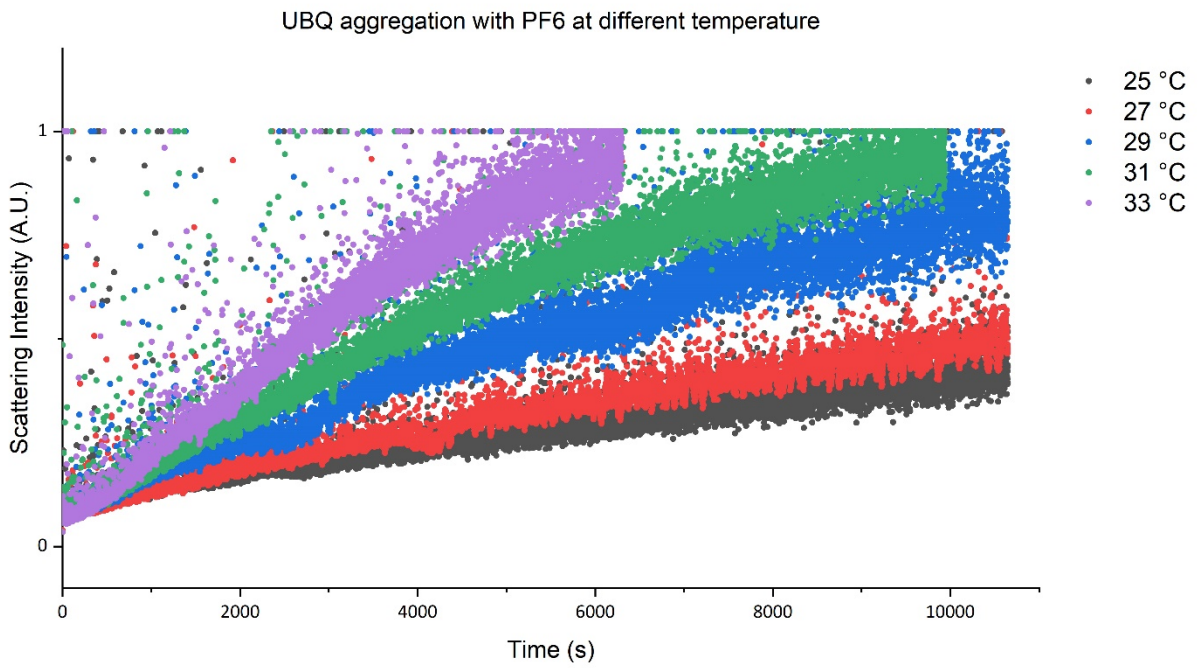


Figure S2 The aggregation rate of ubiquitin with PF_6^- at five different temperatures at pH = 2.3.

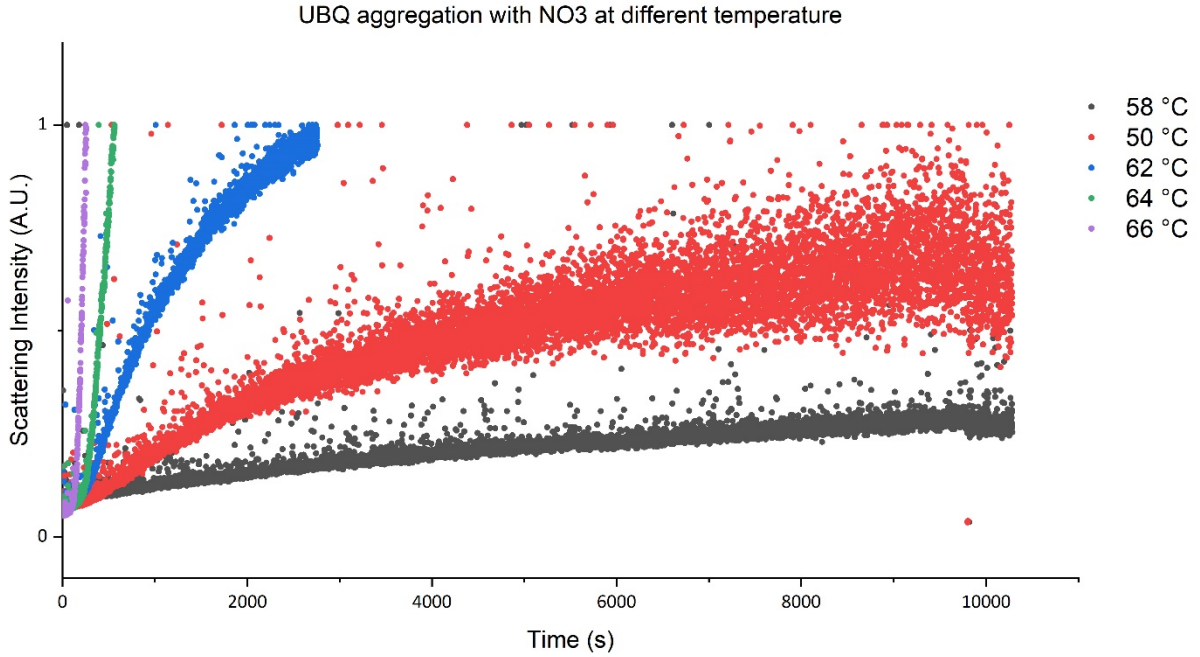


Figure S3 The aggregation rate of ubiquitin with NO_3^- at five different temperatures at pH = 2.3.

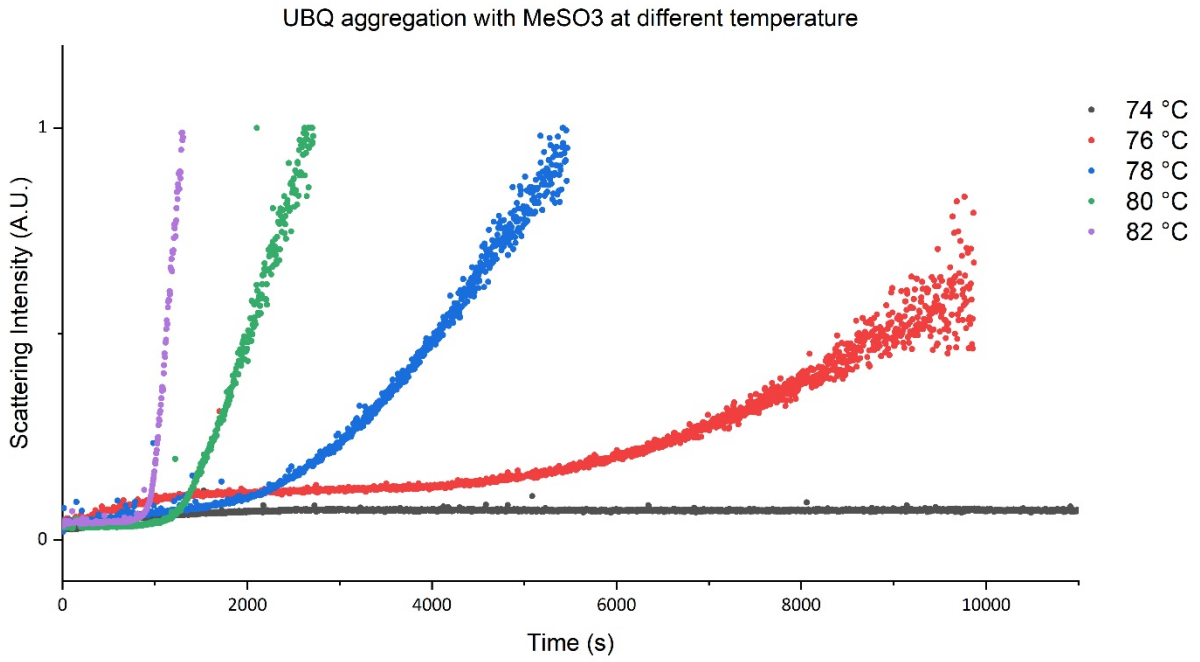


Figure S4 The aggregation rate of ubiquitin with $MeSO_3^-$ at five different temperatures at pH = 2.3.

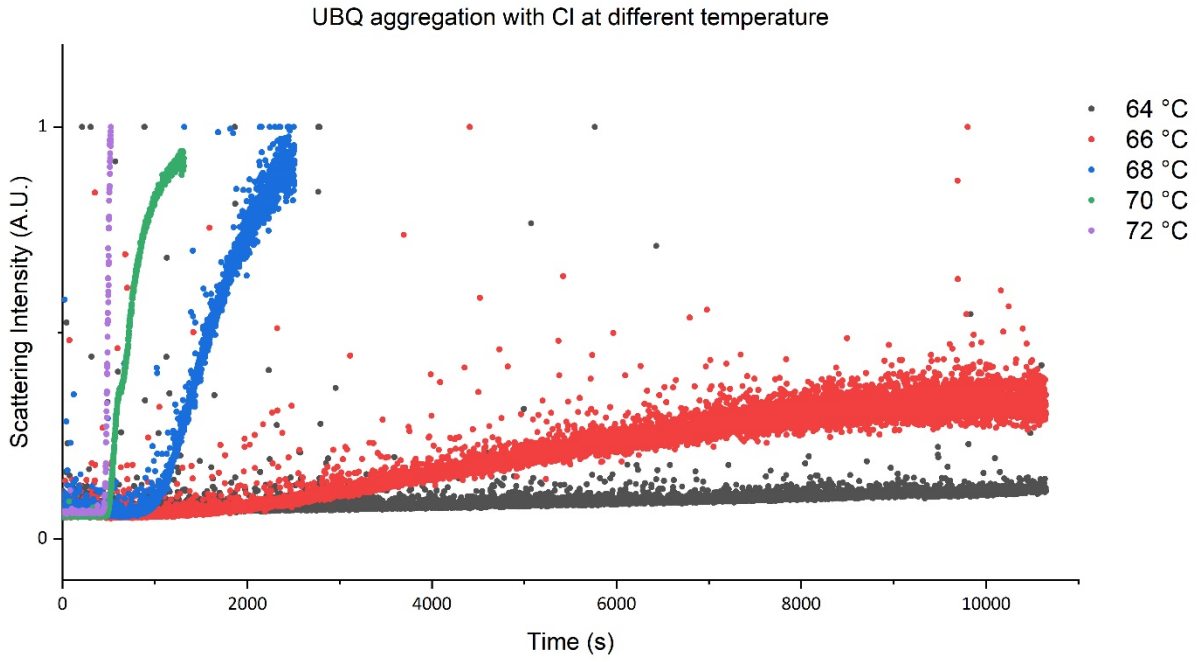


Figure S5 The aggregation rate of ubiquitin with Cl^- at five different temperatures at pH = 2.3.

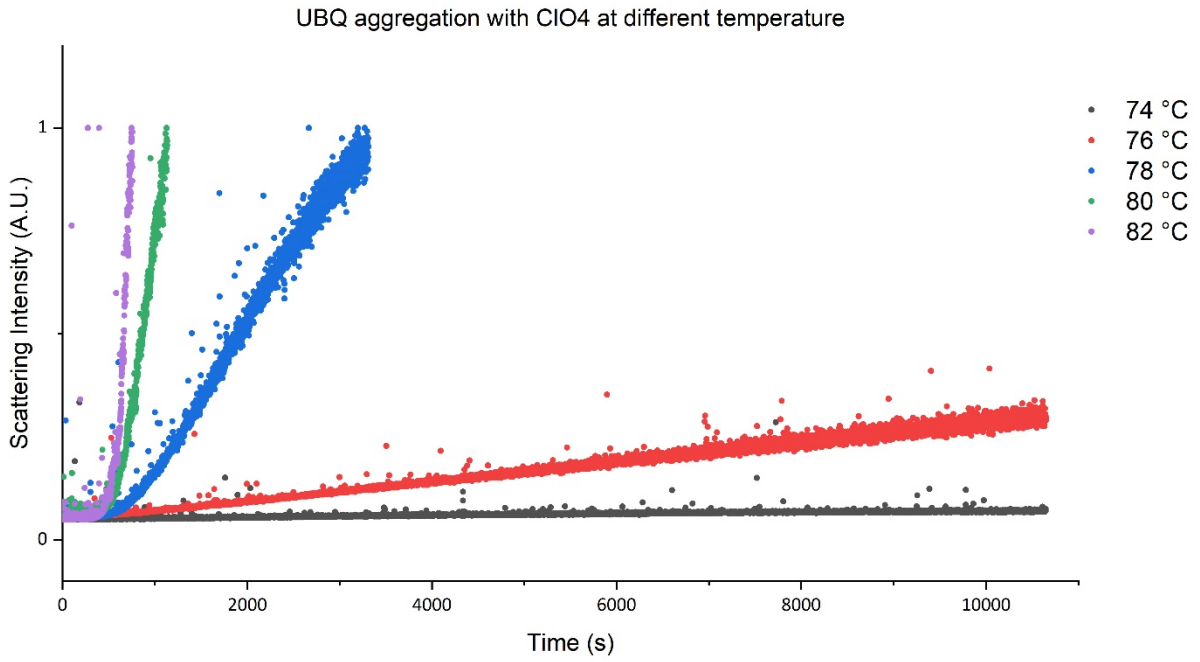


Figure S6 The aggregation rate of ubiquitin with ClO_4^- at five different temperatures at pH = 2.3.

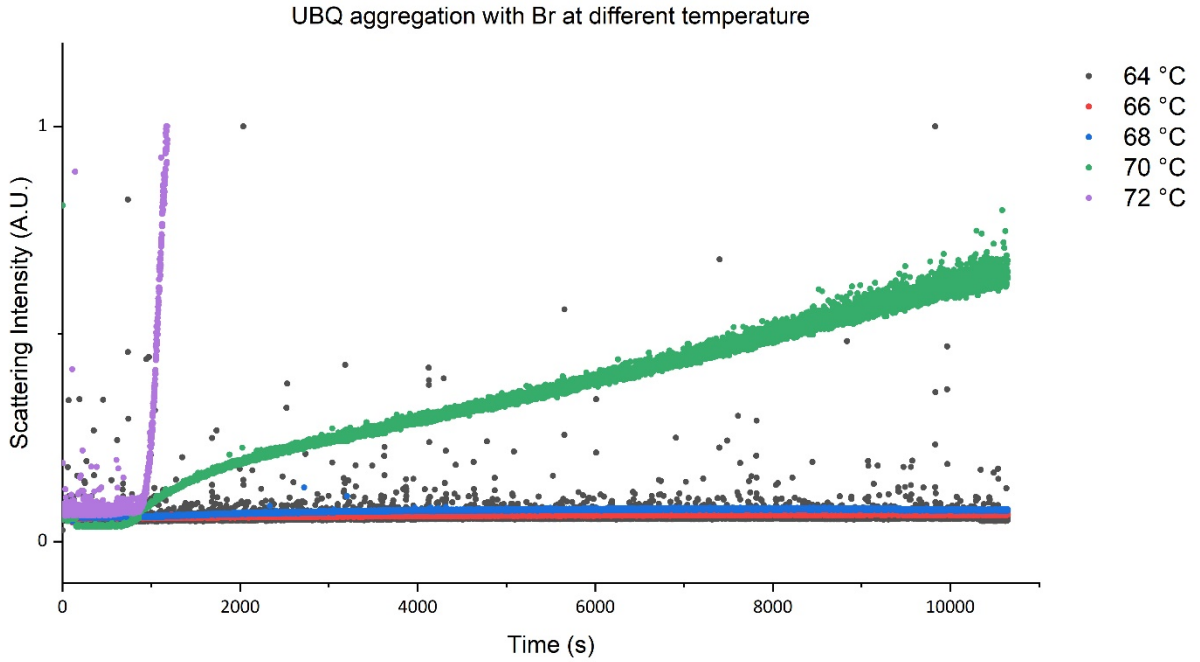


Figure S7 The aggregation rate of ubiquitin with Br^- at five different temperatures at pH = 2.3.

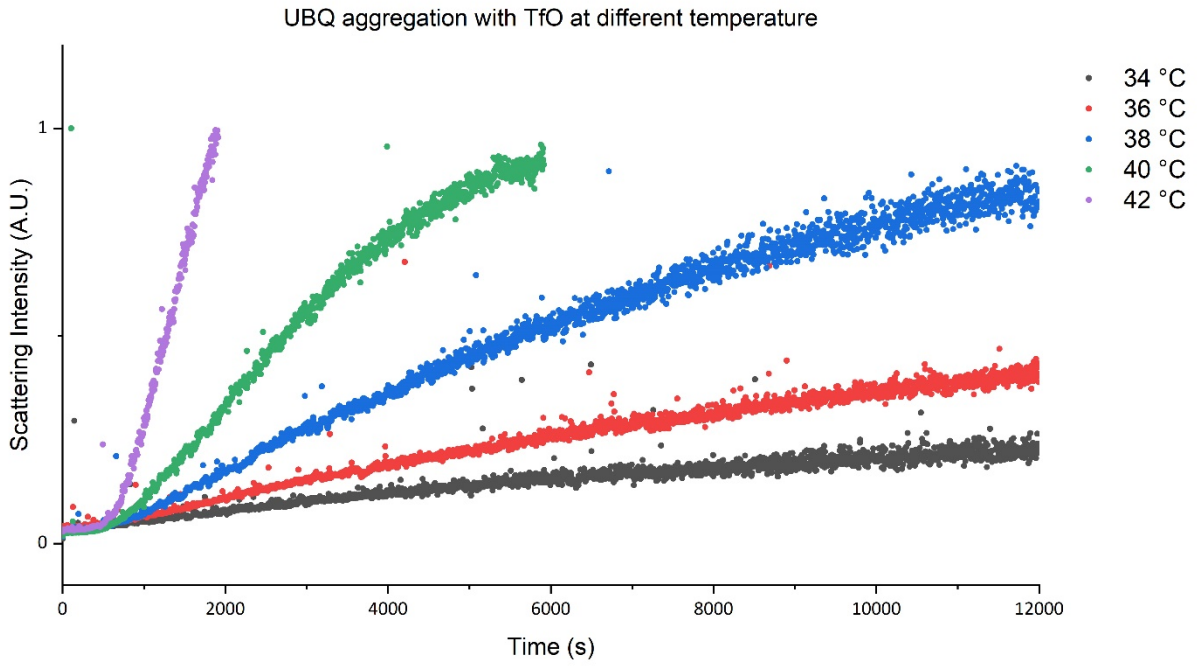


Figure S8 The aggregation rate of ubiquitin with TfO^- at five different temperatures at pH = 2.3.

2.2. SLS aggregation studies at different pH values

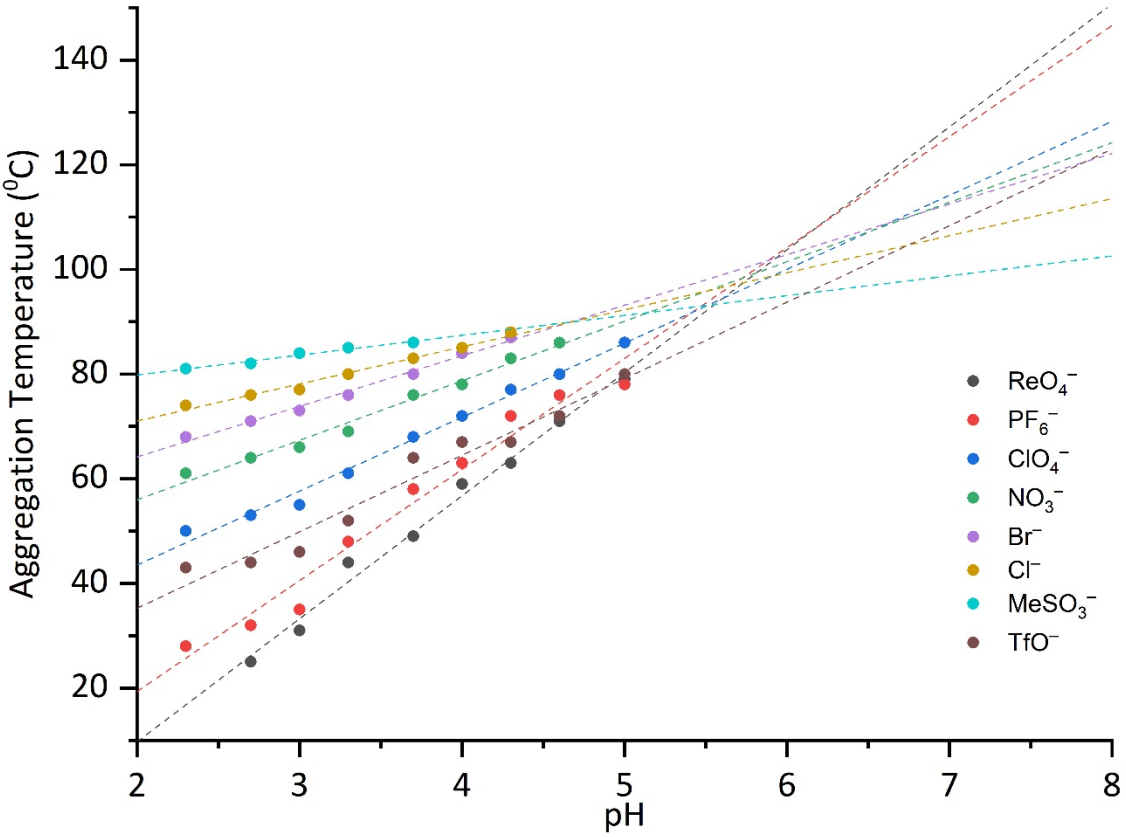
Eight anions were selected to determine how anions affect aggregation of ubiquitin at different pH values: ReO_4^-, PF_6^-, TfO^-, ClO_4^-, NO_3^-, Br^-, Cl^- and $MeSO_3^-$. In the absence of salts, at all pH values we saw no evidence of aggregation (data not shown). For this set of anions, nine sets of experiment were set up at different pH values ranging from 2.3 to 5.0.

Based on preliminary study results, the concentration of ubiquitin is set to 1mg/mL, and salt concentration 1 M. All ubiquitin samples were prepared at 2 mg/mL stock solution and diluted to the desired concentration by 10 mM phosphate buffer. 1mL of ubiquitin solution was mixed thoroughly with 1 mL of salt solution in 1 cm pathlength capped cuvette. The buffer solution was subtracted as baseline. The temperature ramp experiments were carried out from 25 - 90 ˚C, over 7 h. All experiments were run in duplicate or triplicate.

Figure S9 shows the cumulative SLS data for all salts across all pH values. The results suggested a strong linear correlation ($R^2 > 0.95$) between the aggregation temperature with pH for all anions. This provides a good model for further work to predict the aggregation temperature at different pH values. According to the Protein Calculator (http://protcalc.sourceforge.net/) we have the following charges (in parenthesis) at the following pH values: 2.3 (12.8), 2.6 (12.6), 3.0 (12.1), 4.0 (9.0), 4.3 (7.2), 4.5 (5.9), 5.0 (3.2).

A representative example of the raw and processed data at each condition is shown below (Figure S11, Figure S13, Figure S15, Figure S17, Figure S19, Figure S21, Figure S23, Figure S25, and Figure S27), along with the grouped (processed) data at each pH value (Figure S10, Figure S12, Figure S14, Figure S16, Figure S18, Figure S20, Figure S22, Figure S24, and Figure S26). Processing involved: 1) removal of the signal change after the ND filter was switched from 0.0 to 0.5; 2) smoothing of the data by removal of outlier data (3 σ) using a moving set of 20 data points as the median, and; 3) converting the raw scattering intensity data (the weight average Mw) to the degree of aggregation M_w/M_0 (where M_0 is the monomer weight).

To quantify aggregation, we defined the T_{agg} values as the intersection of the straight line (slope) of each SLS spike (curve) with the X-axis. Note that there is no unique T_{agg} for protein aggregation. In other words, the 'T_{agg}' is not a fundamental property, but rather depends on the temperature ramp rate and other independent variables in the experiment (protein concentration, nature of buffer and concentration, and salt concentration)



Equation	y = a + b*x							
Plot	ReO4	PF6	ClO4	NO3	Br	Cl	MeSO3	TfO
Weight	No Weighting							
Intercept	-37.27318 ± 3	-23.09952 ± 5	15.22809 ± 2.	33.20432 ± 2.	44.8361 ± 1.	56.85503 ± 1	72.22069 ± 1.	5.98319 ± 4.3
Slope	23.50279 ± 1.	21.21264 ± 1.	14.13213 ± 0.	11.3751 ± 0.5	9.66297 ± 0.	7.08218 ± 0.	3.7931 ± 0.38	14.62466 ± 1.
Residual Sum of	27.96872	92.37976	14.29467	9.47436	3.0771	2.51339	0.51034	60.96722
Pearson's r	0.99439	0.98467	0.99457	0.99198	0.99472	0.992	0.98505	0.9789
R-Square (COD)	0.98881	0.96957	0.98918	0.98402	0.98946	0.98406	0.97033	0.95825
Adj. R-Square	0.98695	0.96523	0.98763	0.98136	0.98735	0.98088	0.96044	0.95228

Figure S9 The linear regression analysis of the aggregation temperature of ubiquitin as a function of pH for different salts (all 1 M). All fitting lines were extended to pH 8.

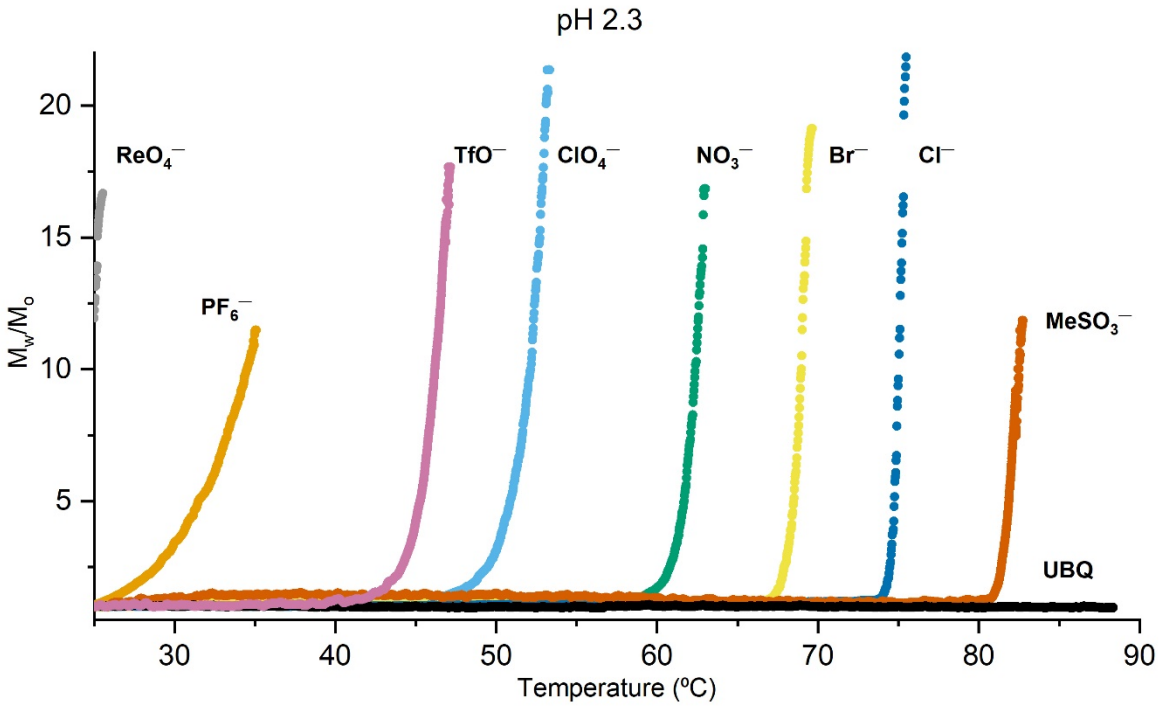


Figure S10 The grouped aggregation curve data for ubiquitin in the presence of different salt (all 1 M) at pH = 2.3.

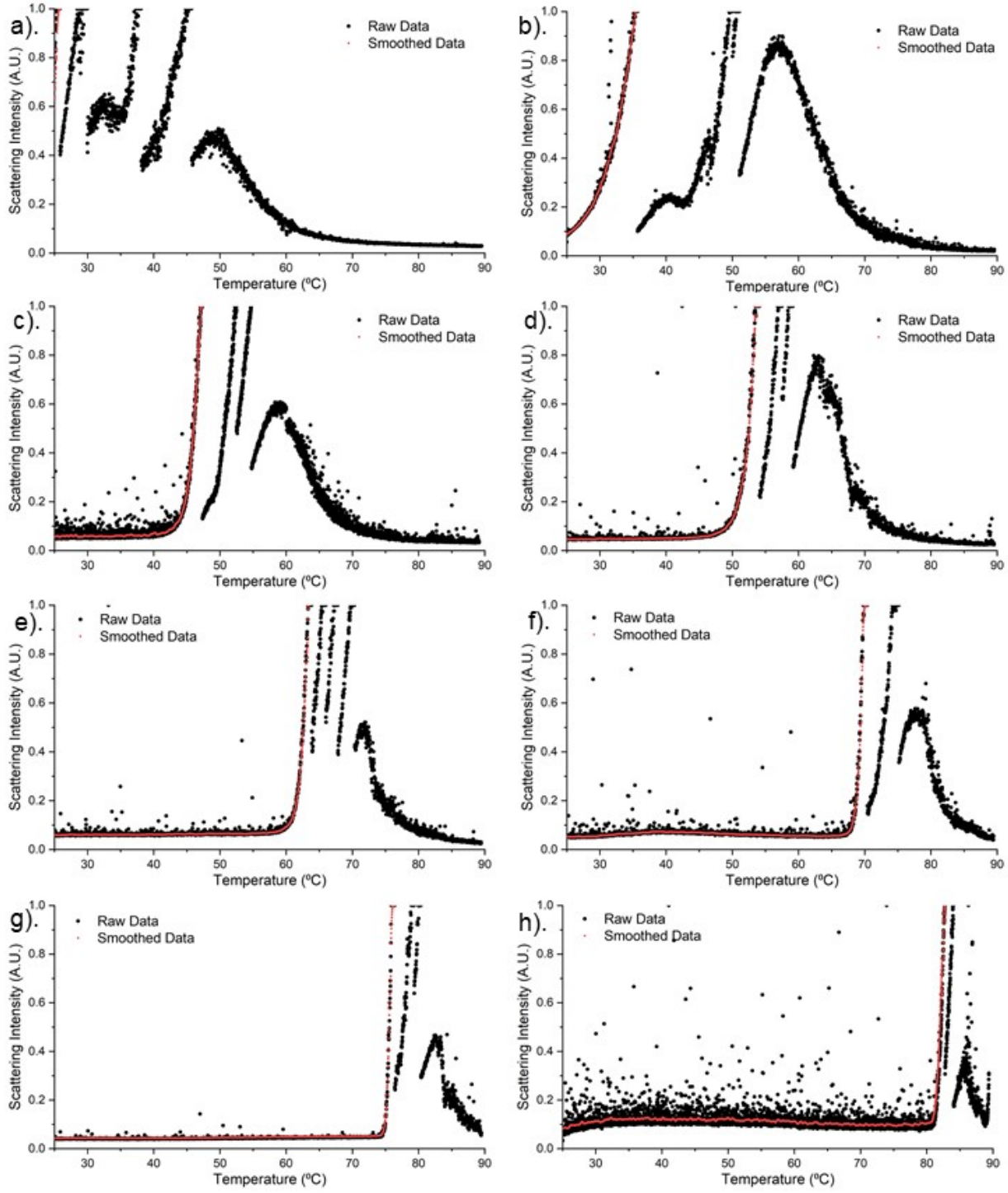


Figure S11 The raw data and processed data of the aggregation of ubiquitin in the presence of different salts (all 1M) at pH = 2.3: a). ReO_4^-; b). PF_6^-; c). TfO^-; d). ClO_4^-; e). NO_3^-; f). Br^-; g). Cl^-; h). $MeSO_3^-$.

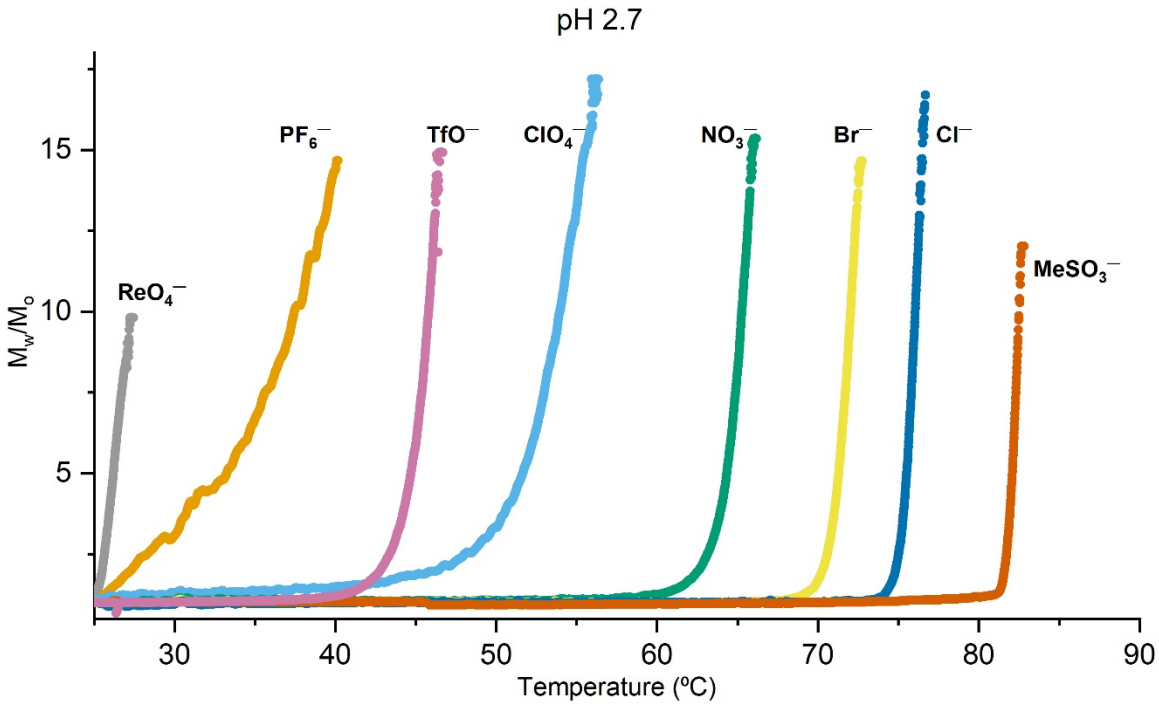


Figure S12 The grouped aggregation curve data for ubiquitin in the presence of different salt (all 1 M) at pH = 2.3.

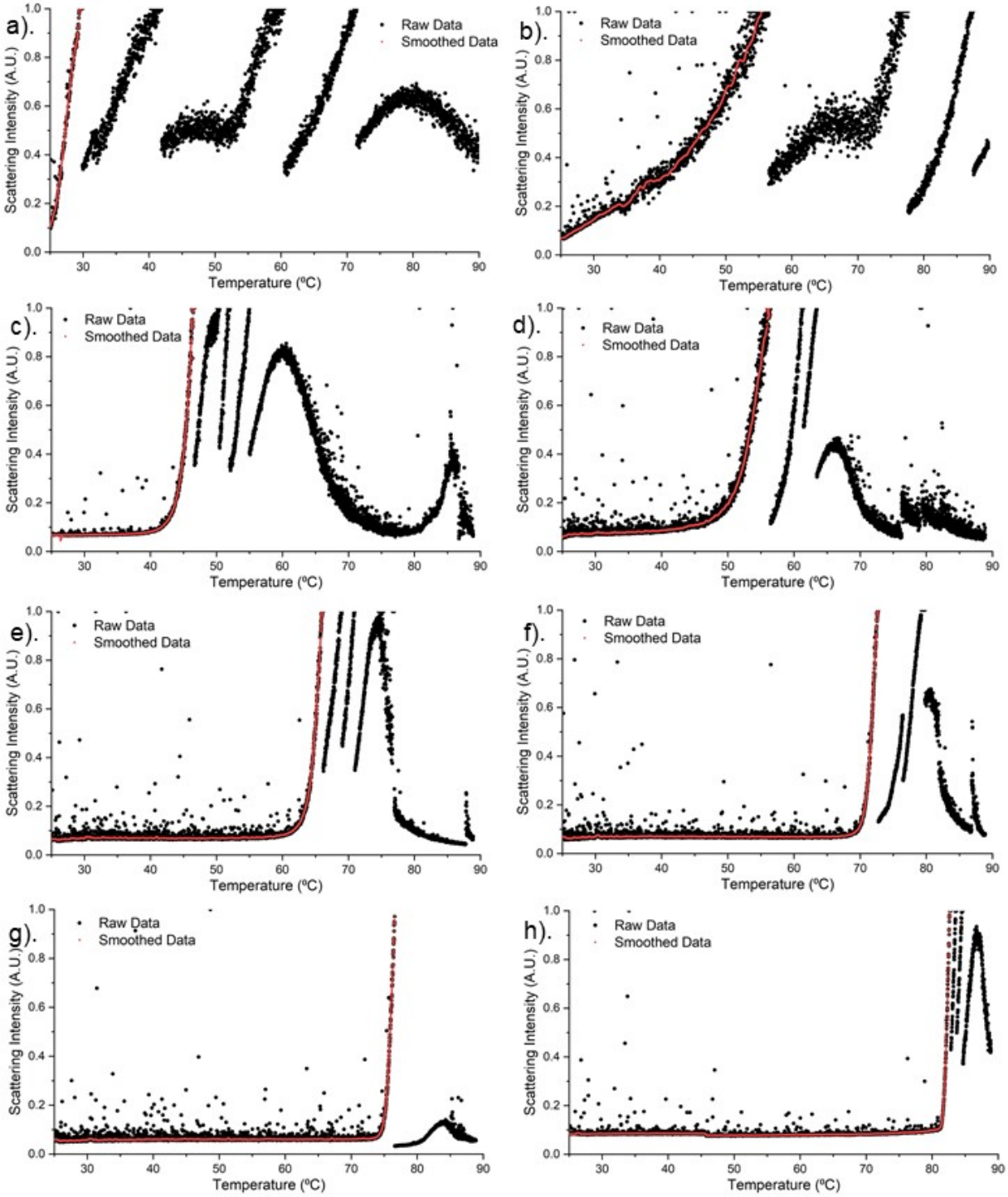


Figure S13 The raw data and processed data of the aggregation of ubiquitin in the presence of different salts (all 1M) at pH = 2.7: a). ReO_4^-; b). PF_6^-; c). TfO^-; d). ClO_4^-; e). NO_3^-; f). $Br;^-$ g). Cl^-; h). $MeSO_3^-$.

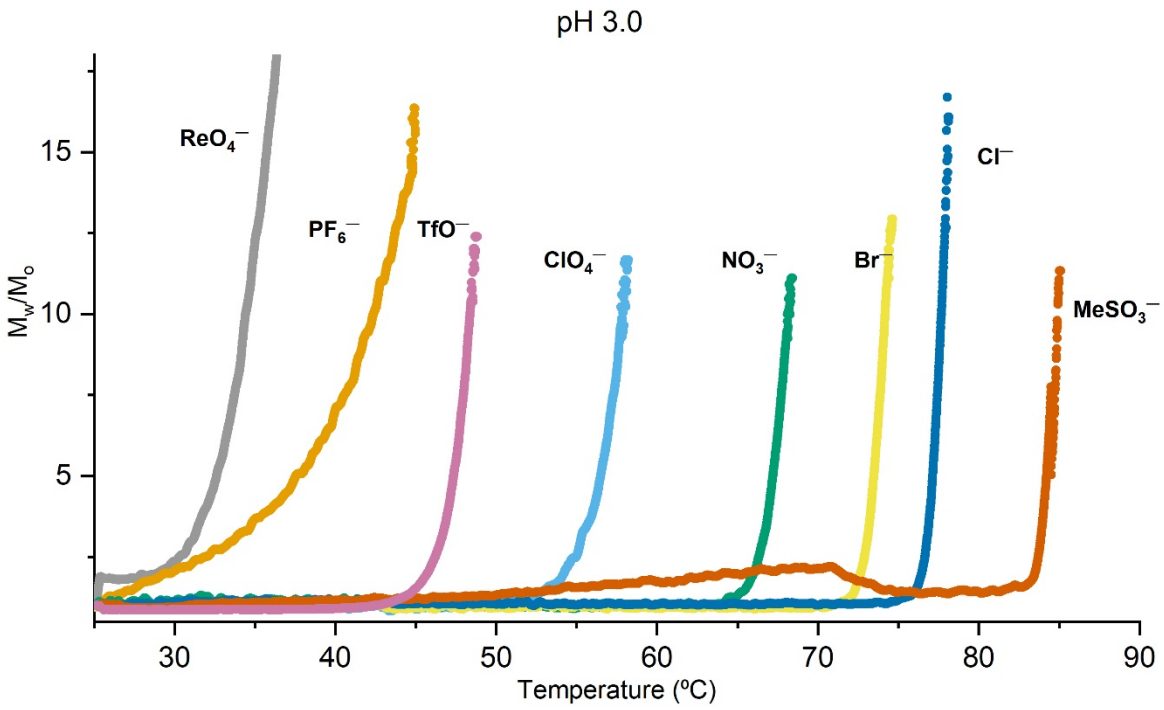


Figure S14 The grouped aggregation curve data for ubiquitin in the presence of different salt (all 1 M) at pH = 3.0.

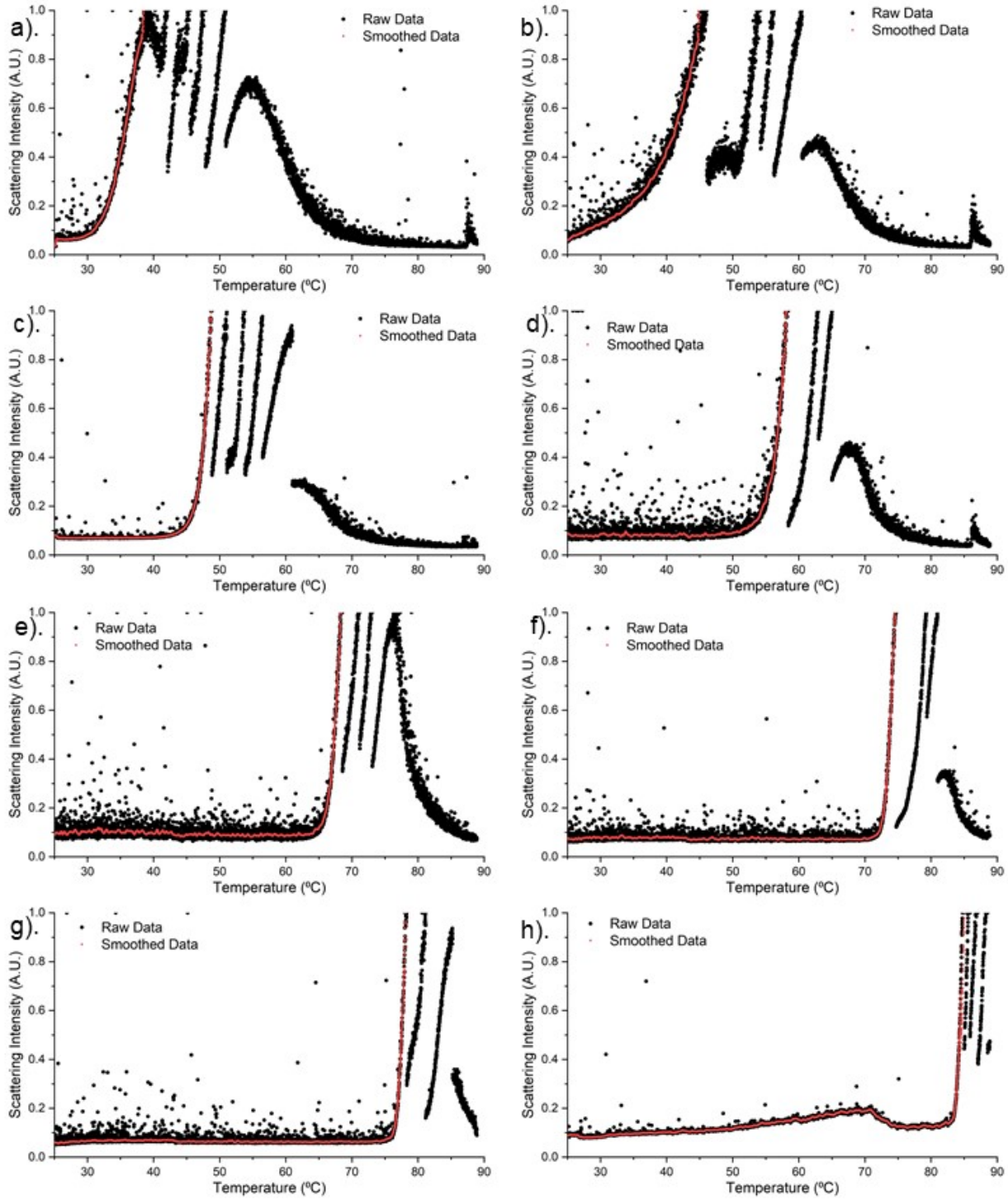


Figure S15 The raw data and processed data of the aggregation of ubiquitin in the presence of different salts (all 1M) at pH = 3.0: a). ReO_4^-; b). PF_6^-; c). TfO^-; d). ClO_4^-; e). NO_3^-; f). $Br;^-$ g). Cl^-; h). $MeSO_3^-$.

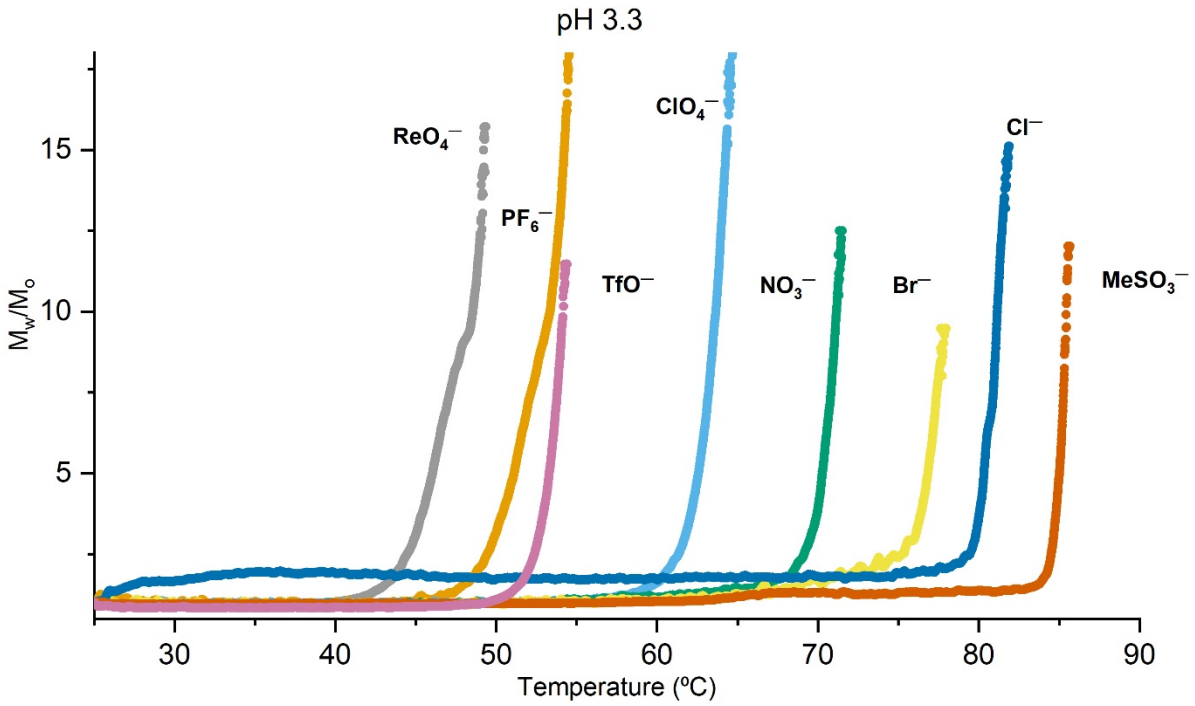


Figure S16 The grouped aggregation curve data for ubiquitin in the presence of different salt (all 1 M) at pH = 3.3.

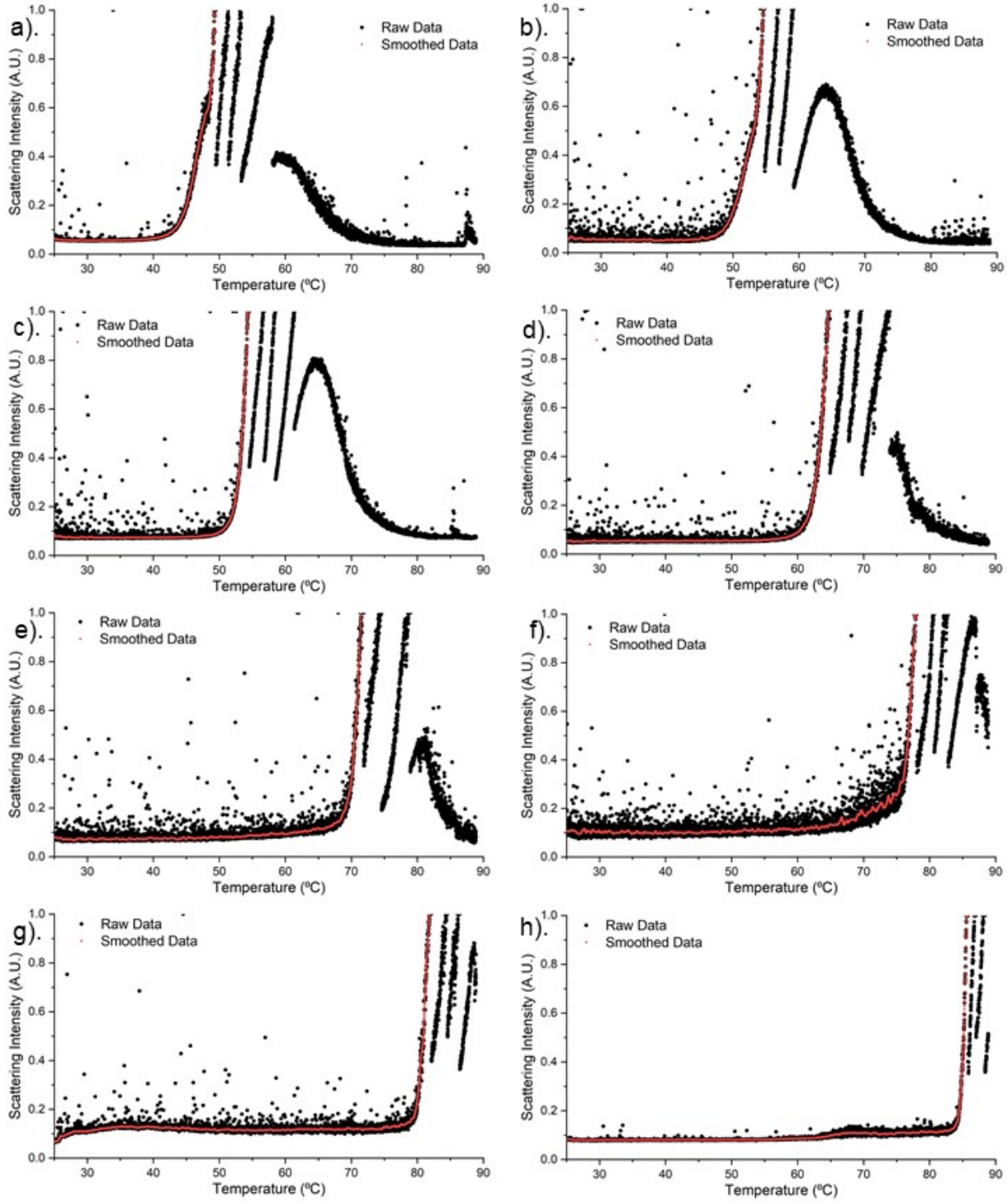


Figure S17 The raw data and processed data of the aggregation of ubiquitin in the presence of different salts (all 1M) at pH = 3.3: a). ReO_4^-; b). PF_6^-; c). TfO^-; d). ClO_4^-; e). NO_3^-; f). Br^-; g). Cl^-; h). $MeSO_3^-$.

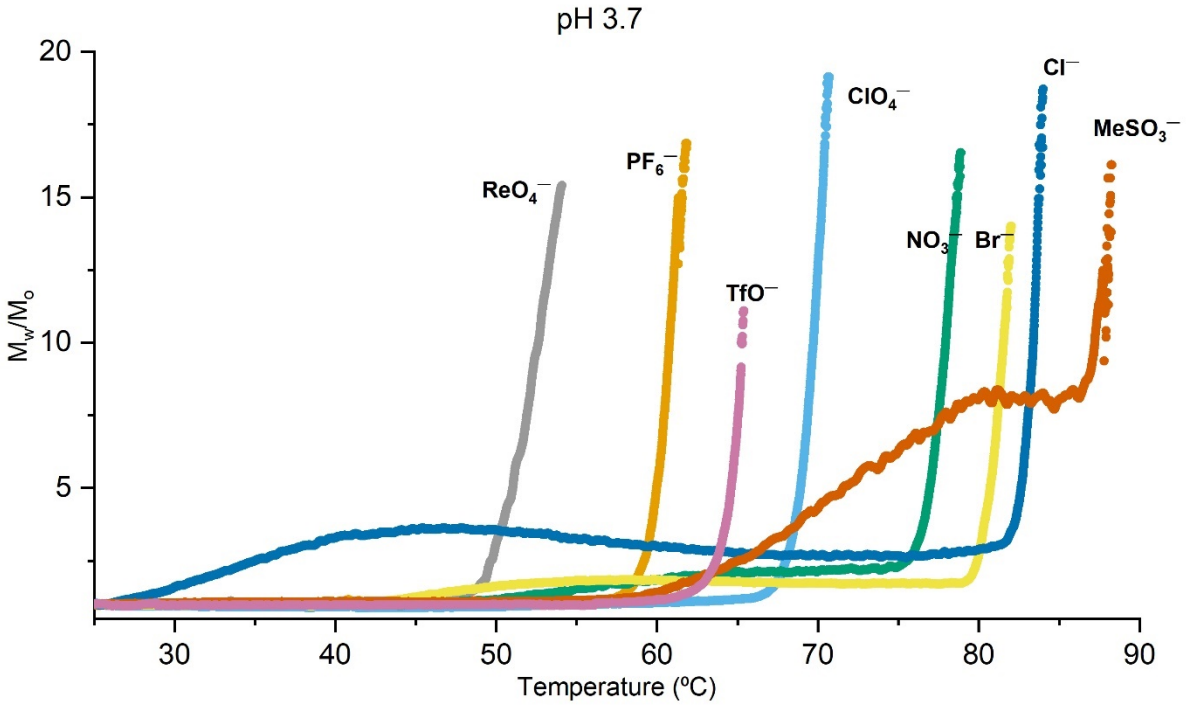


Figure S18 The grouped aggregation curve data for ubiquitin in the presence of different salt (all 1 M) at pH = 3.7.

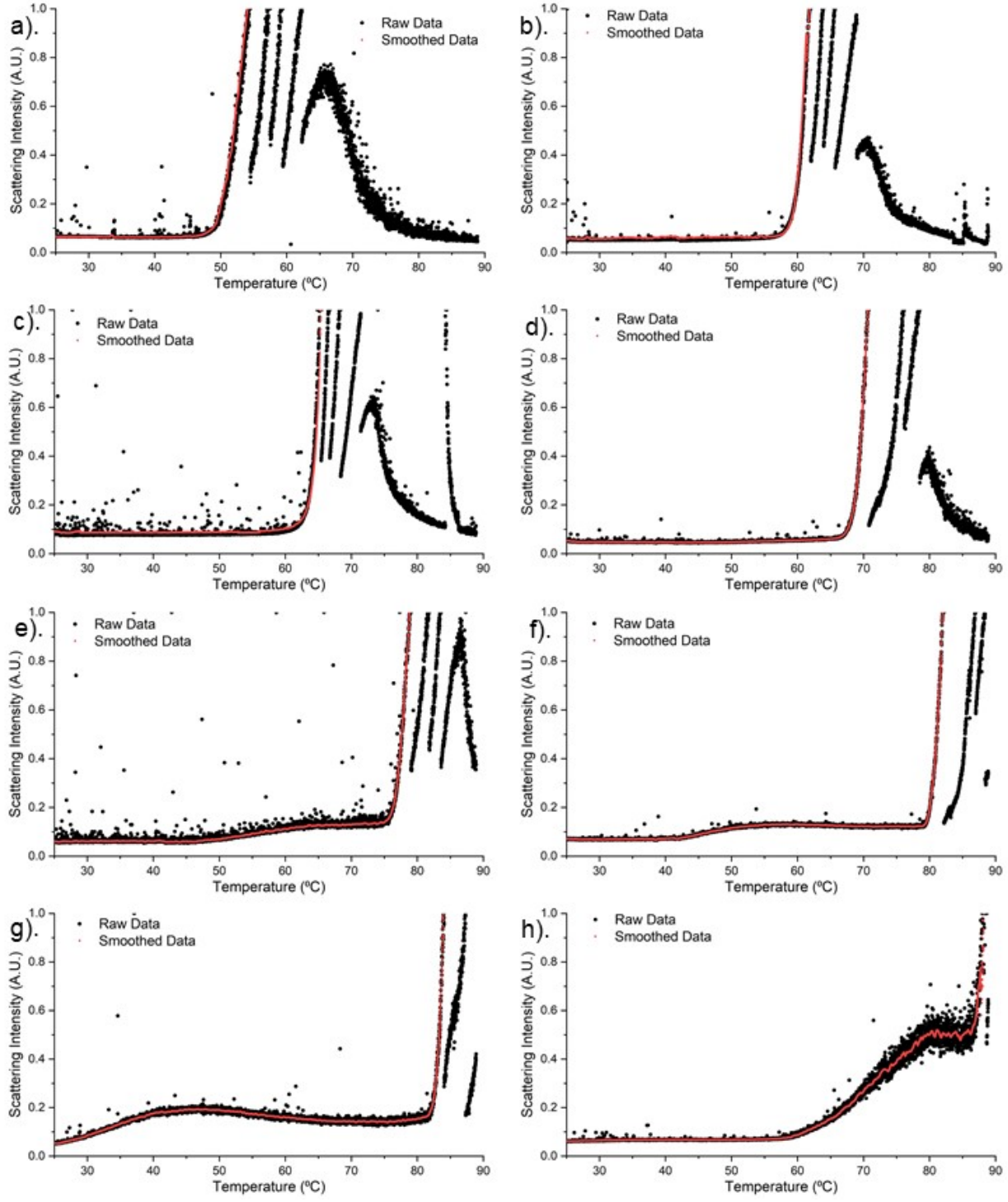


Figure S19 The raw data and processed data of the aggregation of ubiquitin in the presence of different salts (all 1M) at pH = 3.7: a). ReO_4^-; b). PF_6^-; c). TfO^-; d). ClO_4^-; e). NO_3^-; f). $Br;^-$ g). Cl^-; h). $MeSO_3^-$.

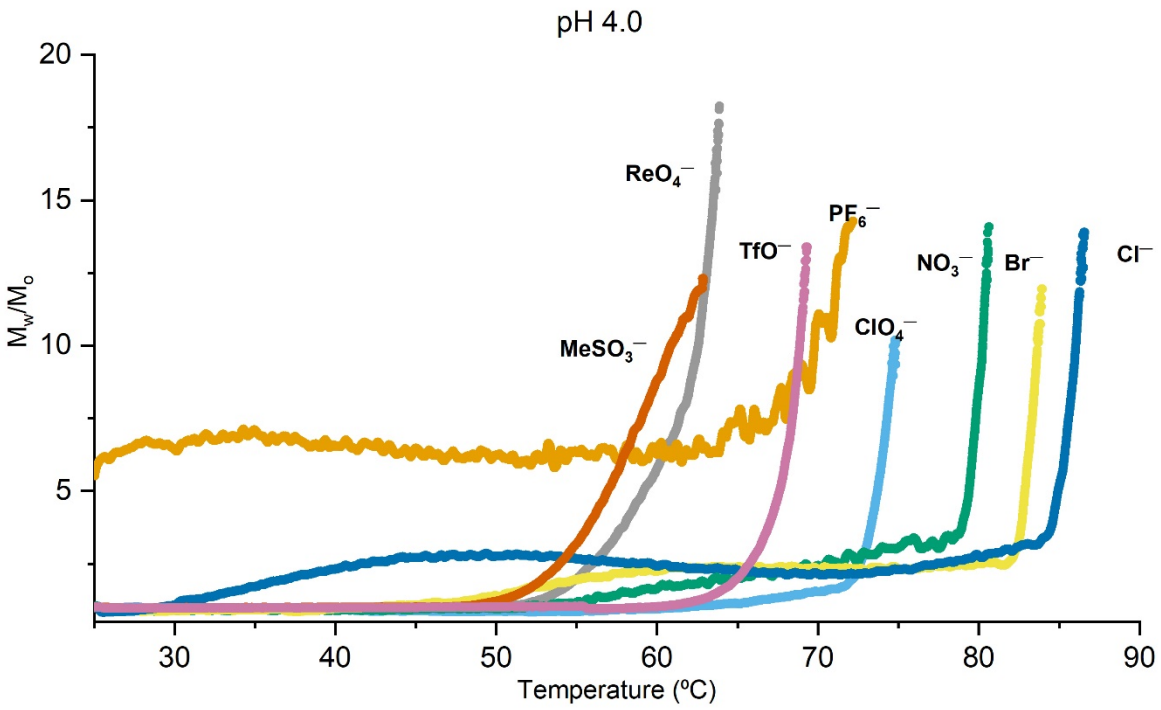


Figure S20 The grouped aggregation curve data for ubiquitin in the presence of different salt (all 1 M) at pH = 4.0.

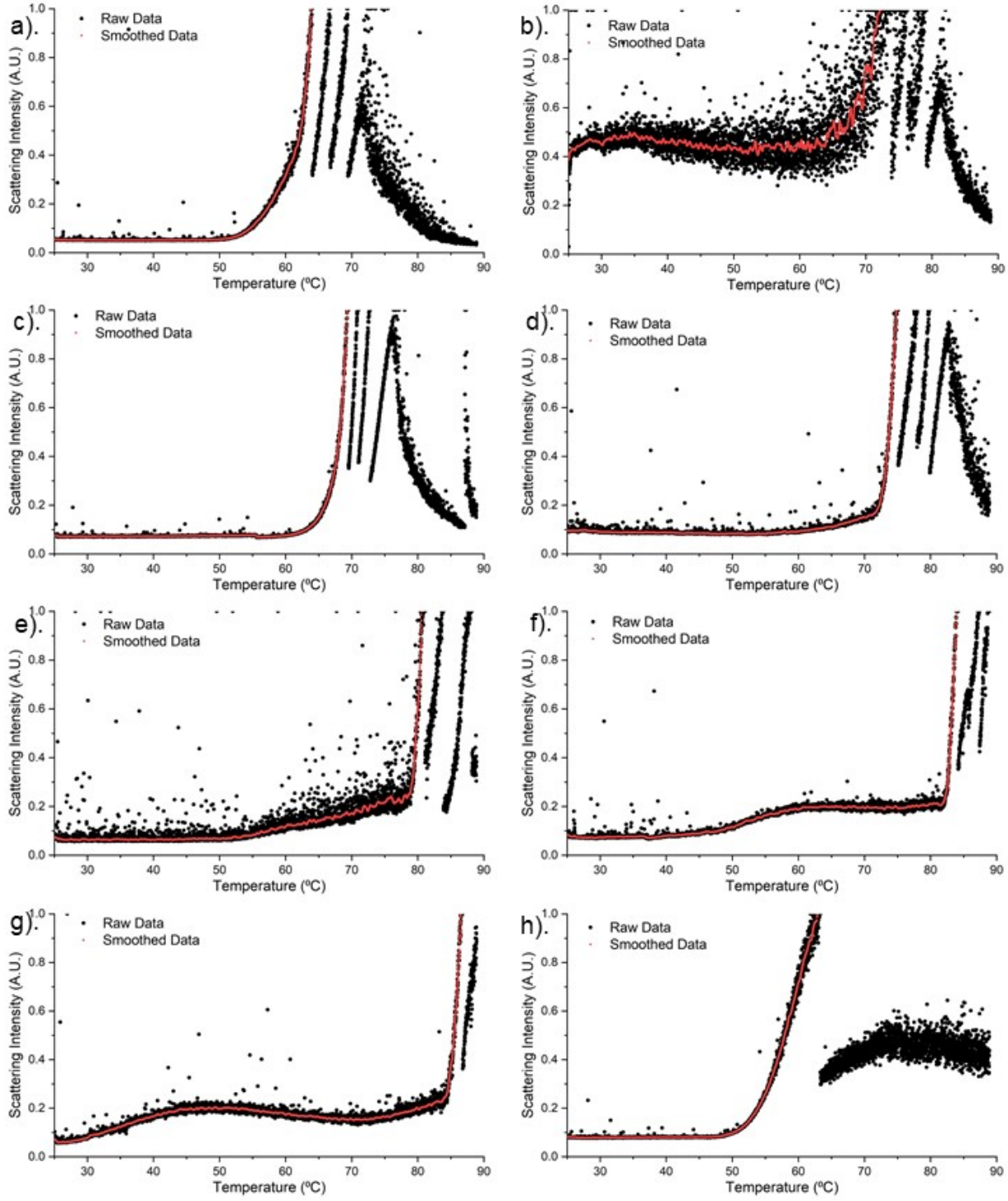


Figure S21 The raw data and processed data of the aggregation of ubiquitin in the presence of different salts (all 1M) at pH = 4.0: a). ReO_4^-; b). PF_6^-; c). TfO^-; d). ClO_4^-; e). NO_3^-; f). $Br;^-$ g). Cl^-; h). $MeSO_3^-$.

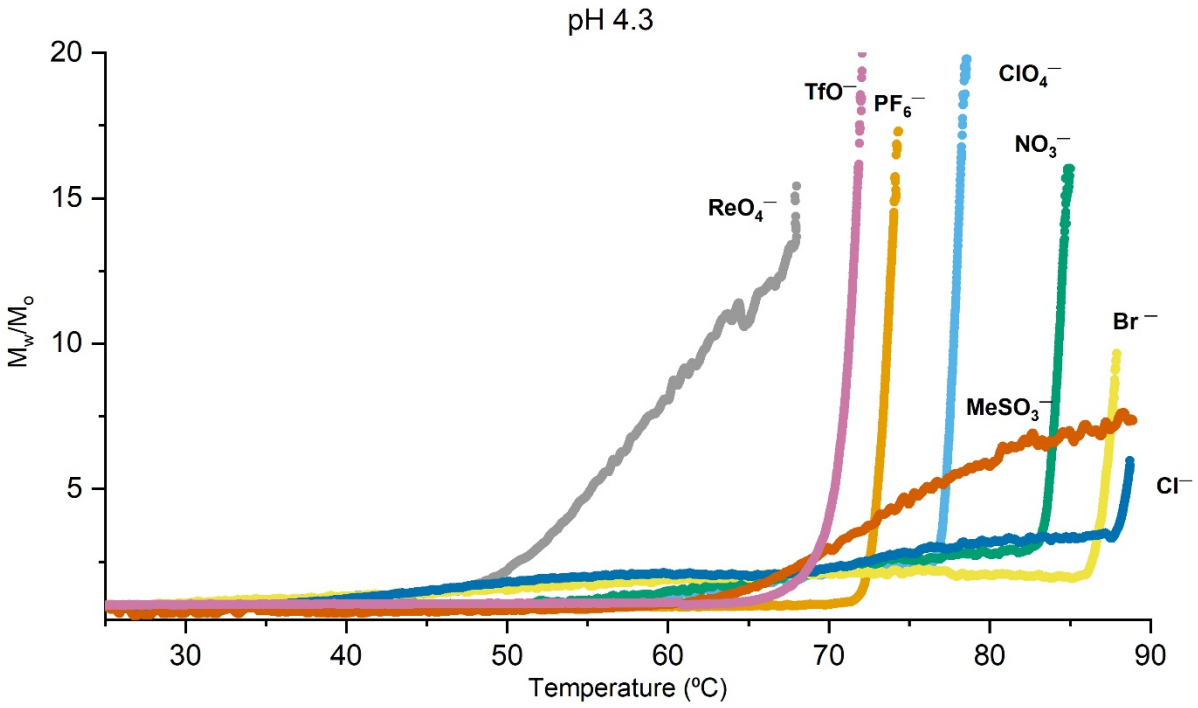


Figure S22 The grouped aggregation curve data for ubiquitin in the presence of different salt (all 1 M) at pH = 4.3.

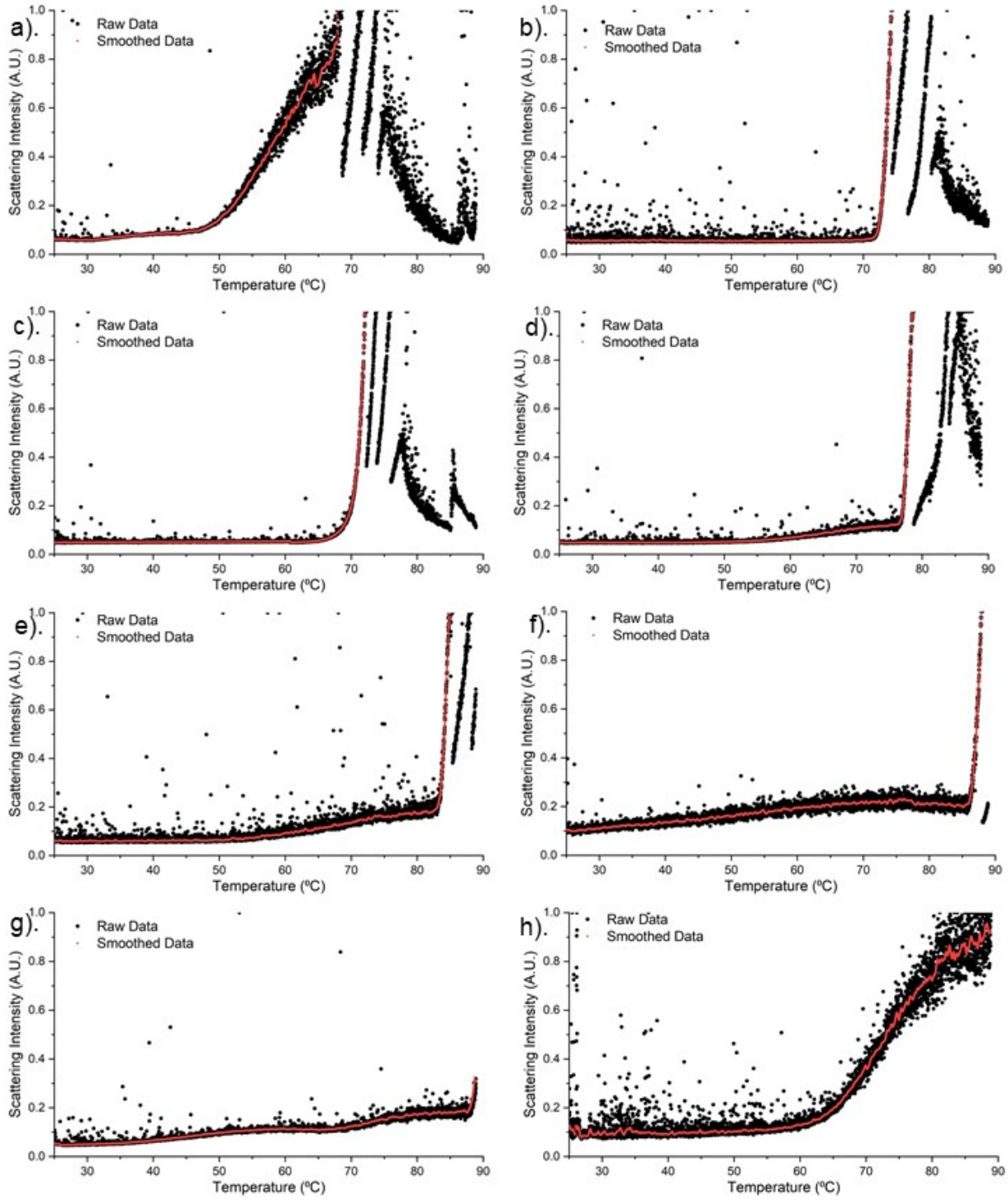


Figure S23 The raw data and processed data of the aggregation of ubiquitin in the presence of different salts (all 1M) at pH = 4.3: a). ReO_4^-; b). PF_6^-; c). TfO^-; d). ClO_4^-; e). NO_3^-; f). $Br;^-$ g). Cl^-; h). $MeSO_3^-$.

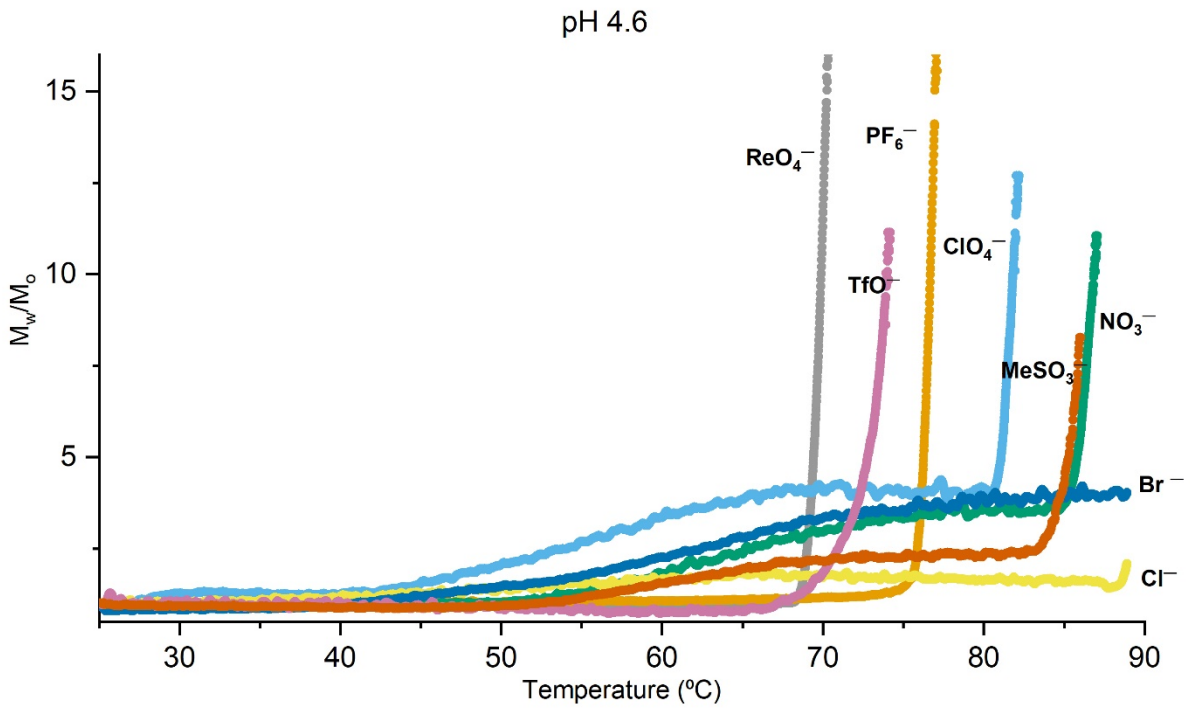


Figure S24 The grouped aggregation curve data for ubiquitin in the presence of different salt (all 1 M) at pH = 4.6.

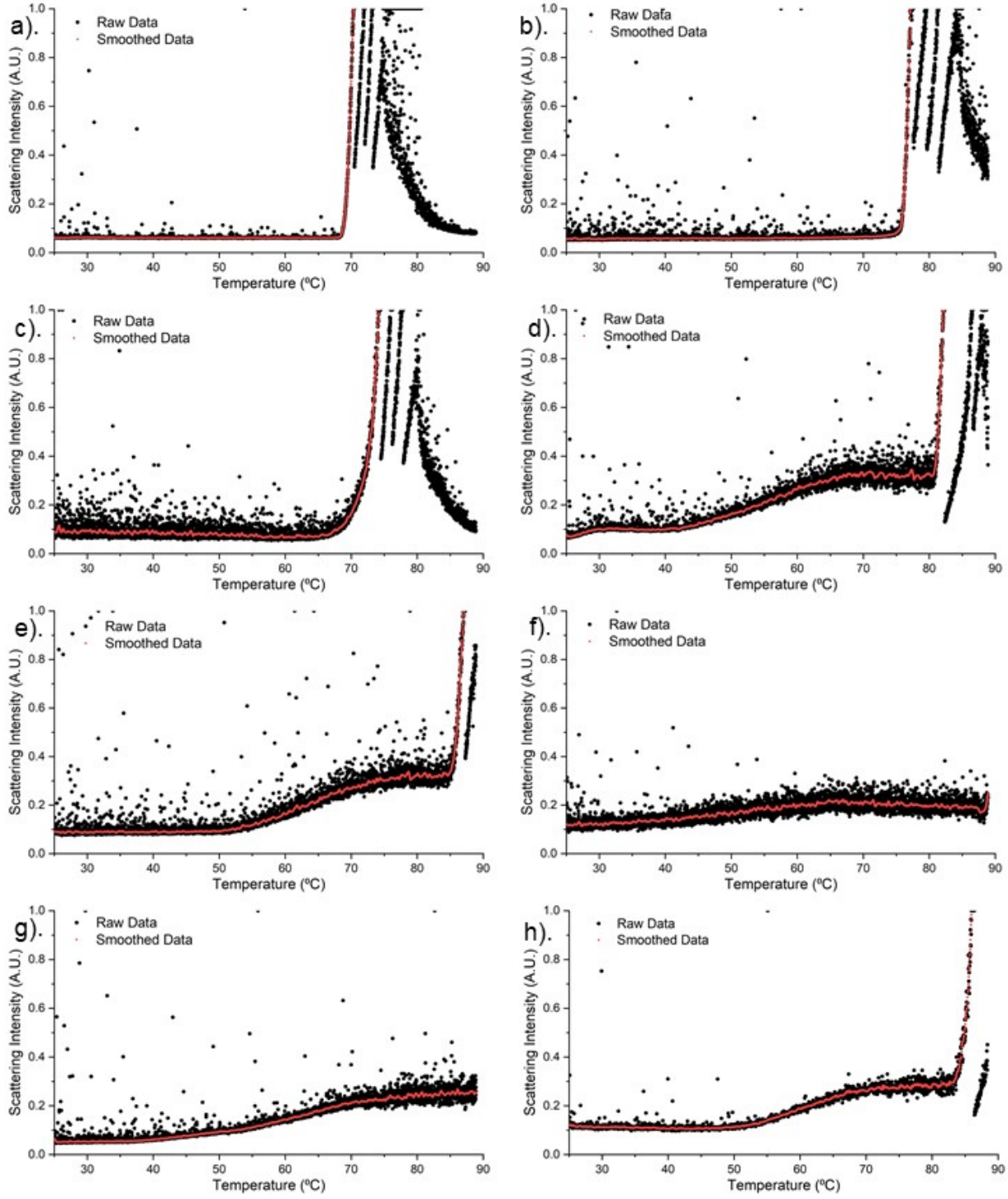


Figure S25 The raw data and processed data of the aggregation of ubiquitin in the presence of different salts (all 1M) at pH = 4.0: a). ReO_4^-; b). PF_6^-; c). TfO^-; d). ClO_4^-; e). NO_3^-; f). $Br;^-$ g). Cl^-; h). $MeSO_3^-$.

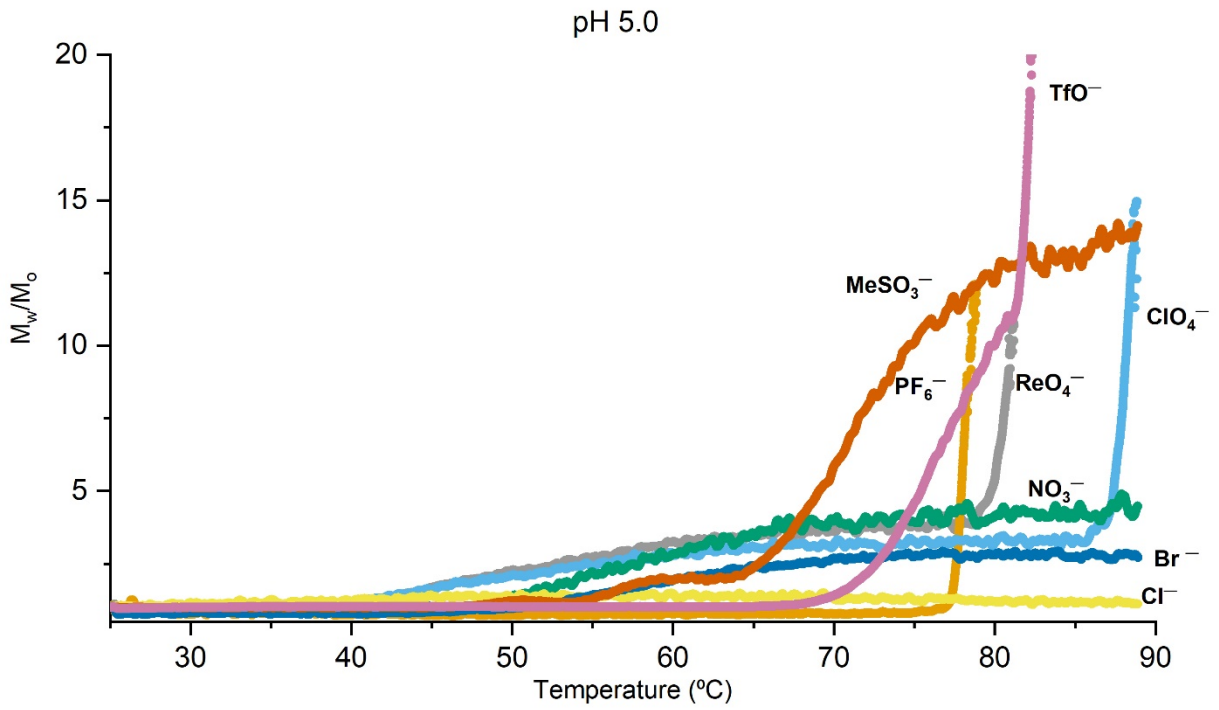


Figure S26 The grouped aggregation curve data for ubiquitin in the presence of different salt (all 1 M) at pH = 5.0.

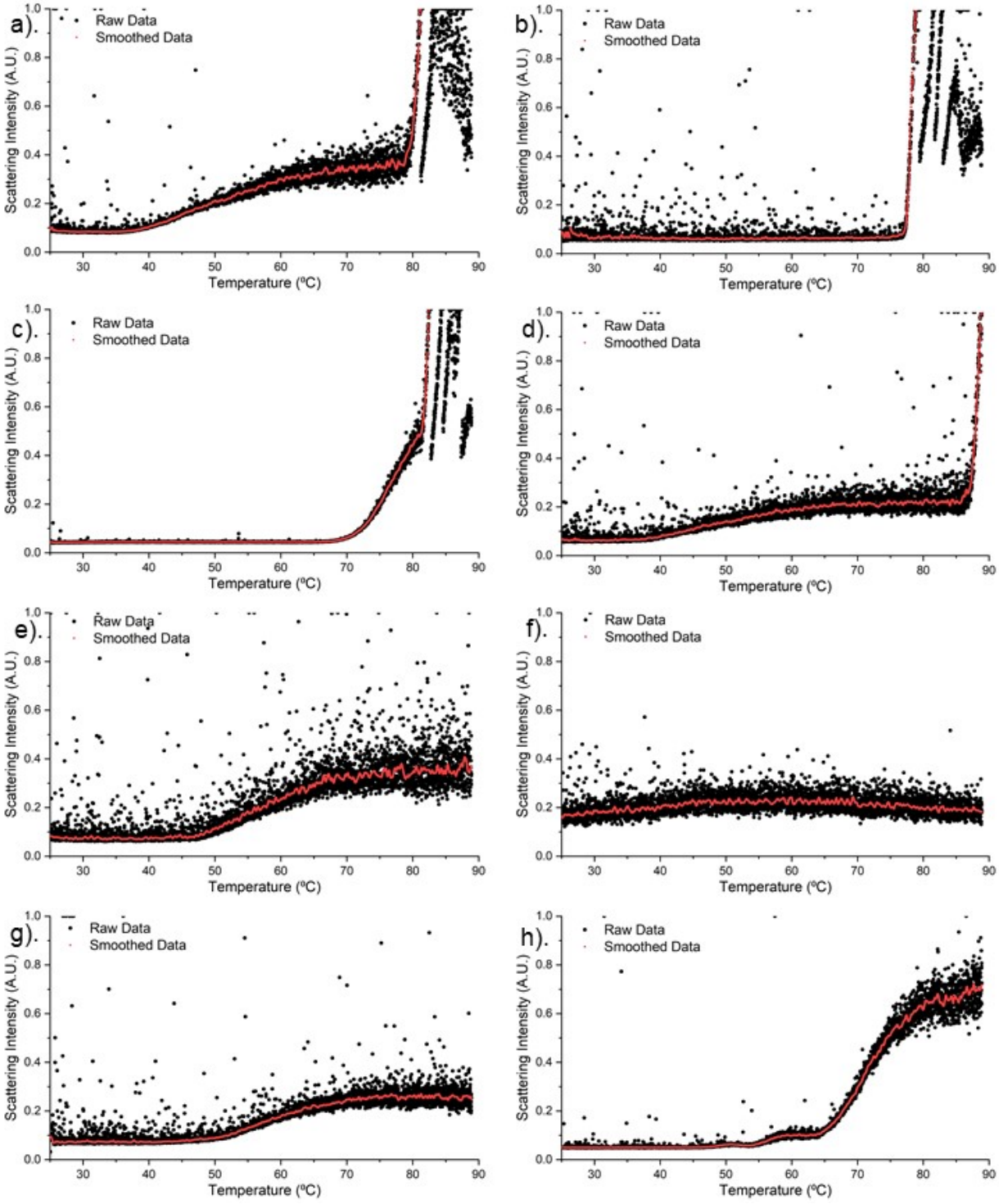


Figure S27 The raw data and processed data of the aggregation of ubiquitin in the presence of different salts (all 1M) at pH = 5.0: a). ReO_4^-; b). PF_6^-; c). TfO^-; d). ClO_4^-; e). NO_3^-; f). Br^-; g). Cl^-; h). $MeSO_3^-$.

3. Circular Dichroism (CD) Experiments

In order to determine the secondary structure change with different salts, CD spectra of ubiquitin in 400 mM of Cl^-, ClO_4^-, $MeSO_3^-$, PF_6^-, and TfO^- were measured at 25 ˚C. The wavelength range was 190 to 250 nm.[1] The effects of three anions: Br^-, NO_3^-, ReO_4^-, could not be measured because of strong adsorption from the salt solution interrupting the signal from the ubiquitin.

Ubiquitin samples were prepared at 2 mg/mL stock solution in 10 mM phosphate buffer. Then 200 µL of 800 mM salt solution was combined with 200 µL stock ubiquitin solution to give a final solution of 1 mg/mL of ubiquitin and 400 mM of salt mixed solution. The mixed CD sample solution was loaded in a 1 mm optical path length quartz cuvette whilst high purity N_2 gas was purged into the spectropolarimeter and the sample chamber during the measurements. The spectra of each sample was the average of five scans collected at a scan speed of 50 nm/min over the far-UV range (190 – 250 nm). The salt solutions were subtracted as baseline in corresponding protein/salt mixed solution. Data were processed by Origin 2019.

The CD results indicates that at rt, the interaction of anion to the surface of ubiquitin did not disrupt the native structure of ubiquitin.

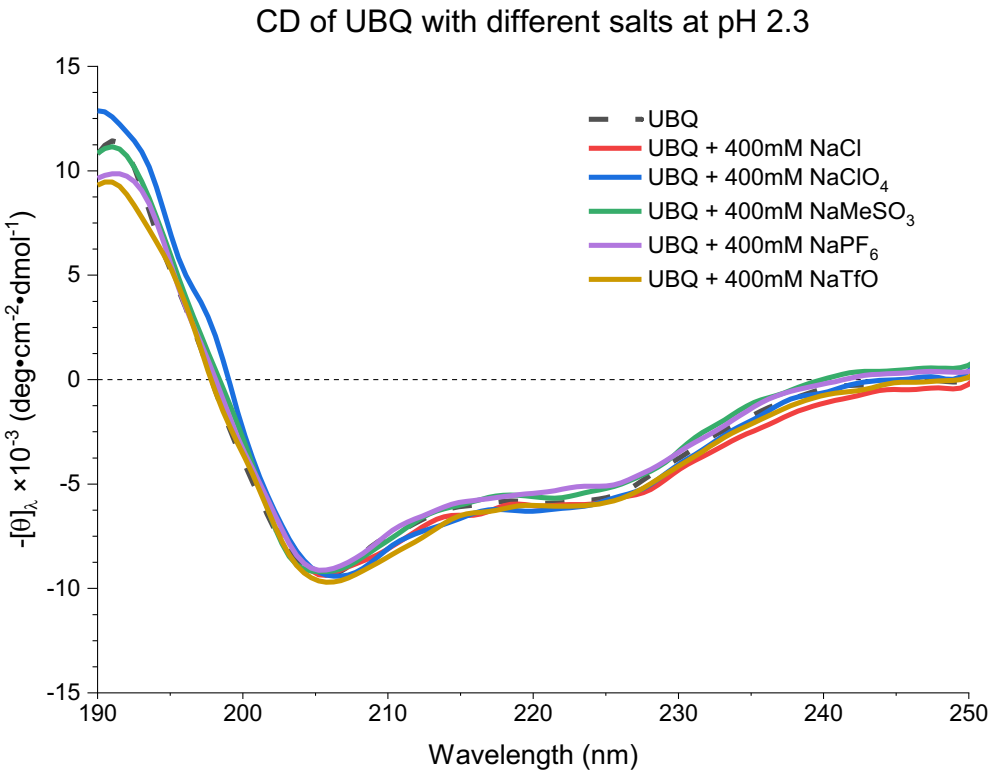


Figure S28 Stacked CD spectra of ubiquitin with different salts at pH 2.3

4. Differential Scanning Calorimetry (DSC) Experiments

All ubiquitin samples were prepared in 10 mM phosphate buffer as 2 mg/mL stock solution and diluted to the desired concentration. All protein/salt mixed solution were loaded into the sample cell (250 µL) and the salt solution loaded in the reference cell. All experiments were collected from 25-110 °C or 40-130 °C, depending on the sample unfolding temperature from preliminary tests. The temperature ramping rate was at a 90 °C /hour under 60 ± 5 psi pressure. Each experimental result was the average of two or three measurements, in which the salt reference was subtracted from the corresponding protein/salt solution. Data processing used the instrument packaged software, and was plot with OriginPro 2019 and Matlab R2018a.

4.1. Anion effect

To study the anion effect on the thermostability of ubiquitin at pH 2.3, eight different anions, i.e. ReO_4^-, PF_6^-, TfO^-, ClO_4^-, NO_3^-, Br^-, Cl^- and $MeSO_3^-$, were used.

Based on preliminary studies, the concentration of ubiquitin was set to 1 mg/mL. Thus, the ubiquitin samples were prepared at 2 mg/mL stock solution and mixed with the salt solution to give a final concentration of 1 mg/mL. Data was corrected using the respective buffer and salt solutions devoid of protein. The temperature ramp experiments were carried out from 25 to 110 °C. For each test, the resulting data was the average of at least two trials. Figure S29 shows the cumulative data, whilst Figure S30 to Figure S37 show representative examples pf thermograms for individual salts.

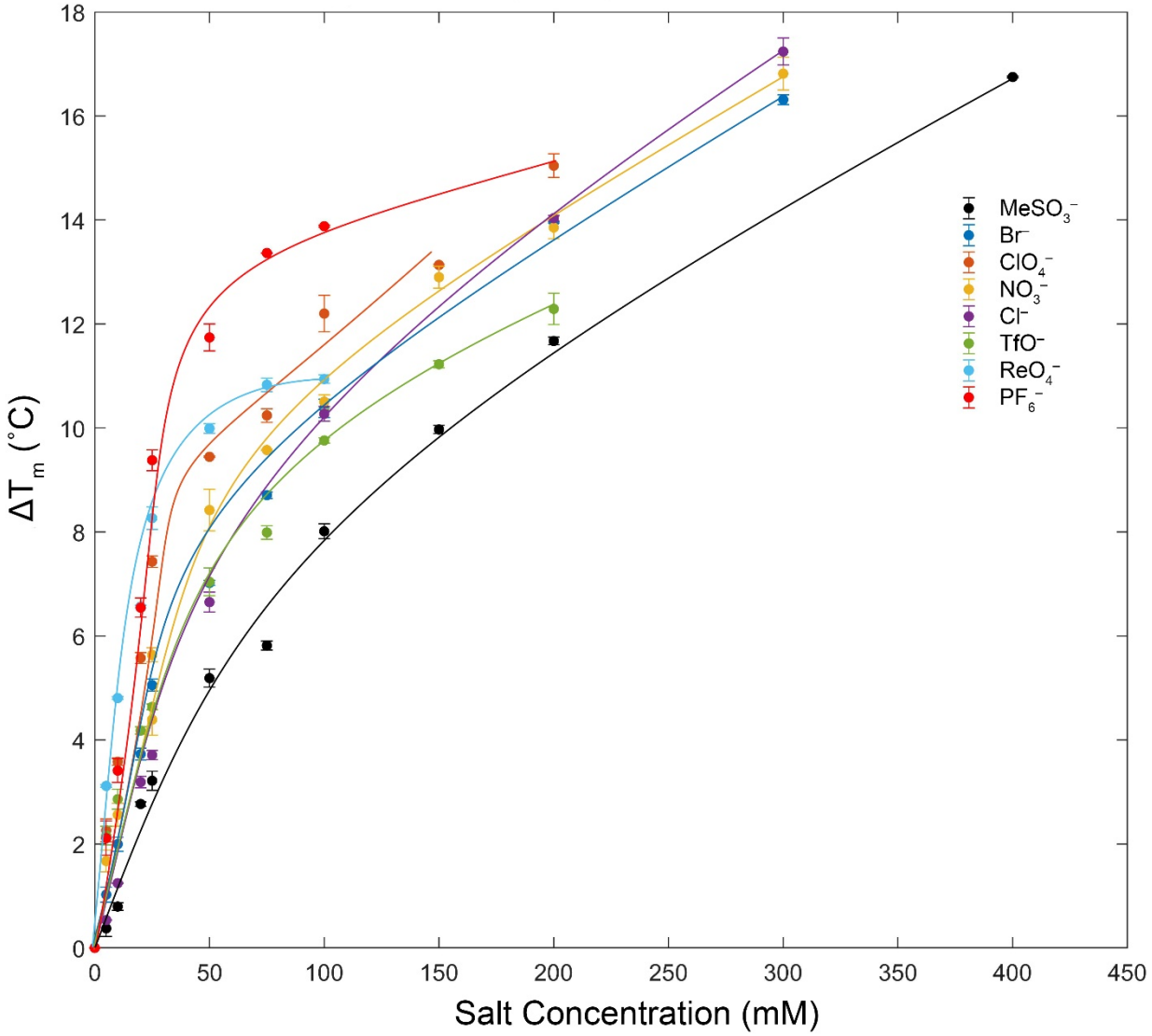


Figure S29 The concentration dependent melting point temperature (ΔT_m) of ubiquitin as a function of different salts at pH = 2.3.

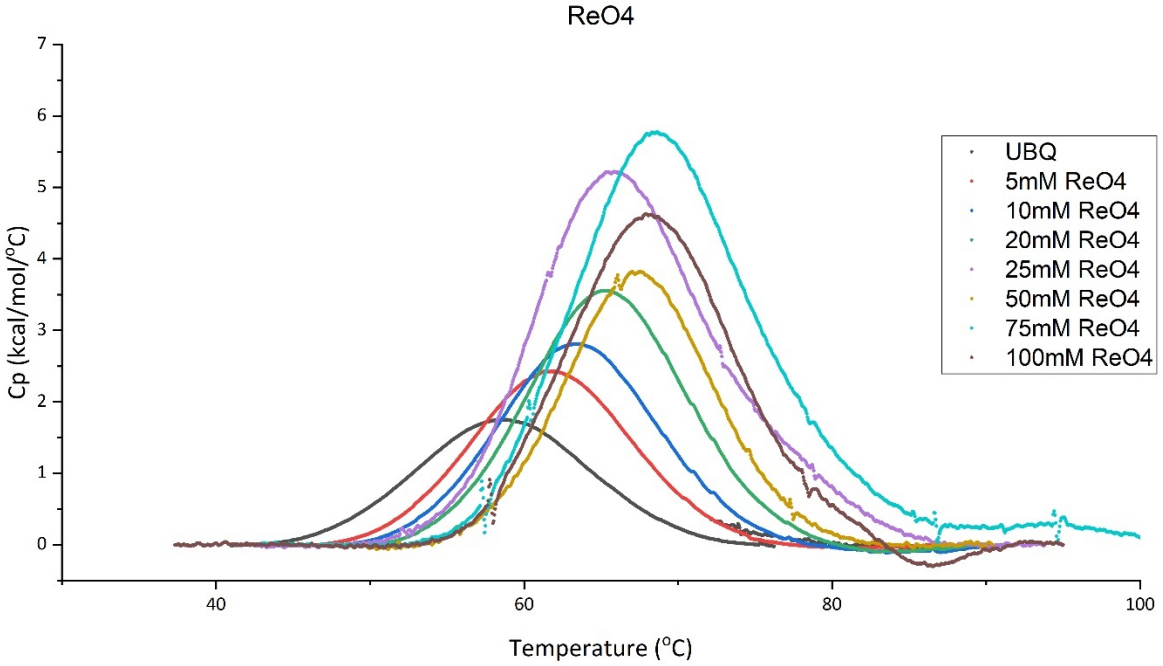


Figure S30 The DSC thermogram of ubiquitin with different concentration of sodium perrhenate at pH = 2.3.

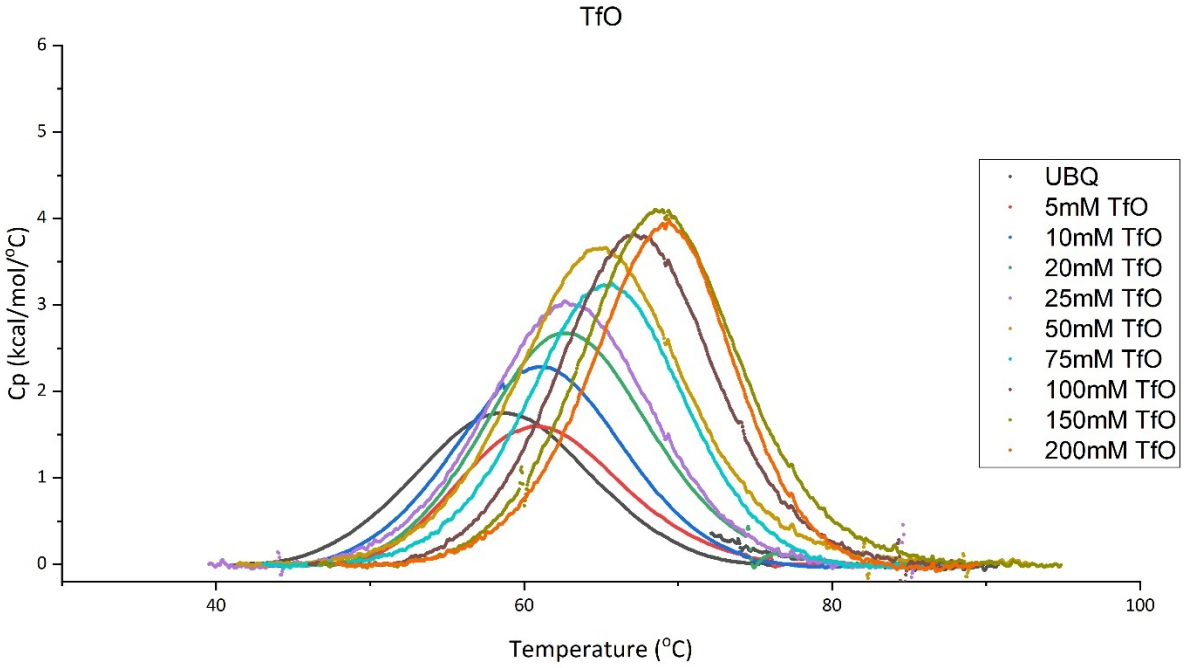


Figure S31 The DSC thermogram of ubiquitin with different concentration of sodium triflate at pH = 2.3.

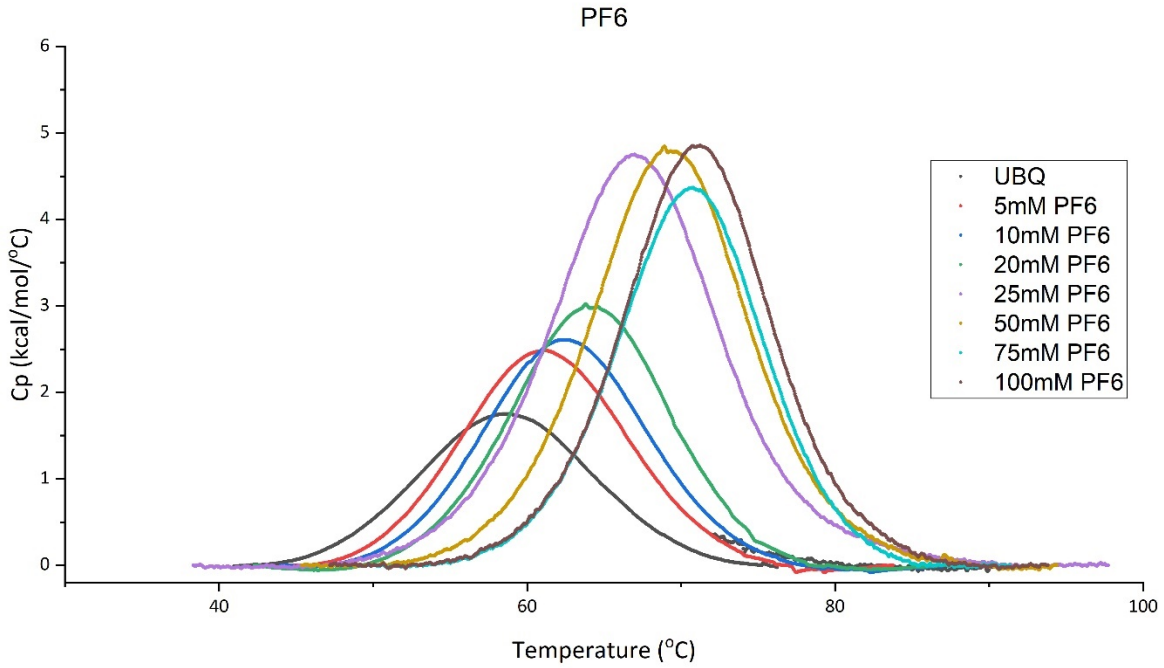


Figure S32 The DSC thermogram of ubiquitin with different concentration of sodium hexafluorophosphate at pH = 2.3.

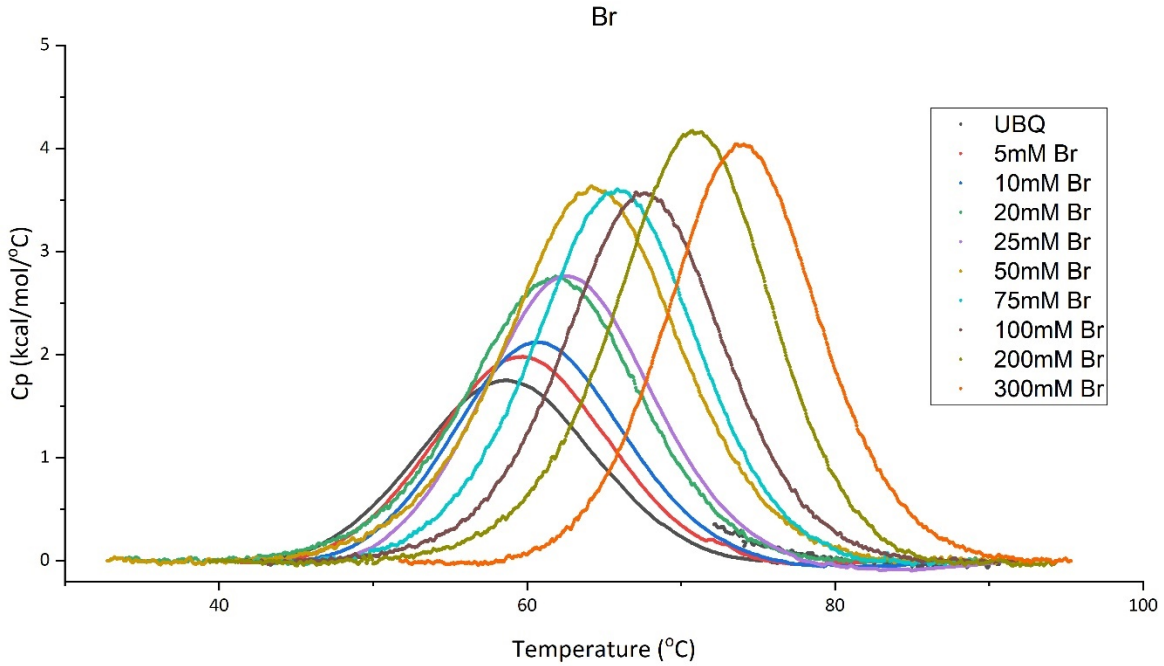


Figure S33 The DSC thermogram of ubiquitin with different concentration of sodium bromide at pH = 2.3.

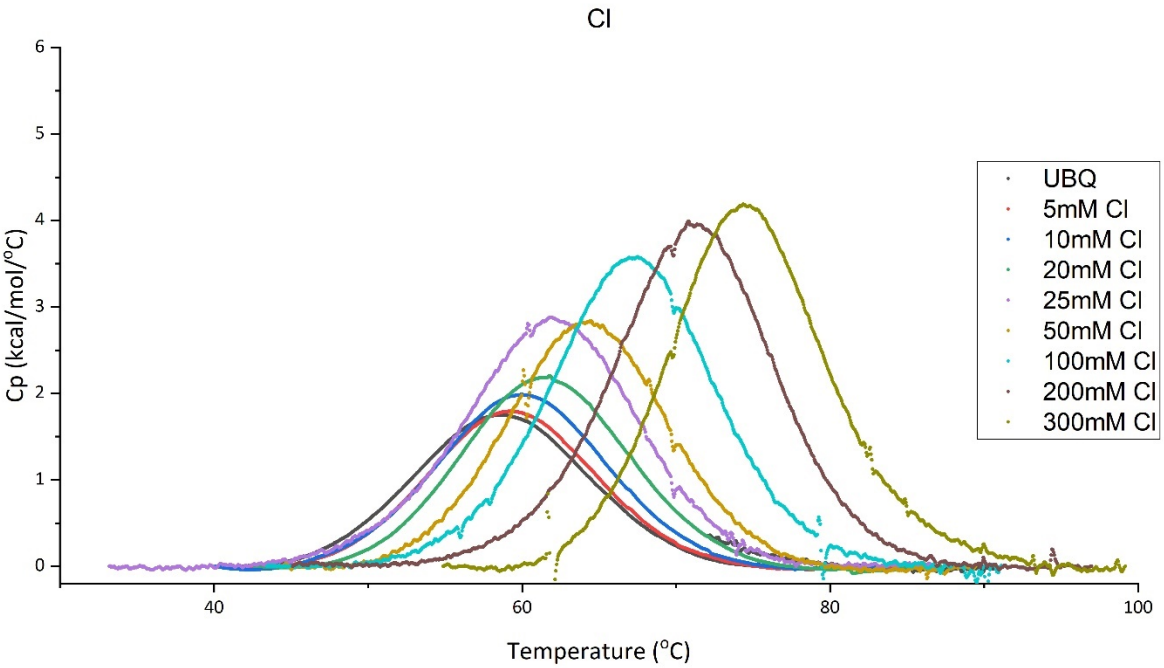


Figure S34 The DSC thermogram of ubiquitin with different concentration of sodium chloride at pH = 2.3.

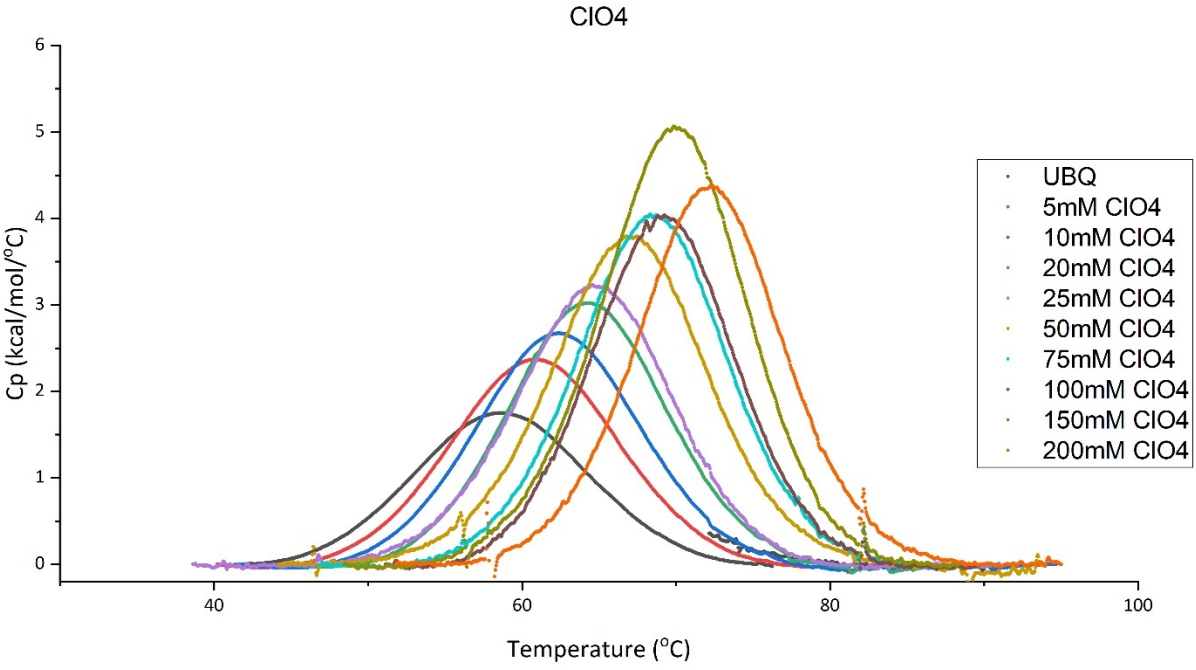


Figure S35 The DSC thermogram of ubiquitin with different concentration of sodium perchlorate at pH = 2.3.

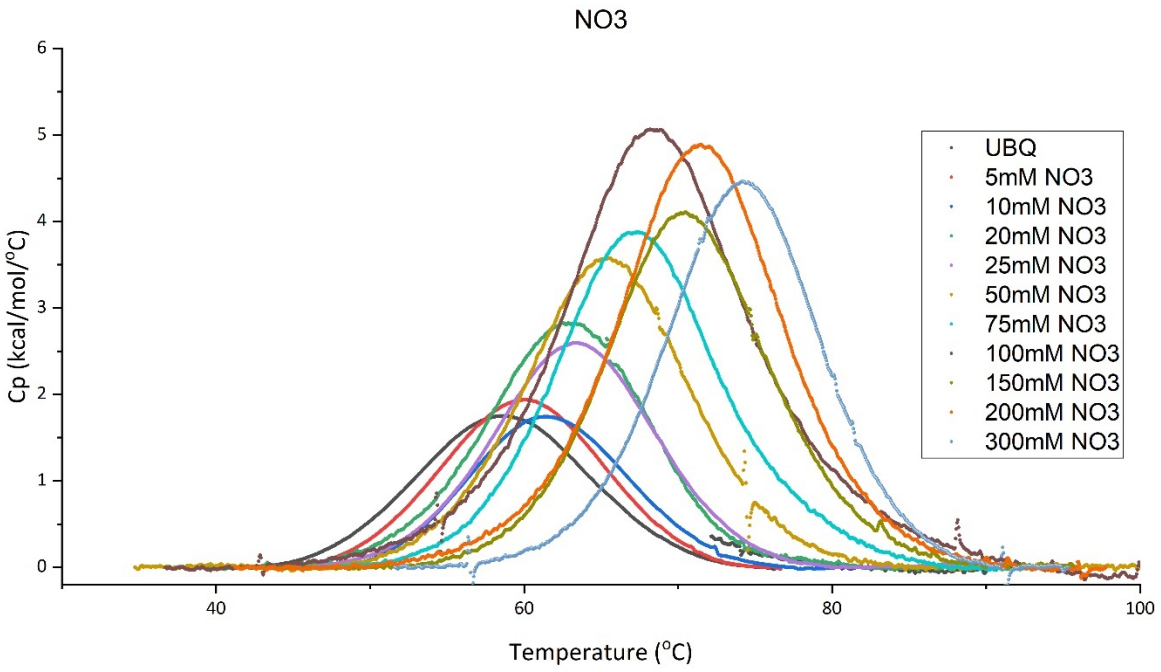


Figure S36 The DSC thermogram of ubiquitin with different concentration of sodium nitrate at pH = 2.3.

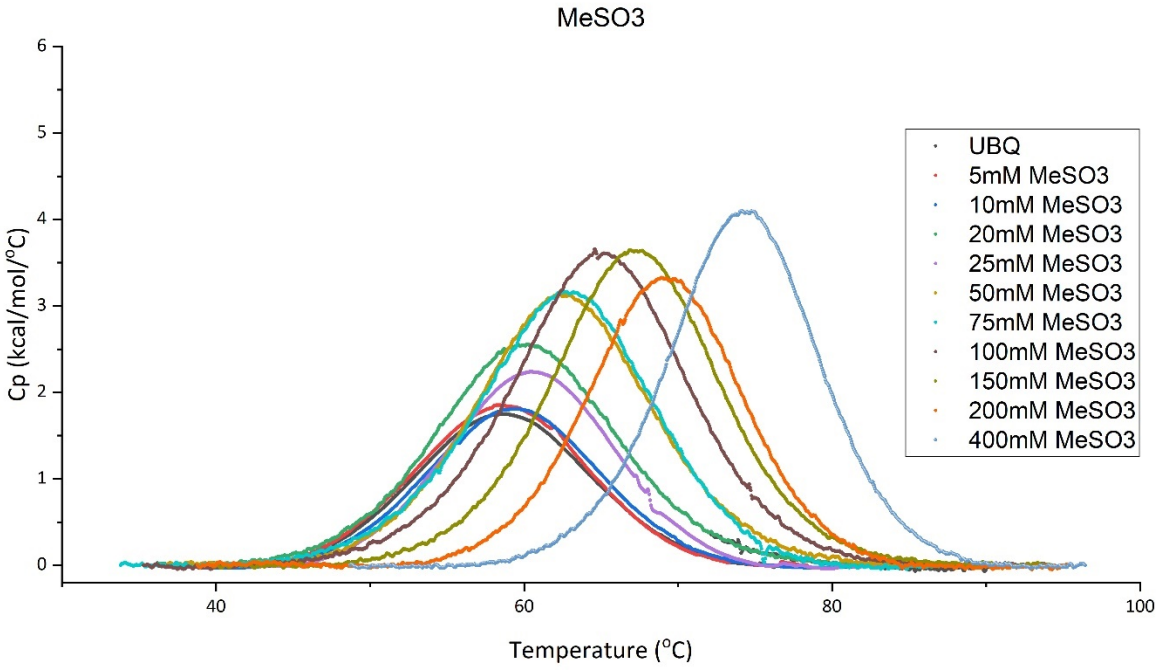


Figure S37 The DSC thermogram of ubiquitin with different concentration of sodium methanesulfonate at pH = 2.3.

4.2. DSC pH study of $NaClO_4$

The thermostability of ubiquitin (ΔT_m) in the presence of varying concentrations of $NaClO_4$ was examined over up over the pH range 2.3 – 5.0. Above pH 5, the minor unfolding peak corresponding to aggregated protein began to affect the symmetry of the primary peak (and hence the T_m value). Hence data above this pH value was not recorded. All runs were duplicated or triplicated, with the error (standard deviation) less than < 0.4˚C.

Positive values of ΔT_m at low pH indicate a stabilizing of ubiquitin, while negative values at high pH reflects the normal Hofmeister effect on the thermostability of ubiquitin. According to the Protein Calculator (http://protcalc.sourceforge.net/) we have the following charges (in parenthesis) at the following pH values: 2.3 (12.8), 2.6 (12.6), 3.0 (12.1), 4.0 (9.0), 4.3 (7.2), 4.5 (5.9), 5.0 (3.2).

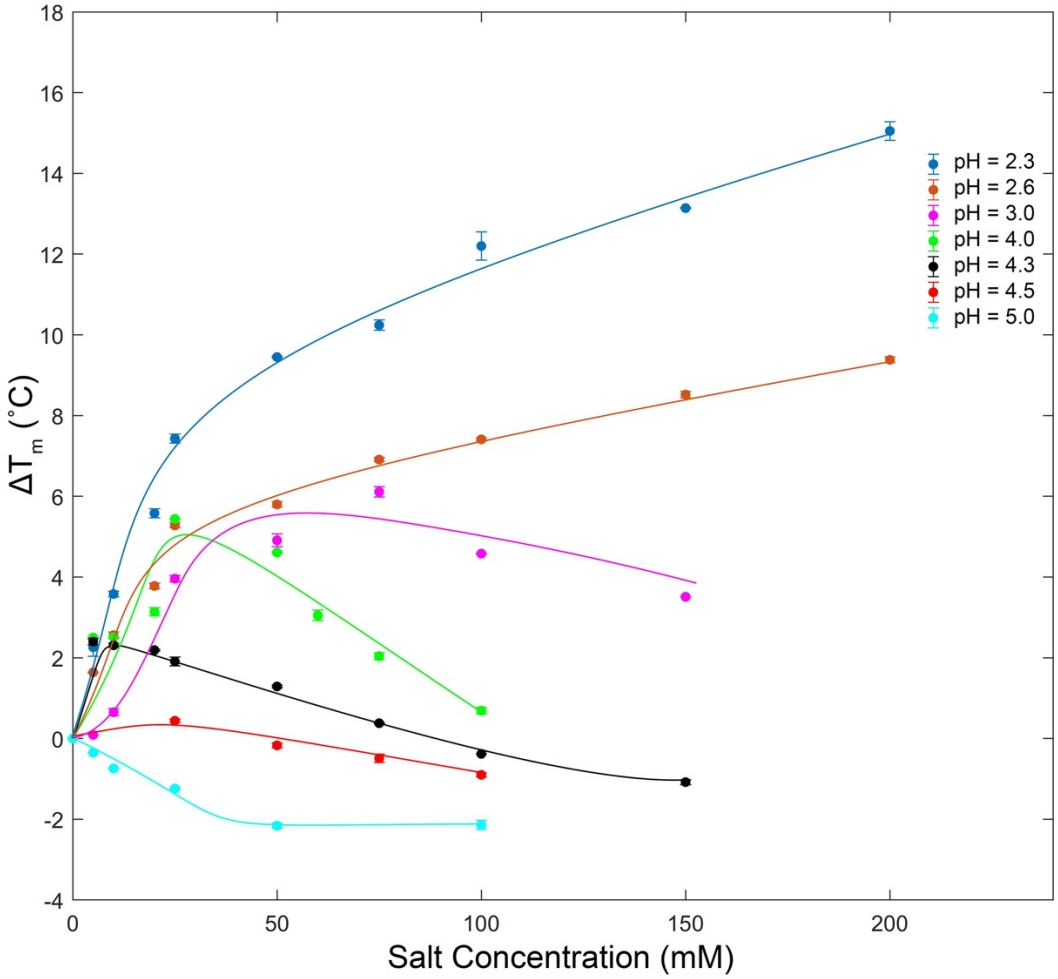


Figure S38 The $NaClO_4$ concentration dependent melting point temperature (ΔT_m) of ubiquitin as a function of pH.

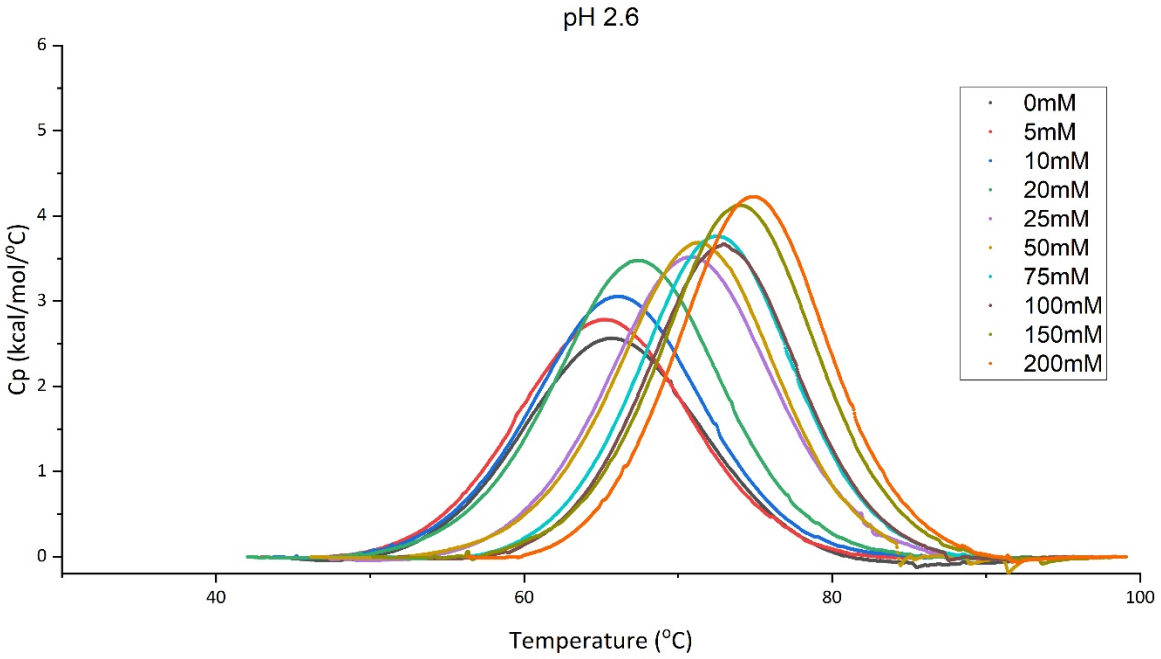


Figure S39 The DSC thermograms of ubiquitin with different concentration of sodium perchlorate at pH = 2.6.

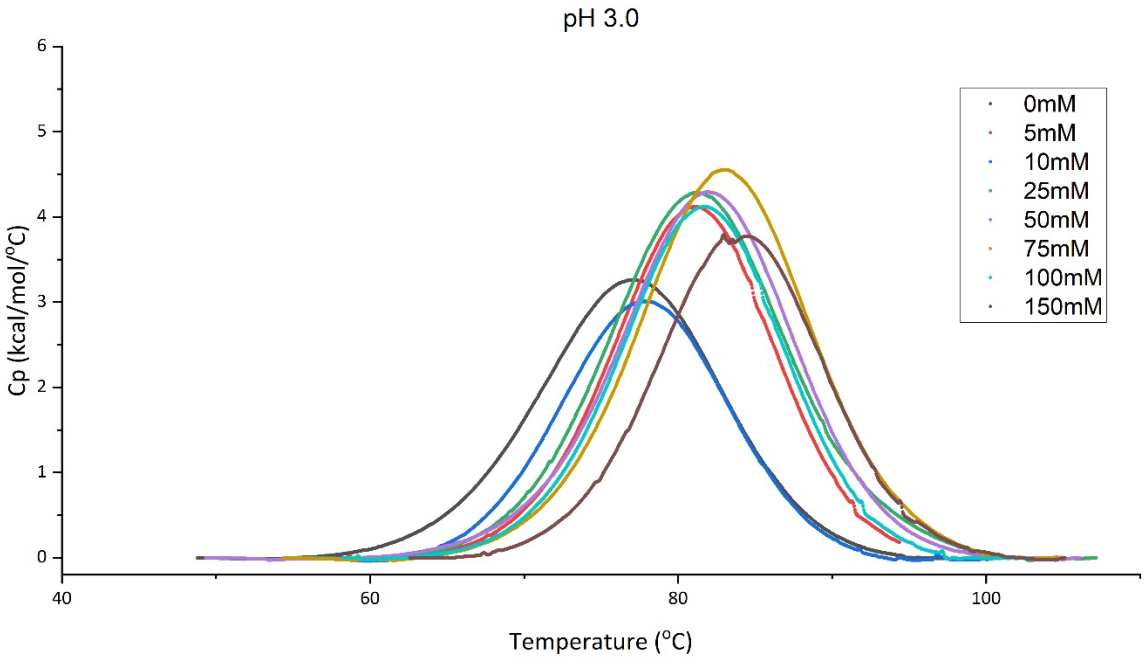


Figure S40 The DSC thermograms of ubiquitin with different concentration of sodium perchlorate at pH = 3.0

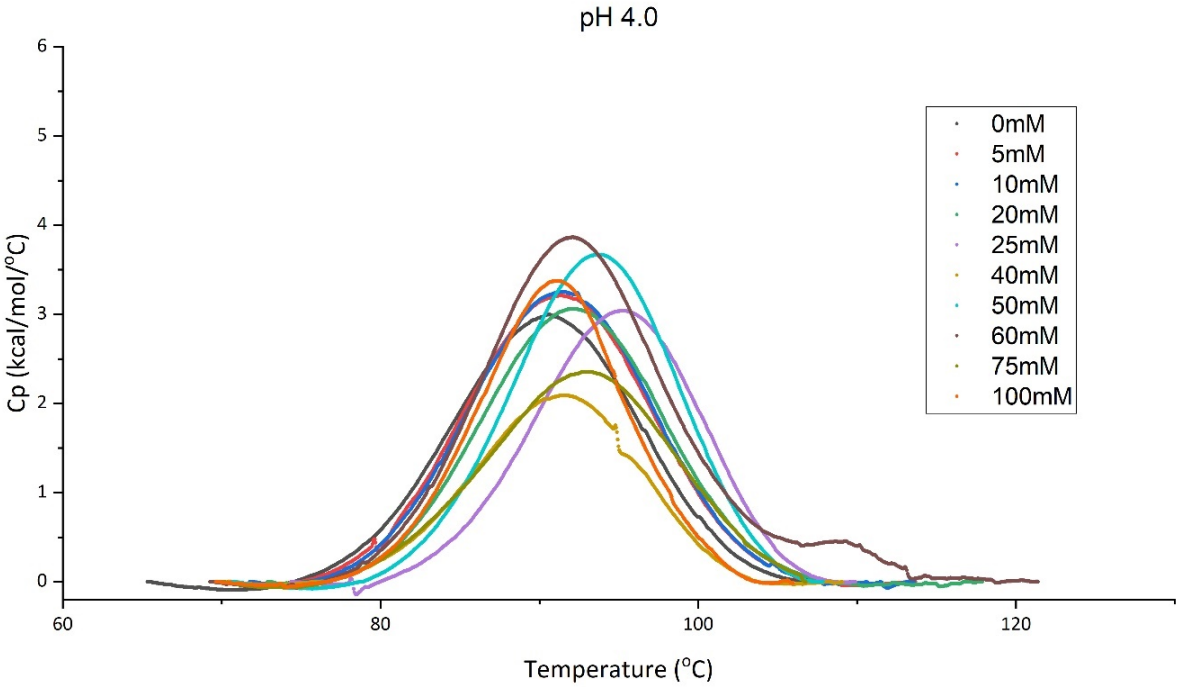


Figure S41 The DSC thermograms of ubiquitin with different concentration of sodium perchlorate at pH = 4.0

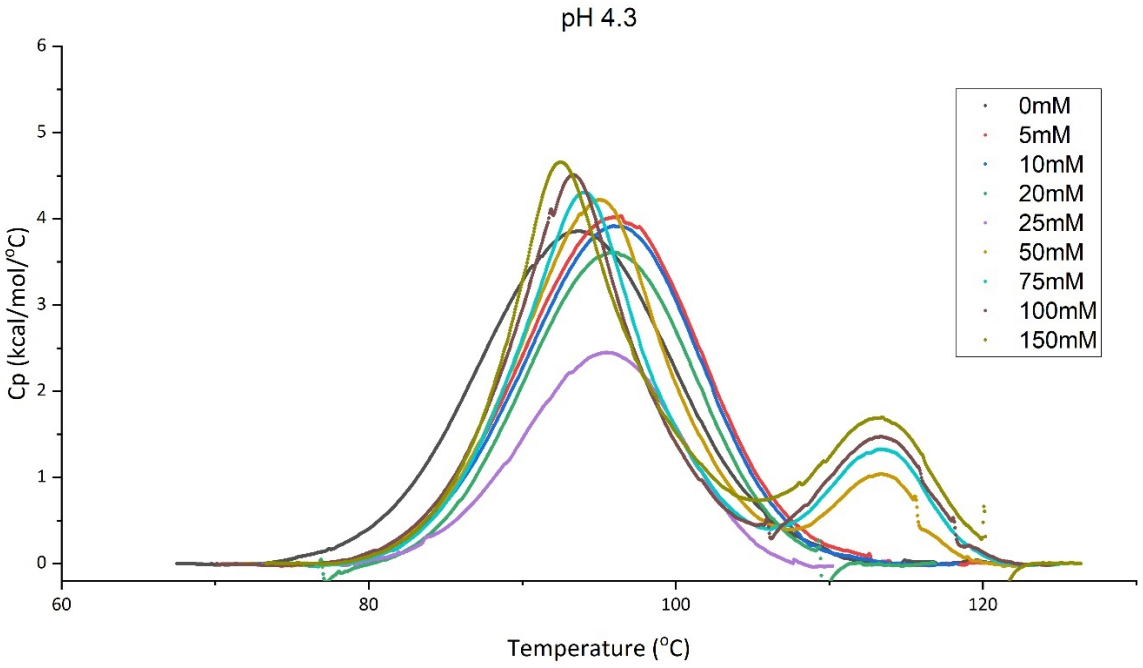


Figure S42 The DSC thermograms of ubiquitin with different concentration of sodium perchlorate at pH = 4.3

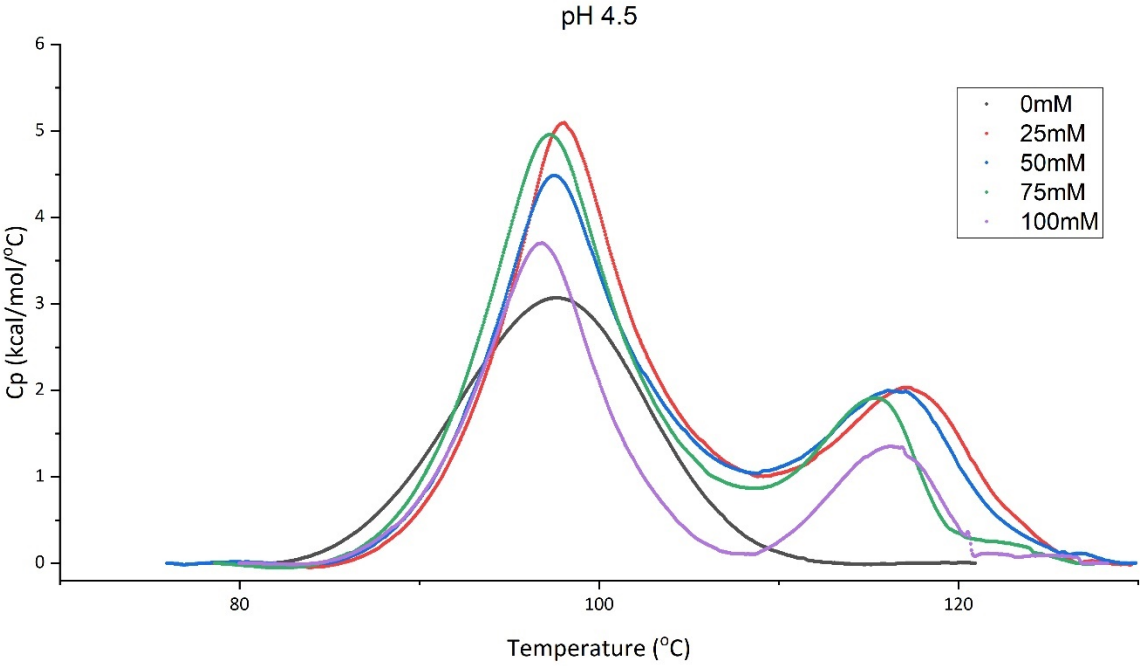


Figure S43 The DSC thermograms of ubiquitin with different concentration of sodium perchlorate at pH = 4.5

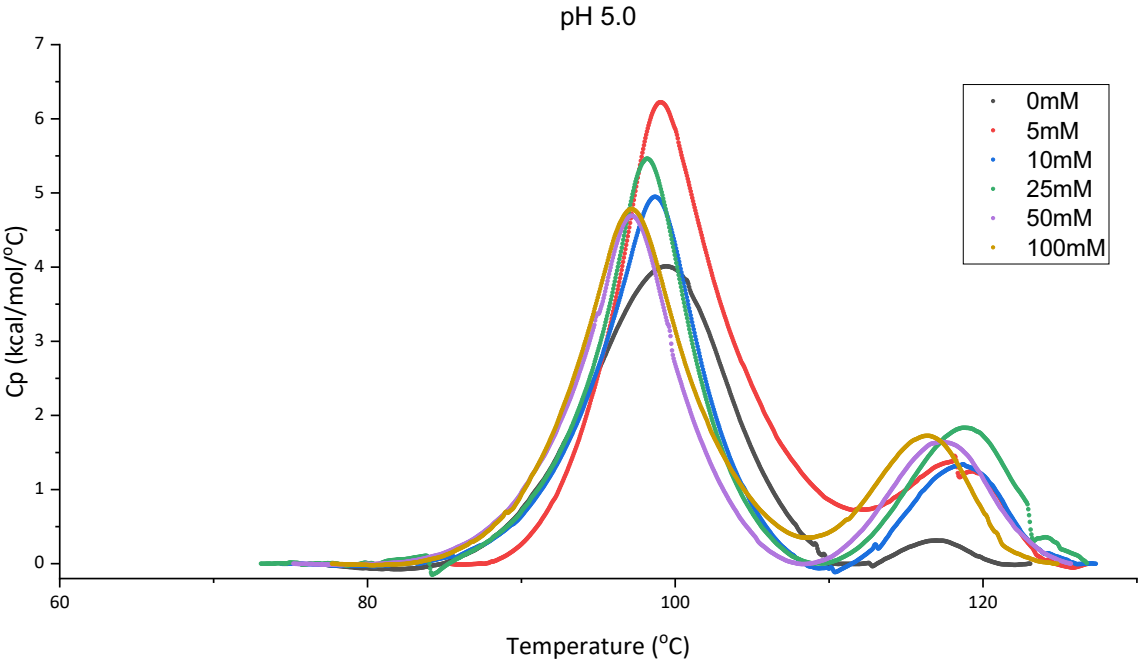


Figure S44 The DSC thermograms of ubiquitin with different concentration of sodium perchlorate at pH = 5.0.

4.3. Anion effects at high pH

We also preliminarily explored the unfolding of Ub from pH 7 to 12. A significant amount of aggregation was observed from pH 7 to 11. However, at pH 12 where the protein is anionic we observed that ClO_4^- and Cl^- weakly stabilized Ub, whereas ReO_4^- destabilizer the protein (Figure S45-S48). We view this as weak cation binding to the negative Ub tending to stabilize Ub, but the relatively strongly associating ReO_4^- counter this and inducing a slight salting-in effect.

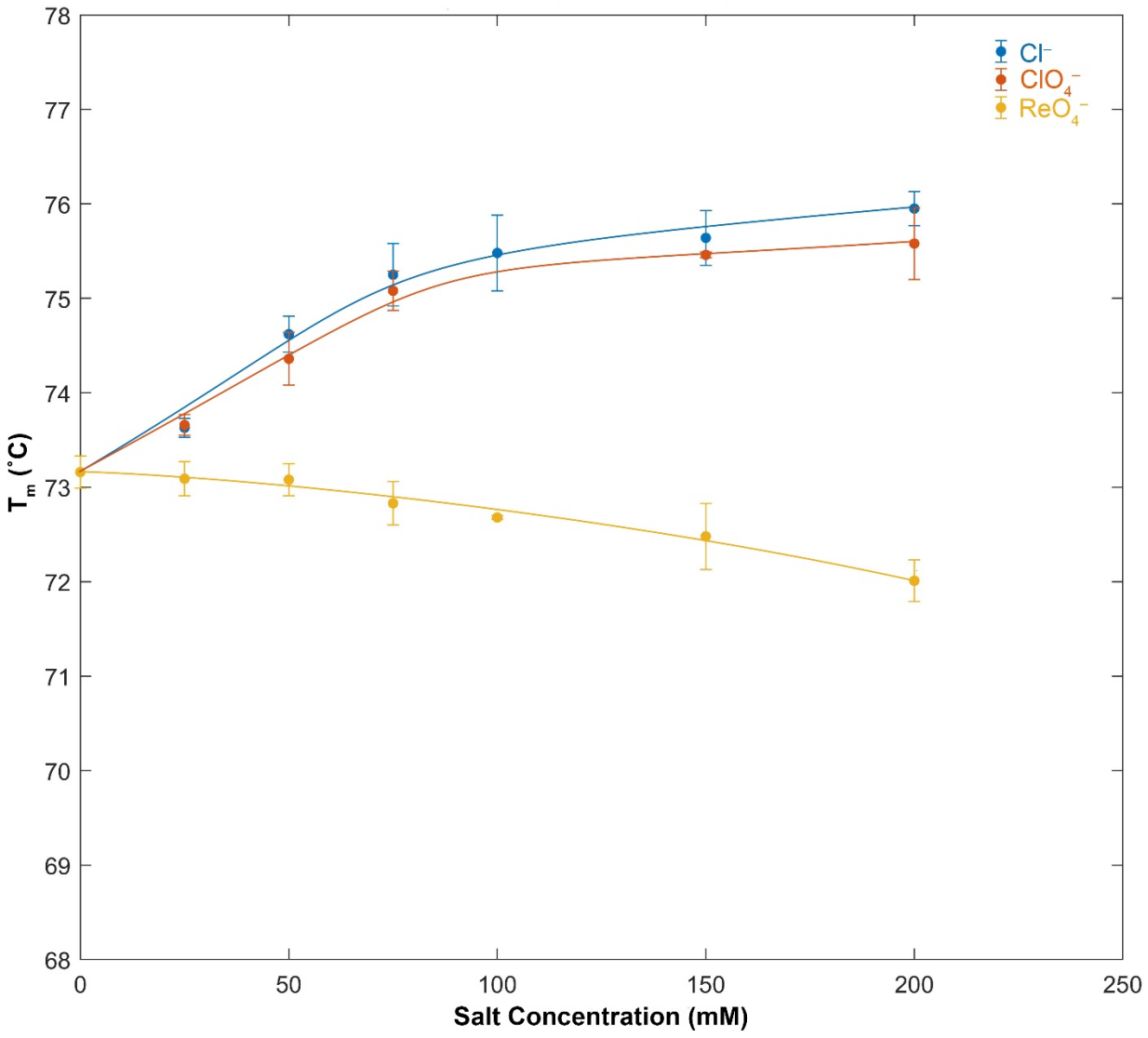


Figure S45 The concentration dependent melting point temperature (ΔT_m) of ubiquitin as a function of different salts at pH = 12.

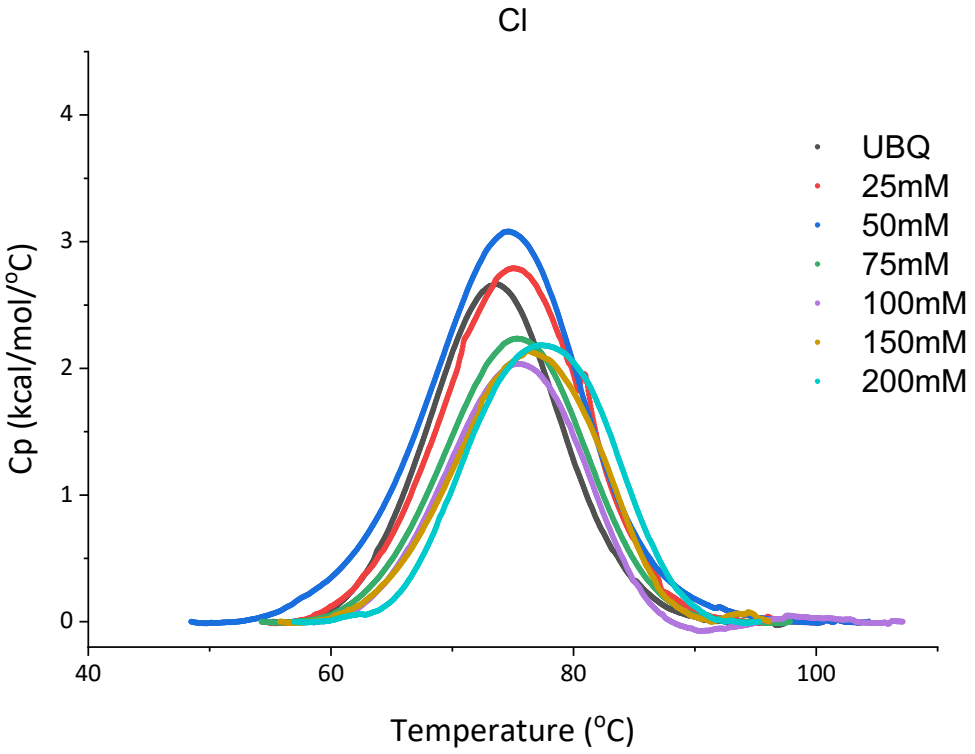


Figure S46 The DSC thermograms of ubiquitin with different concentration of sodium chloride at pH = 12.

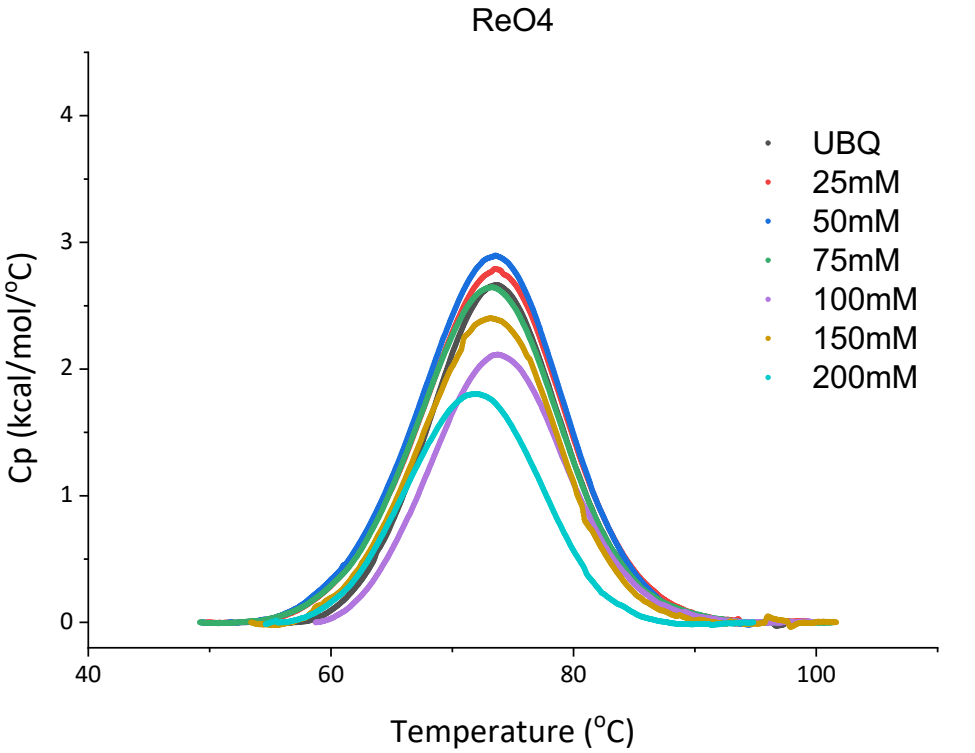


Figure S47 The DSC thermograms of ubiquitin with different concentration of sodium perrhenate at pH = 12.

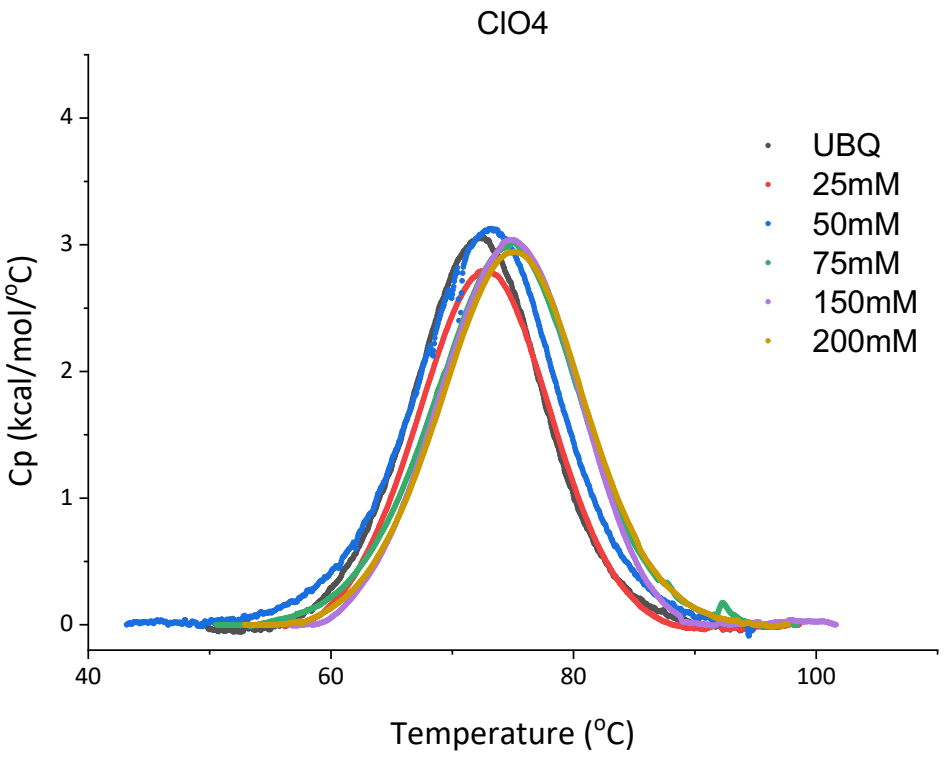


Figure S48 The DSC thermograms of ubiquitin with different concentration of sodium perchlorate at pH = 12.

5. ^{1}H-^{15}N HSQC NMR Experiments

^{15}N-enrich human ubiquitin (98 atom %, recombinant expressed in *E. coli*) was purchased from Sigma-Aldrich and used without further purification. All samples were prepared from 100 mg/mL stock solution and diluted to the desired concentration and pH using 50 mM phosphate buffer in 90% H_2O/ 10% D_2O. All experiments were carried out in a Shigemi NMR tube. The salt solutions were prepared at 2 M or 4 M concentrations in buffer.

The experiments were carried out on a 500 MHz Bruker NMR with a BBI probe. The temperature was control at 25 ˚C. The use of chemical shift perturbation (CSP) to identify the protein-ligand interaction by ^{1}H-^{15}N HSQC NMR has been widely reported.[2-4] The Δδ value for each titration point is derived from the adjusted chemical perturbation of the chemical shifts of ^{1}H and ^{15}N, Eq. S2:

$$\Delta\delta = \sqrt{(\Delta\delta_H)^2 + c(\Delta\delta_N)^2} \qquad \text{Eq. S2}$$

In which $\Delta\delta_H$ and $\Delta\delta_N$ are the chemical shifts of the N–H signals along the H- and N- dimensions, and c is a scaling factor (0.2).[4] Six residues, M1, P19, E24, P37, P38, G53, either have no N–H group (P) or no apparent ^{1}H-^{15}N HSQC signal.[5,6]

1H-15N HSQC NMR Spectroscopy

Each point in a titration curve required approximately 8-10 hours of data acquisition. Thus, only 8-10 points per titration curve were recorded to keep each titration experiment to less that 100 hours of acquisition time.

NH signals that generally underwent non-linear shifts greater than 0.18 ppm (Fig S69) were associated with an anion binding site and fitted to a 1:1 binding model using non-linear regression analysis. Because of long acquisition times, reproducibility of affinity data was confirmed by comparison between similar pH vales rather than repetition at individual pH values (e.g., data at pH 2.3 and 2.8). Based on this data the errors in affinity for the six identified binding sites are respectively (Sites 1-6): < 5%, 30%, < 5%, 17%, 23%, 6%.

5.1. Spectrum of ubiquitin at pH 5.8

The ^{1}H-^{15}N HSQC spectrum has been previously reported and assigned.[5] To validate the method and protocol for the further titration work, the HSQC of ubiquitin at pH 5.8 was duplicated (

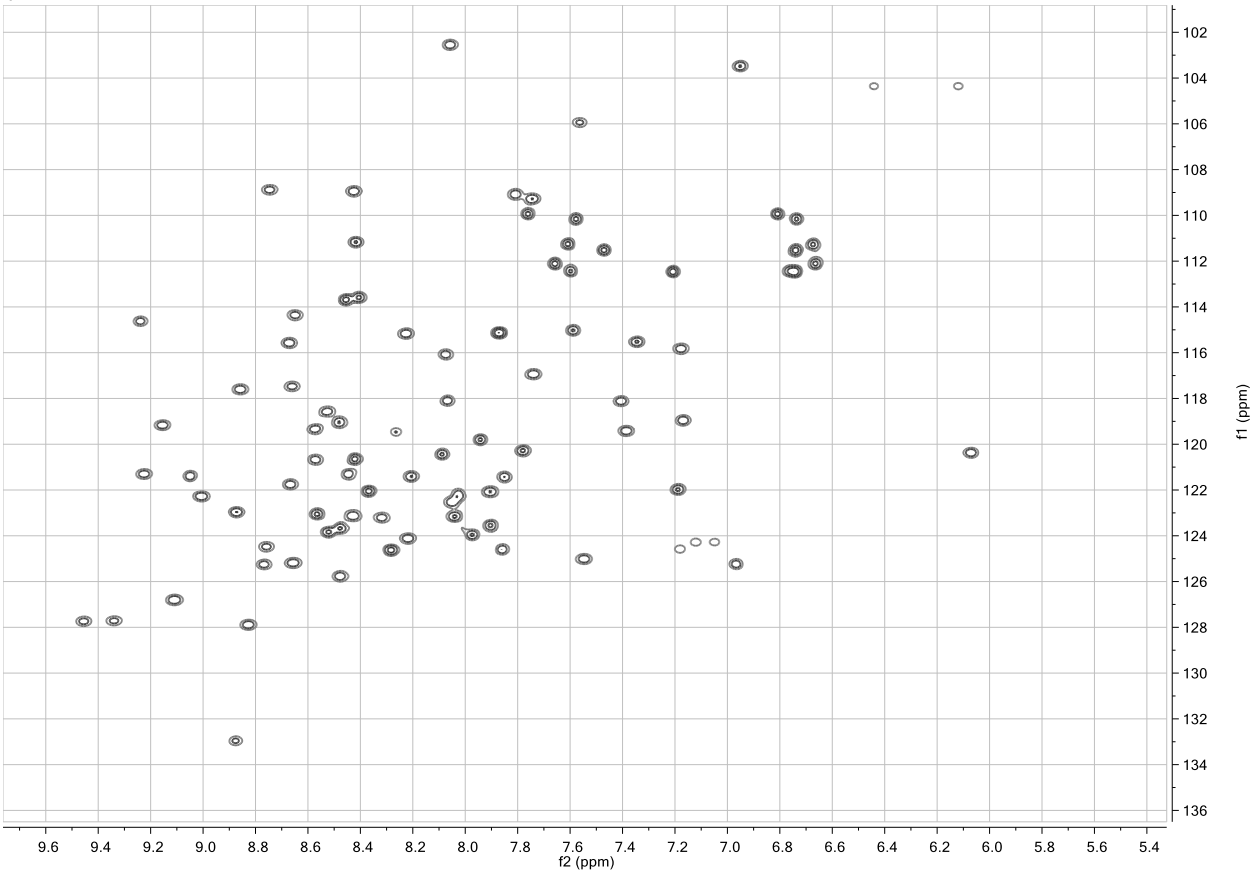


Figure S49). The spectrum was found to be essentially identical to previous reports. The values from this spectrum (Table S2) provide the baseline for the HSQC spectra shifts under the other conditions investigated.

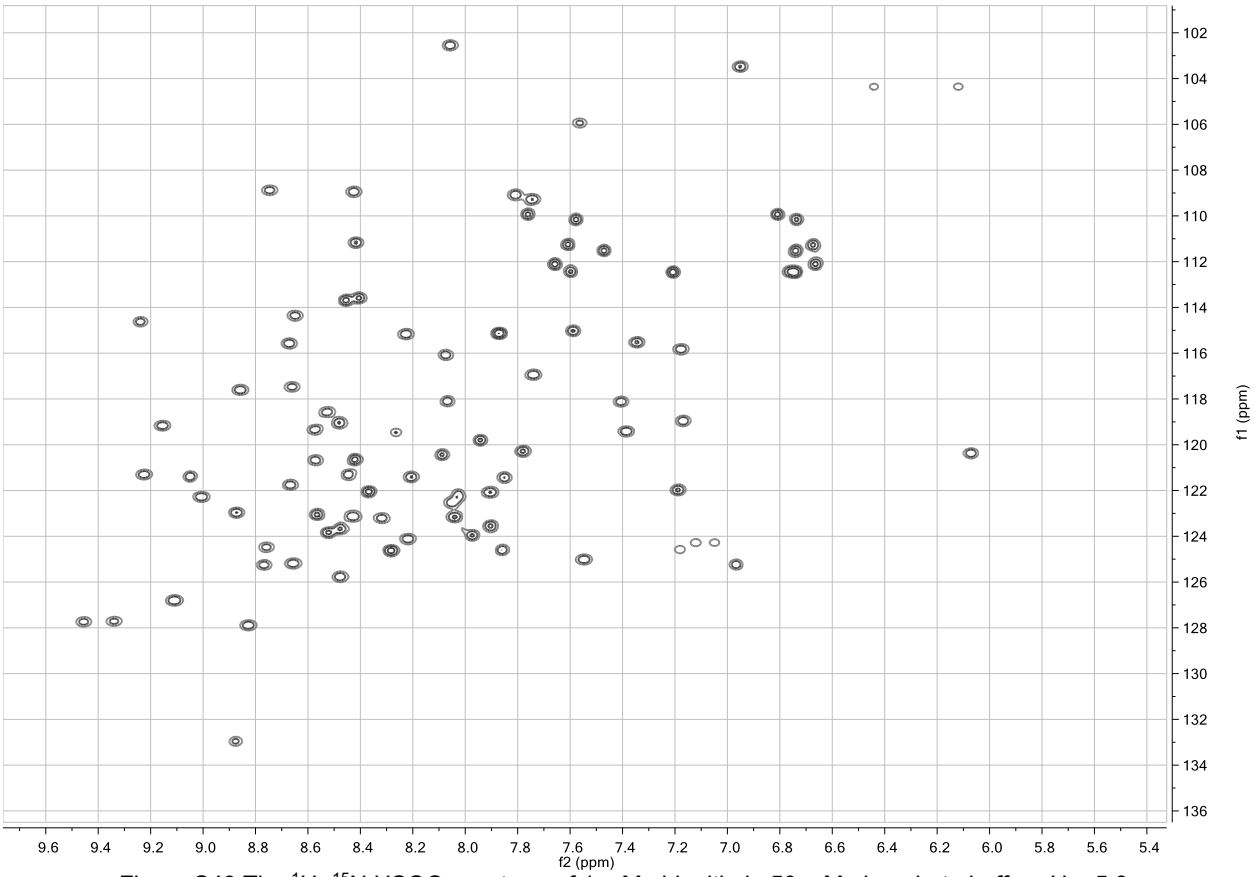


Figure S49 The ^{1}H -^{15}N HSQC spectrum of 1 mM ubiquitin in 50 mM phosphate buffer pH = 5.8.

Table S2: ^{1}H -^{15}N HSQC chemical shift of 1 mM ubiquitin in 50 mM phosphate buffer, pH = 5.8.

Amino Acid #.	^{1}H	^{15}N	Amino Acid #.	^{1}H	^{15}N
M1*	–	–	**D39**	8.45	113.68
Q2	8.87	122.97	**Q40**	7.74	116.94
I3	8.23	115.16	**Q41**	7.41	118.11
F4	8.53	118.57	**R42**	8.43	123.13
V5	9.23	121.3	**L43**	8.76	124.48
K6	8.83	127.89	**I44**	9.01	122.28
T7	8.67	115.57	**F45**	8.77	125.25
L8	9.05	121.39	**A46**	8.88	132.96
T9	7.56	105.94	**G47**	8.06	102.55
G10	7.74	109.28	**K48**	7.9	122.08
K11	7.19	121.98	**Q49**	8.57	123.05
T12	8.57	120.67	**L50**	8.48	125.77
I13	9.46	127.74	**E51**	8.32	123.21
T14	8.67	121.76	**D52**	8.09	120.44
L15	8.66	125.18	**G53***	–	–
E16	8.05	122.52	**R54**	7.39	119.42
V17	8.86	117.6	**T55**	8.75	108.88
E18	8.57	119.33	**L56**	8.07	118.09
P19*	–	–	**S57**	8.41	113.59
S20	6.95	103.48	**D58**	7.86	124.6
D21	7.97	123.96	**Y59**	7.18	115.82
T22	7.81	109.08	**N60**	8.07	116.07
I23	8.45	121.3	**I61**	7.17	118.96
E24*	–	–	**Q62**	7.55	125.01
N25	7.85	121.43	**K63**	8.42	120.65
V26	8.03	122.29	**E64**	9.24	114.62
K27	8.48	119.04	**S65**	7.59	115.02
A28	7.9	123.55	**T66**	8.66	117.47
K29	7.78	120.28	**L67**	9.34	127.72
I30	8.21	121.41	**H68**	9.15	119.17
Q31	8.48	123.68	**L69**	8.22	124.11
D32	7.94	119.8	**V70**	9.11	126.8
K33	7.35	115.52	**L71**	8.04	123.16
E34	8.65	114.35	**R72**	8.52	123.83
G35	8.43	108.95	**L73**	8.28	124.62
I36	6.07	120.37	**R74**	8.37	122.05
P37*	–	–	**G75**	8.42	111.17
P38*	–	–	**G76**	7.87	115.13

Note: *The signal of this residue does not exist or has not been observed by ^{1}H-^{15}N HSQC

5.2. Titration study of six salts at pH = 2.8.

Titration experiments were carried out to determine which residues were more sensitive to the anion, i.e., to provide information about anion binding. All ubiquitin and salt solutions were prepared with the method discussed above at pH = 2.8. For each titration data set the following is shown: 1) A stack of (eight to ten) HSQC spectra, which showed the raw data from the titration of each salt; 2) A table of the chemical shift data (^{1}H and ^{15}N) highlighting signal positions at both the beginning (0 equiv.) and the end of the titration (300 – 800 equiv. depending on the anion), and the adjusted chemical perturbation value Δδ); 3) the bar graph from Δδ value of each titration, and; 4) A representation of the ubiquitin surface showing (on the structure of PDB ID: 1UBQ[7]) the location of anion binding. A color scale of white to dark red corresponds to a shift of 0 to 0.5 ppm was applied for all representation because most of the chemical shift perturbation values falls in the range from 0.1 to 0.5 ppm.

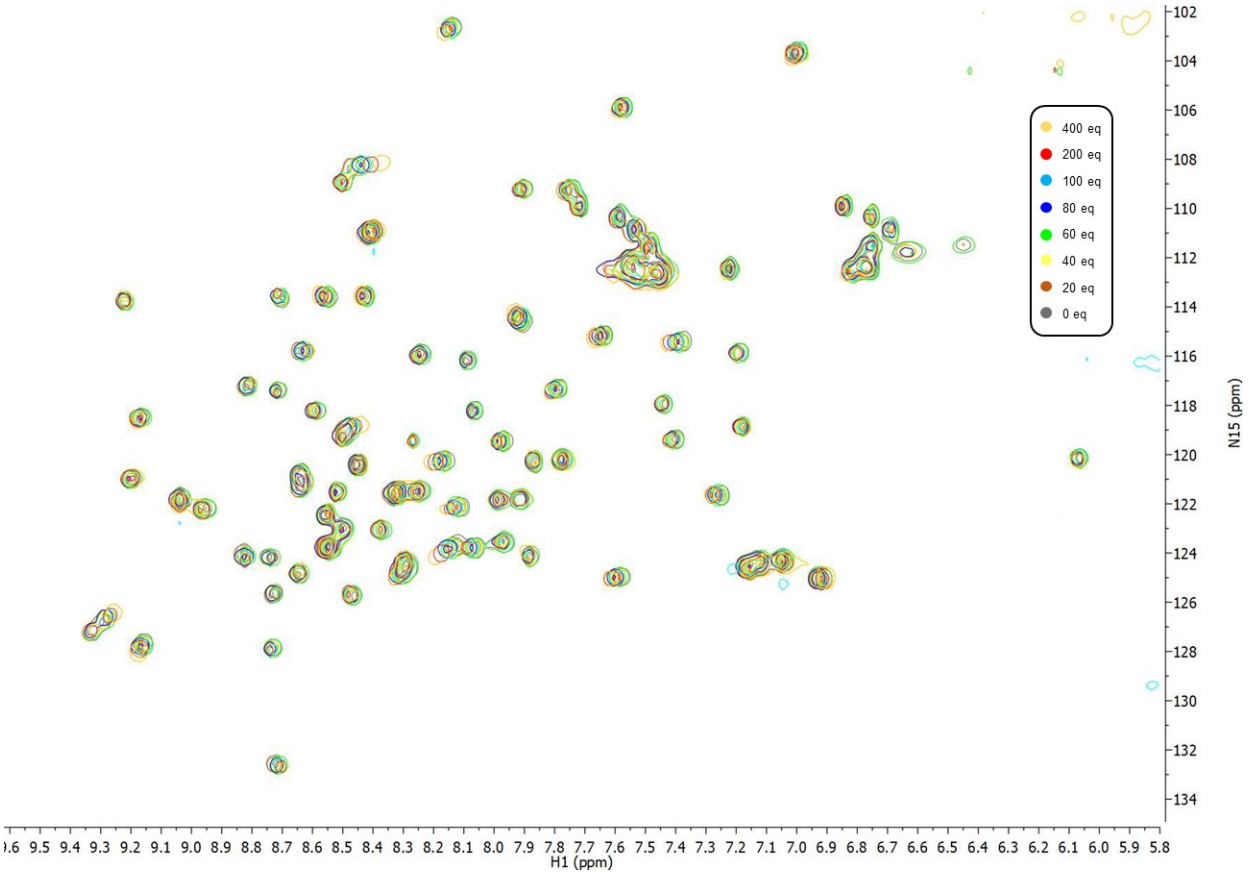


Figure S50 Stack of ^{1}H -^{15}N HSQC titration spectra of 1 mM ubiquitin with Cl^- (from 0 to 400 equiv.), in 50 mM phosphate buffer 90% H_2O/10% D_2O, pH = 2.8.

Table S3: ^{1}H -^{15}N HSQC chemical shift data from the titration of 1 mM ubiquitin with 2 M NaCl, pH = 2.8

Amino Acid #.	0 equiv.		400 equiv.		Δδ
	^{1}H	15N	^{1}H	^{15}N	
M1					
Q2	8.83	123.99	8.82	124.40	0.181
I3	8.24	115.88	8.25	116.04	0.072
F4	8.58	118.22	8.60	118.25	0.021
V5	9.21	121.02	9.19	120.93	0.042
K6	8.73	127.84	8.75	127.98	0.063
T7	8.64	115.78	8.61	115.81	0.039
L8	9.04	121.73	9.04	121.94	0.095
T9	7.57	105.87	7.59	105.97	0.050
G10	7.74	109.24	7.77	109.25	0.025
K11	7.25	121.66	7.28	121.64	0.035
T12	8.64	120.79	8.63	121.02	0.100
I13	9.30	126.80	9.25	126.41	0.180
T14	8.64	120.79	8.63	121.02	0.100
L15	8.65	124.87	8.64	124.67	0.091
E16	8.11	122.13	8.16	122.23	0.066
V17	8.82	117.26	8.80	117.04	0.103
E18	8.49	119.16	8.44	118.78	0.176
P19					
S20	6.99	103.61	7.01	103.75	0.061
D21	7.97	123.57	7.97	123.48	0.038
T22	7.90	109.19	7.92	109.17	0.025
I23	8.52	121.51	8.52	121.48	0.012
E24					
N25	7.86	120.34	7.87	120.17	0.077
V26	7.99	121.84	7.98	121.82	0.016
K27	8.49	119.16	8.50	119.32	0.074
A28	8.06	123.79	8.10	123.80	0.039
K29	7.78	120.18	7.78	120.26	0.033
I30	8.25	121.47	8.26	121.52	0.027
Q31	8.55	123.79	8.55	123.75	0.018
D32	7.97	119.44	8.00	119.43	0.034
K33	7.37	115.39	7.43	115.49	0.072
E34	8.70	113.67	8.72	113.41	0.121
G35	8.50	108.91	8.50	108.97	0.029
I36	6.06	120.14	6.07	120.27	0.060
P37					
P38					
D39	8.55	113.60	8.58	113.52	0.046
Q40	7.79	117.29	7.81	117.43	0.068

Q41	7.44	117.95	7.45	117.91	0.024
R42	8.50	122.99	8.50	123.25	0.118
L43	8.74	124.16	8.72	124.23	0.038
I44	8.95	122.17	8.97	122.20	0.024
F45	8.74	125.69	8.72	125.66	0.022
A46	8.72	132.56	8.70	132.68	0.060
G47	8.14	102.60	8.16	102.86	0.116
K48	7.91	121.83	7.91	121.73	0.045
Q49	8.55	122.49	8.55	122.36	0.062
L50	8.46	125.74	8.48	125.65	0.046
E51	8.37	123.07	8.38	123.05	0.006
D52	8.16	120.25	8.21	120.32	0.057
G53					
R54	7.40	119.36	7.43	119.47	0.056
T55	8.47	108.38	8.37	108.15	0.146
L56	8.06	118.19	8.07	118.32	0.056
S57	8.42	113.58	8.44	113.54	0.024
D58	7.88	124.19	7.89	124.01	0.083
Y59	7.19	115.84	7.20	115.83	0.014
N60	8.09	116.19	8.09	116.08	0.051
I61	7.18	118.88	7.18	118.88	0.000
Q62	7.58	124.97	7.62	125.04	0.051
K63	8.45	120.42	8.44	120.42	0.011
E64	9.22	113.80	9.22	113.68	0.056
S65	7.63	115.14	7.67	115.24	0.057
T66	8.71	117.37	8.72	117.51	0.062
L67	9.33	127.25	9.32	127.10	0.070
H68	9.17	118.48	9.18	118.56	0.039
L69	8.29	124.56	8.30	124.32	0.108
V70	9.16	127.64	9.17	128.02	0.171
L71	8.12	123.70	8.19	124.18	0.225
R72	8.55	123.79	8.55	123.75	0.018
L73	8.29	124.56	8.33	125.01	0.203
R74	8.33	121.61	8.31	121.51	0.049
G75	8.42	111.03	8.40	110.86	0.080
G76	7.91	114.57	7.93	114.27	0.140

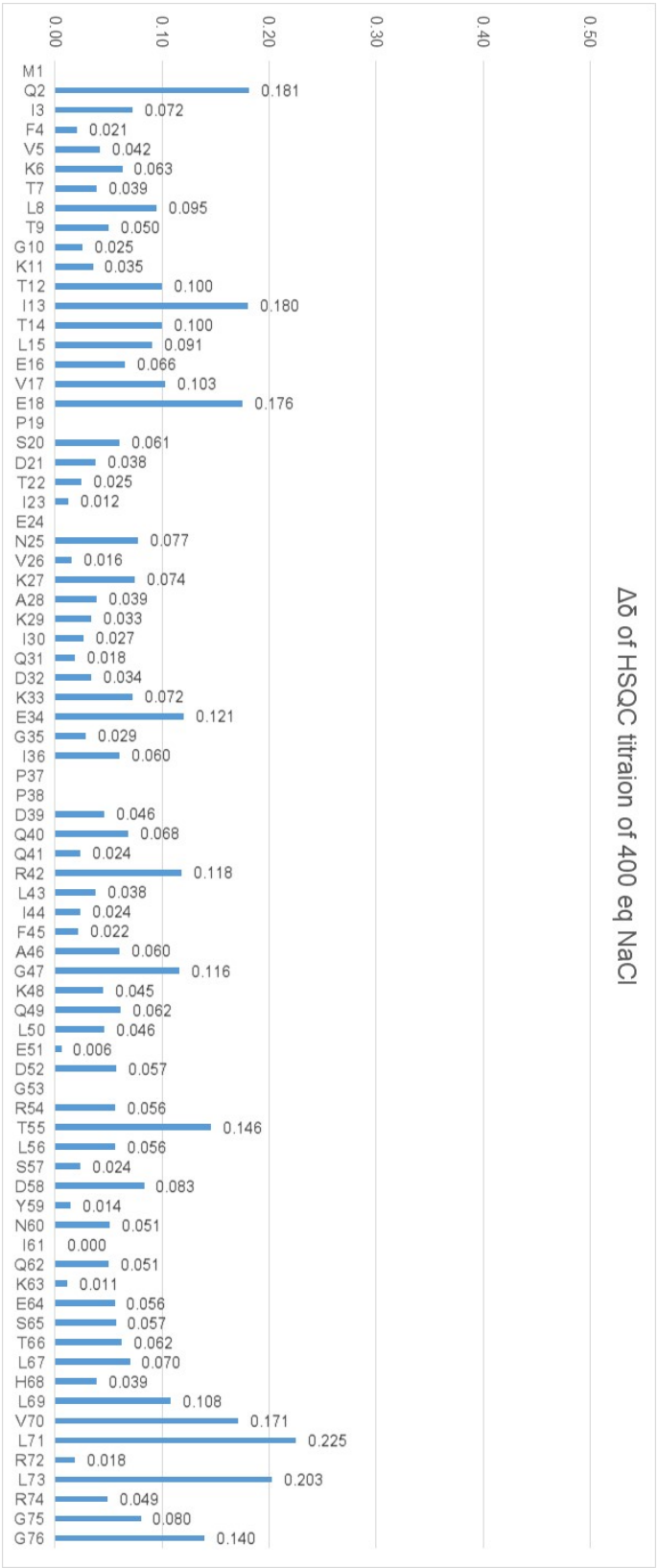


Figure S51 Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with Cl^{-} at 400 equiv. of salt, pH = 2.8.

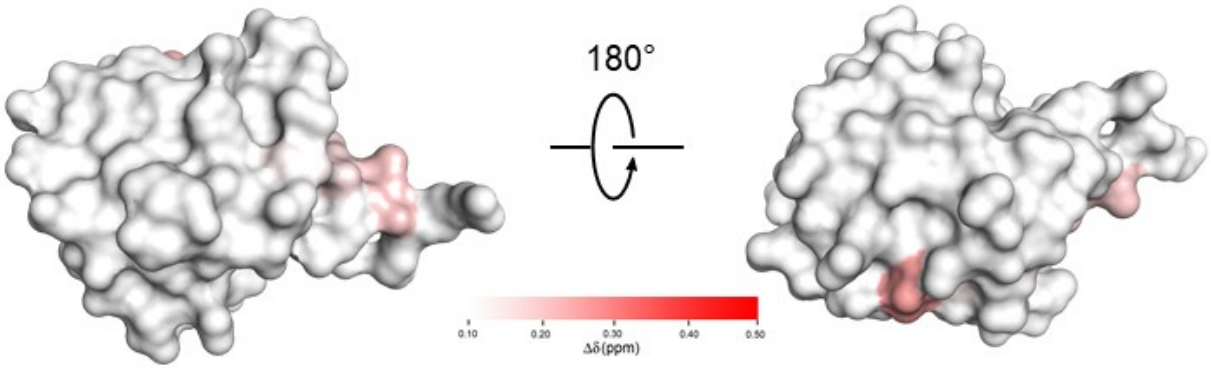


Figure S52 Visualization of the Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with Cl^{-} at 400 equiv. of salt, pH = 2.8.

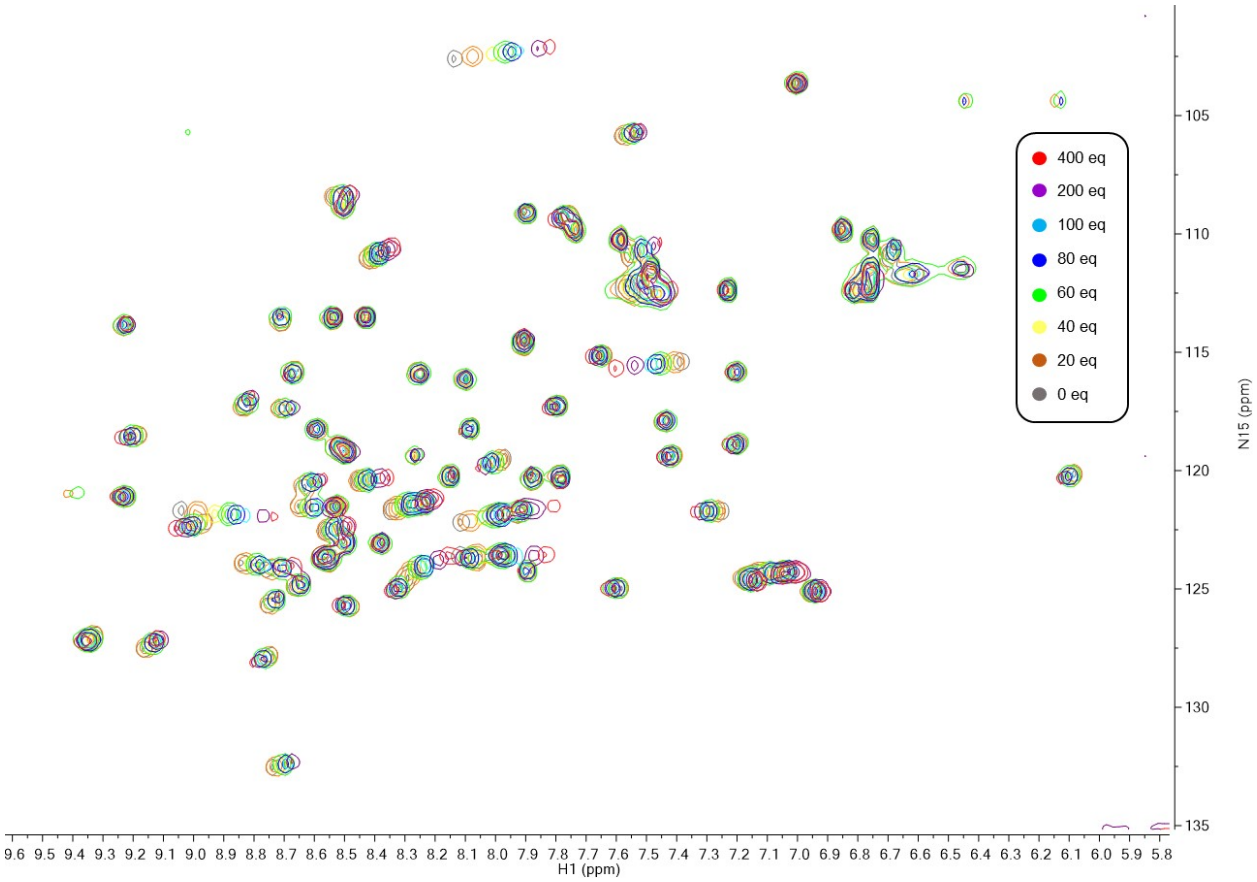


Figure S53 Stack of ^{1}H -^{15}N HSQC titration spectra of 1 mM ubiquitin with ClO_4^- (from 0 to 400 equiv.), in 50 mM phosphate buffer 90% H_2O/10% D_2O, pH = 2.8.

Table S4:^{1}H -^{15}N HSQC chemical shift data from the titration of 1 mM ubiquitin with 2 M $NaClO_4$, pH = 2.8

Amino Acid #.	0 equiv.		400 equiv.		Δδ
	^{1}H	^{15}N	^{1}H	^{15}N	
M1					
Q2	8.84	123.90	8.67	124.02	0.174
I3	8.25	115.82	8.25	115.98	0.072
F4	8.59	118.26	8.61	118.26	0.026
V5	9.22	121.09	9.25	121.12	0.035
K6	8.74	127.81	8.80	128.12	0.155
T7	8.66	115.77	8.70	116.04	0.127
L8	9.04	121.69	8.74	121.95	0.323
T9	7.58	105.88	7.53	105.68	0.101
G10	7.75	109.23	7.80	109.40	0.094
K11	7.25	121.72	7.34	121.76	0.089
T12	8.63	120.74	8.57	120.36	0.179
I13	9.33	127.02	9.39	127.19	0.095
T14	8.65	121.50	8.53	121.56	0.126
L15	8.66	124.94	8.65	124.65	0.129
E16	8.11	122.21	7.81	121.51	0.438
V17	8.83	117.33	8.81	116.80	0.238
E18	8.53	119.06	8.52	118.95	0.050
P19					
S20	7.00	103.63	7.01	103.64	0.010
D21	7.98	123.63	8.00	123.54	0.048
T22	7.89	109.17	7.91	109.03	0.064
I23	8.52	121.51	8.53	121.56	0.024
E24					
N25	7.88	120.45	7.89	120.14	0.139
V26	8.01	121.88	7.97	121.84	0.043
K27	8.50	119.23	8.50	119.23	0.005
A28	8.06	123.76	8.15	123.63	0.117
K29	7.88	120.20	7.79	120.48	0.154
I30	8.25	121.44	8.20	121.15	0.138
Q31	8.53	123.77	8.57	123.50	0.129
D32	7.97	119.50	8.06	119.90	0.199
K33	7.39	115.40	7.60	115.70	0.255
E34	8.71	113.79	8.73	113.37	0.192
G35	8.51	108.92	8.52	108.86	0.026
I36	6.08	120.15	6.13	120.26	0.070
P37					
P38					
D39	8.54	113.62	8.54	113.48	0.061

Q40	7.79	117.23	7.82	117.34	0.063
Q41	7.44	117.96	7.45	117.87	0.040
R42	8.50	122.97	8.51	123.16	0.087
L43	8.76	124.19	8.67	124.02	0.115
I44	8.96	122.18	9.06	122.45	0.157
F45	8.75	125.71	8.72	125.27	0.200
A46	8.74	132.55	8.67	132.29	0.137
G47	8.14	102.61	7.82	102.06	0.398
K48	7.93	121.88	7.81	121.50	0.206
Q49	8.56	122.55	8.48	122.36	0.114
L50	8.48	125.76	8.53	125.70	0.059
E51	8.38	123.11	8.39	123.06	0.026
D52	8.16	120.27	8.14	120.10	0.076
G53					
R54	7.41	119.37	7.45	119.39	0.045
T55	8.54	108.42	8.48	108.30	0.075
L56	8.08	118.20	8.11	118.34	0.077
S57	8.42	113.58	8.44	113.51	0.036
D58	7.89	124.27	7.92	124.23	0.040
Y59	7.20	115.83	7.22	115.87	0.032
N60	8.10	116.19	8.12	116.08	0.052
I61	7.19	118.88	7.23	118.90	0.034
Q62	7.58	124.99	7.63	125.00	0.046
K63	8.46	120.46	8.36	120.30	0.120
E64	9.23	113.91	9.23	113.79	0.054
S65	7.64	115.12	7.68	115.18	0.050
T66	8.72	117.37	8.67	117.42	0.052
L67	9.35	127.20	9.35	127.23	0.014
H68	9.18	118.50	9.24	118.60	0.075
L69	8.29	124.49	8.15	123.63	0.404
V70	9.16	127.55	9.12	127.10	0.205
L71	8.12	123.62	7.84	123.57	0.280
R72	8.56	123.77	8.57	123.60	0.076
L73	8.31	124.68	8.35	125.07	0.181
R74	8.34	121.65	8.23	121.23	0.220
G75	8.42	111.05	8.35	110.56	0.232
G76	7.90	114.70	7.91	114.40	0.135

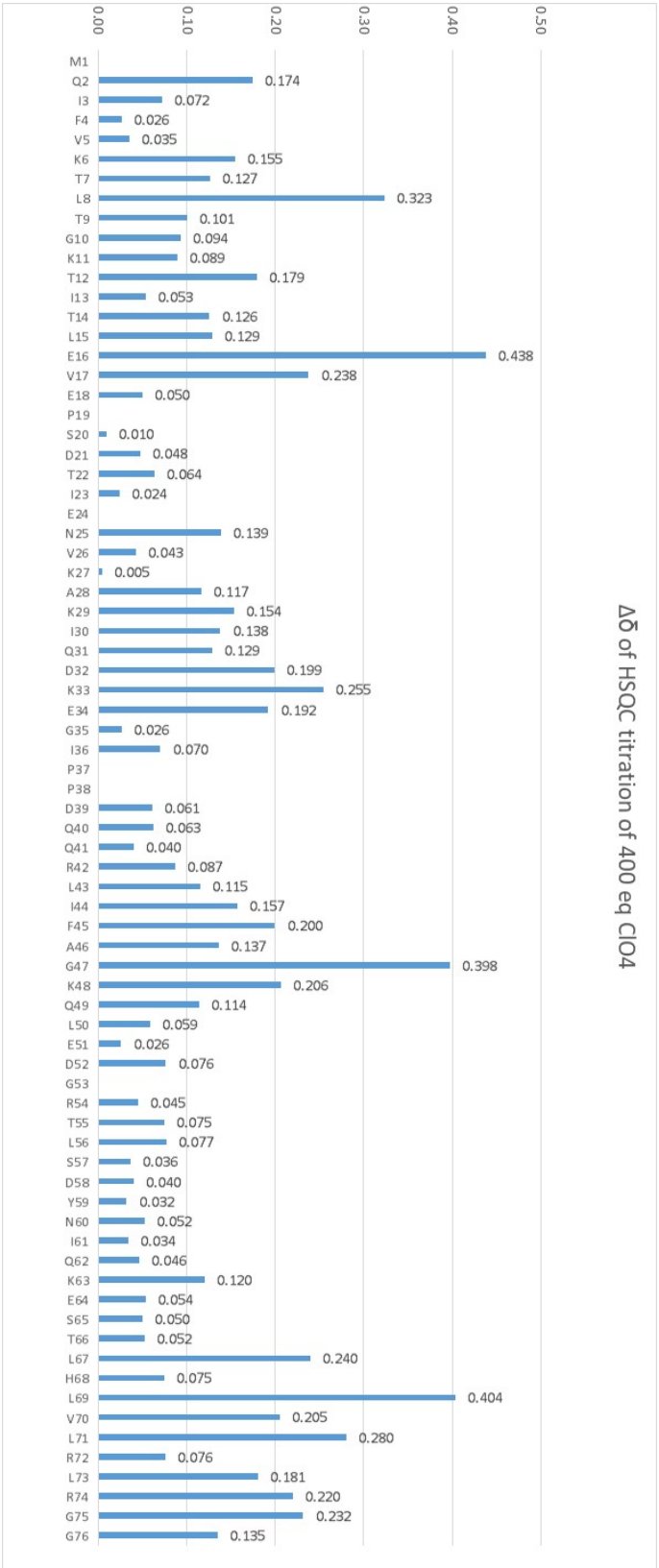


Figure S54 Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ClO_4^- at 400 equiv. of salt, pH = 2.8.

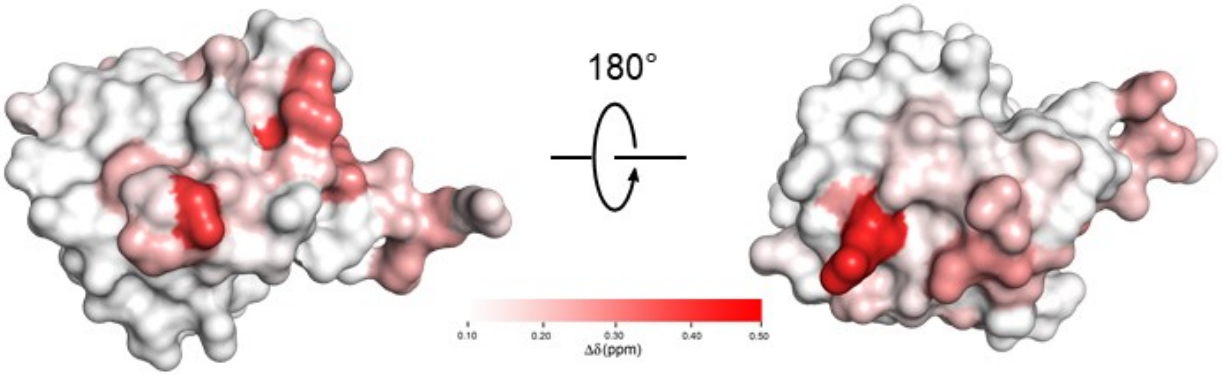


Figure S55 Visualization of the Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ClO_4^- at 400 equiv. of salt, pH = 2.8.

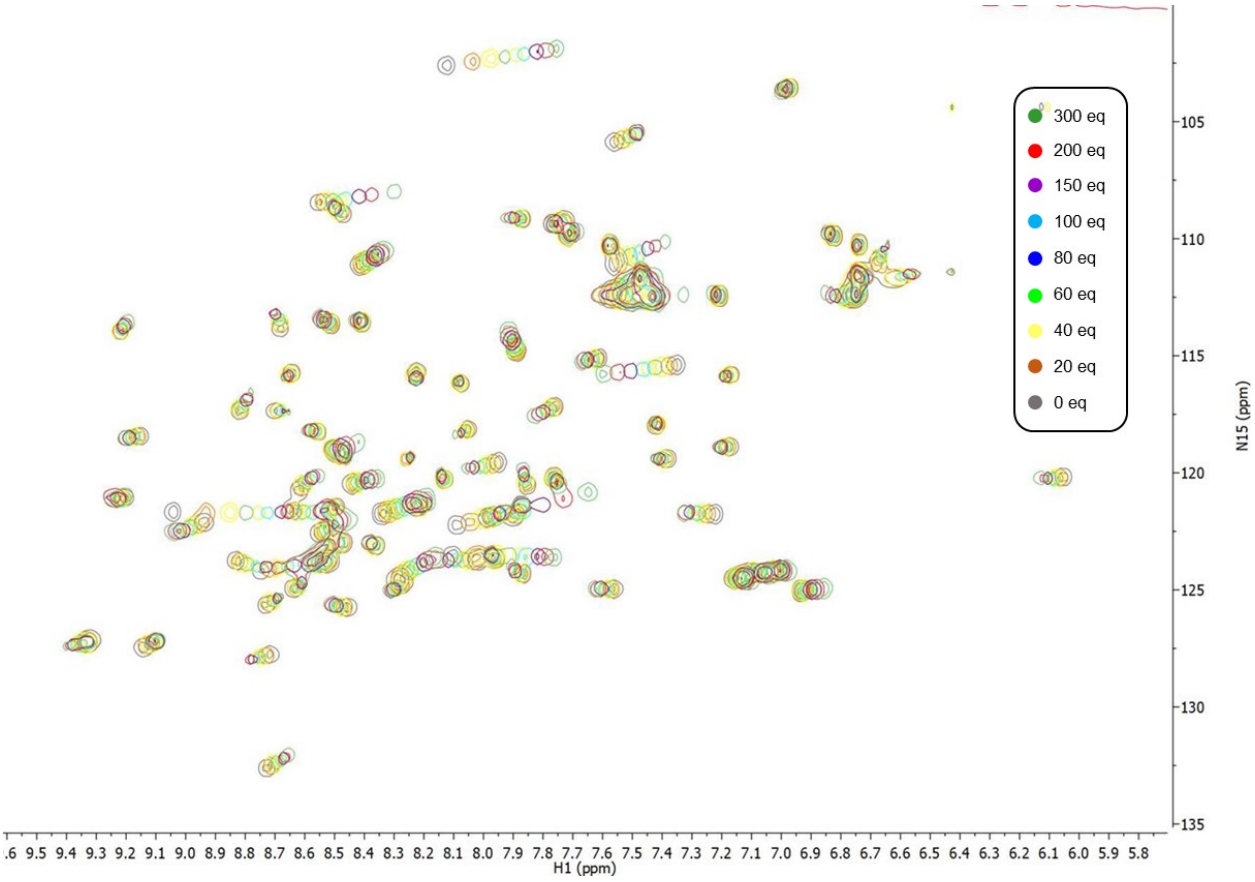


Figure S56 The ^{1}H -^{15}N HSQC titration spectra of 1 mM ubiquitin with PF_6^- (from 0 to 300 equiv.), in 50 mM phosphate buffer 90% H_2O/10% D_2O, pH = 2.8.

Table S5: ^{1}H -^{15}N HSQC chemical shift data from the titration of 1 mM ubiquitin with 2 M $NaPF_6$, pH 2.8

Amino Acid #.	0 equiv.		300 equiv.		Δδ
	^{1}H	^{15}N	^{1}H	^{15}N	
M1					
Q2	8.83	123.75	8.64	124.05	0.234
I3	8.23	115.69	8.23	116.05	0.161
F4	8.56	118.24	8.59	118.18	0.042
V5	9.21	121.07	9.24	121.16	0.049
K6	8.72	127.75	8.79	128.02	0.144
T7	8.65	115.74	8.67	115.96	0.100
L8	9.04	121.68	8.63	121.68	0.409
T9	7.56	105.87	7.48	105.42	0.217
G10	7.73	109.24	7.77	109.39	0.082
K11	7.22	121.76	7.33	121.73	0.104
T12	8.62	120.75	8.56	120.13	0.285
I13	9.33	127.25	9.33	127.18	0.032
T14	8.65	121.57	8.50	121.67	0.155
L15	8.64	124.93	8.61	124.52	0.184
E16	8.09	122.23	7.65	120.83	0.769
V17	8.82	117.35	8.78	116.56	0.353
E18	8.51	118.99	8.42	118.68	0.161
P19					
S20	6.97	103.57	6.99	103.68	0.054
D21	7.96	123.68	8.16	123.63	0.202
T22	7.87	109.15	7.93	109.11	0.064
I23	8.50	121.46	8.50	121.67	0.093
E24					
N25	7.86	120.54	7.87	119.88	0.294
V26	7.99	121.91	7.92	121.60	0.154
K27	8.48	119.16	8.46	119.29	0.061
A28	8.01	123.71	7.97	123.44	0.129
K29	7.76	120.20	7.75	120.50	0.136
I30	8.34	121.74	8.20	121.14	0.305
Q31	8.53	123.78	8.57	123.55	0.113
D32	7.95	119.55	8.06	119.81	0.158
K33	7.35	115.39	7.60	115.77	0.300
E34	8.68	113.84	8.71	113.11	0.324
G35	8.47	108.93	8.50	108.66	0.127
I36	6.05	120.19	6.13	120.20	0.075
P37					
P38					
D39	8.51	113.64	8.55	113.38	0.122
Q40	7.76	117.17	7.83	117.56	0.187

Q41	7.42	117.98	7.43	117.84	0.065
R42	8.47	122.97	8.54	123.28	0.155
L43	8.75	124.19	8.59	123.86	0.216
I44	8.94	122.11	9.04	122.52	0.208
F45	8.73	125.65	8.69	125.29	0.166
A46	8.73	132.61	8.66	132.03	0.267
G47	8.12	102.60	7.75	101.87	0.490
K48	7.90	121.87	7.87	121.31	0.250
Q49	8.55	122.57	8.47	122.01	0.260
L50	8.46	125.75	8.51	125.61	0.080
E51	8.36	123.11	8.38	122.93	0.084
D52	8.13	120.27	8.14	120.01	0.116
G53					
R54	7.39	119.40	7.42	119.33	0.046
T55	8.55	108.44	8.30	108.00	0.321
L56	8.05	118.14	8.09	118.37	0.109
S57	8.41	113.58	8.42	113.51	0.034
D58	7.86	124.32	7.91	124.00	0.150
Y59	7.17	115.81	7.20	115.87	0.042
N60	8.08	116.17	8.09	115.98	0.087
I61	7.17	118.89	7.21	118.88	0.032
Q62	7.56	124.97	7.62	124.96	0.061
K63	8.44	120.47	8.36	120.24	0.131
E64	9.22	113.96	9.19	113.58	0.170
S65	7.61	115.11	7.67	115.24	0.085
T66	8.70	117.35	8.66	117.40	0.048
L67	9.33	127.25	9.39	127.41	0.099
H68	9.15	118.43	9.20	118.52	0.061
L69	8.29	124.58	8.16	123.63	0.446
V70	9.14	127.46	9.10	127.20	0.128
L71	8.10	123.55	7.77	123.61	0.337
R72	8.53	123.78	8.57	123.55	0.113
L73	8.29	124.58	8.31	125.03	0.200
R74	8.22	121.45	8.20	121.14	0.143
G75	8.41	111.10	8.34	110.56	0.255
G76	7.89	114.78	7.92	113.94	0.375

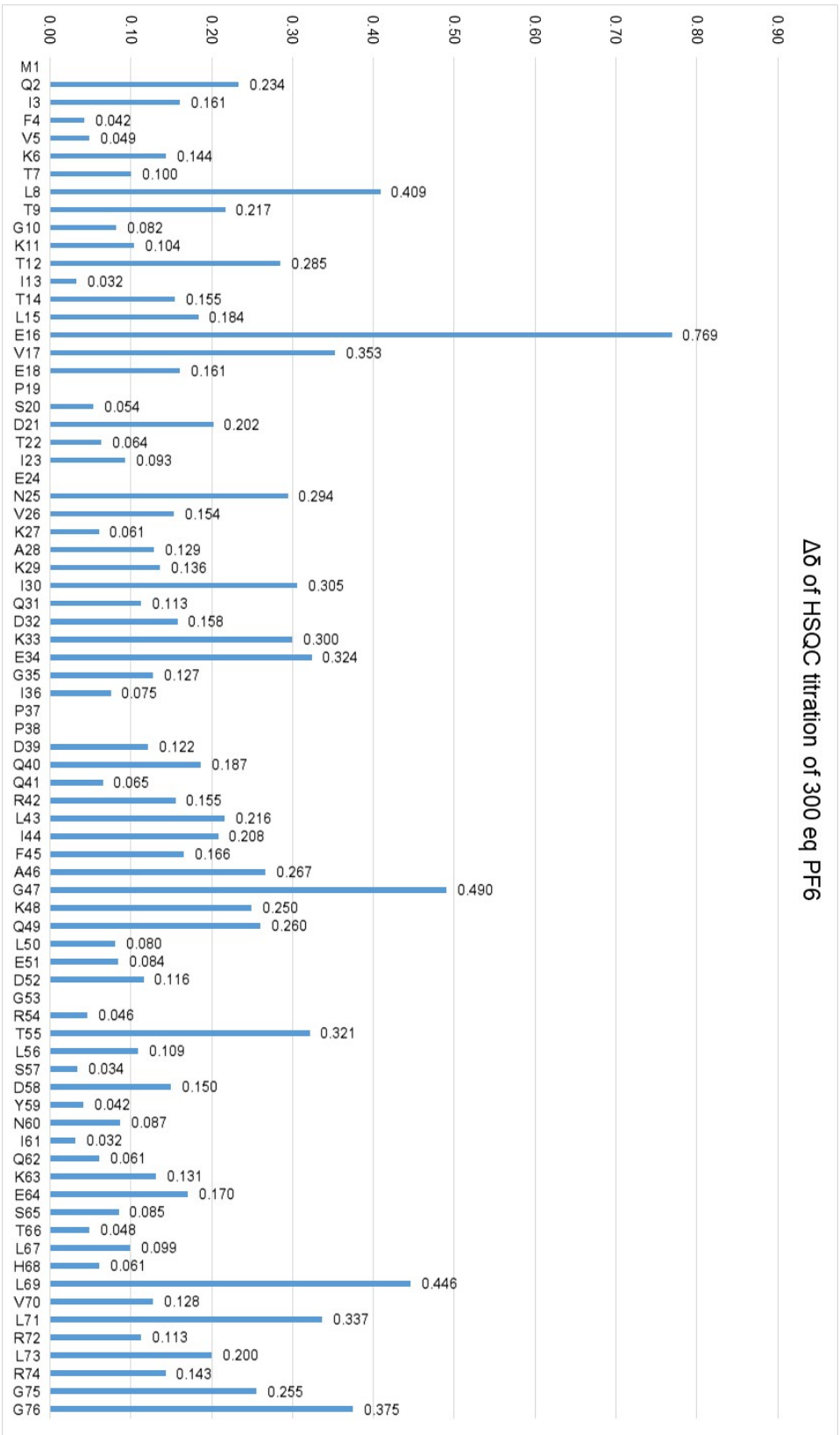


Figure S57 The Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with PF_6^- at 300 equiv. of salt, pH = 2.8.

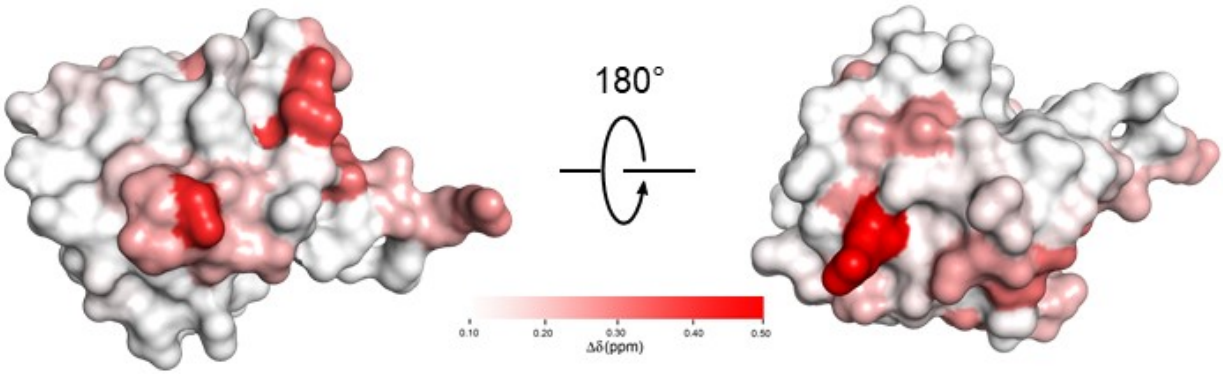


Figure S58 Visualization of the Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with PF_6^- at 300 equiv. of salt, pH = 2.8.

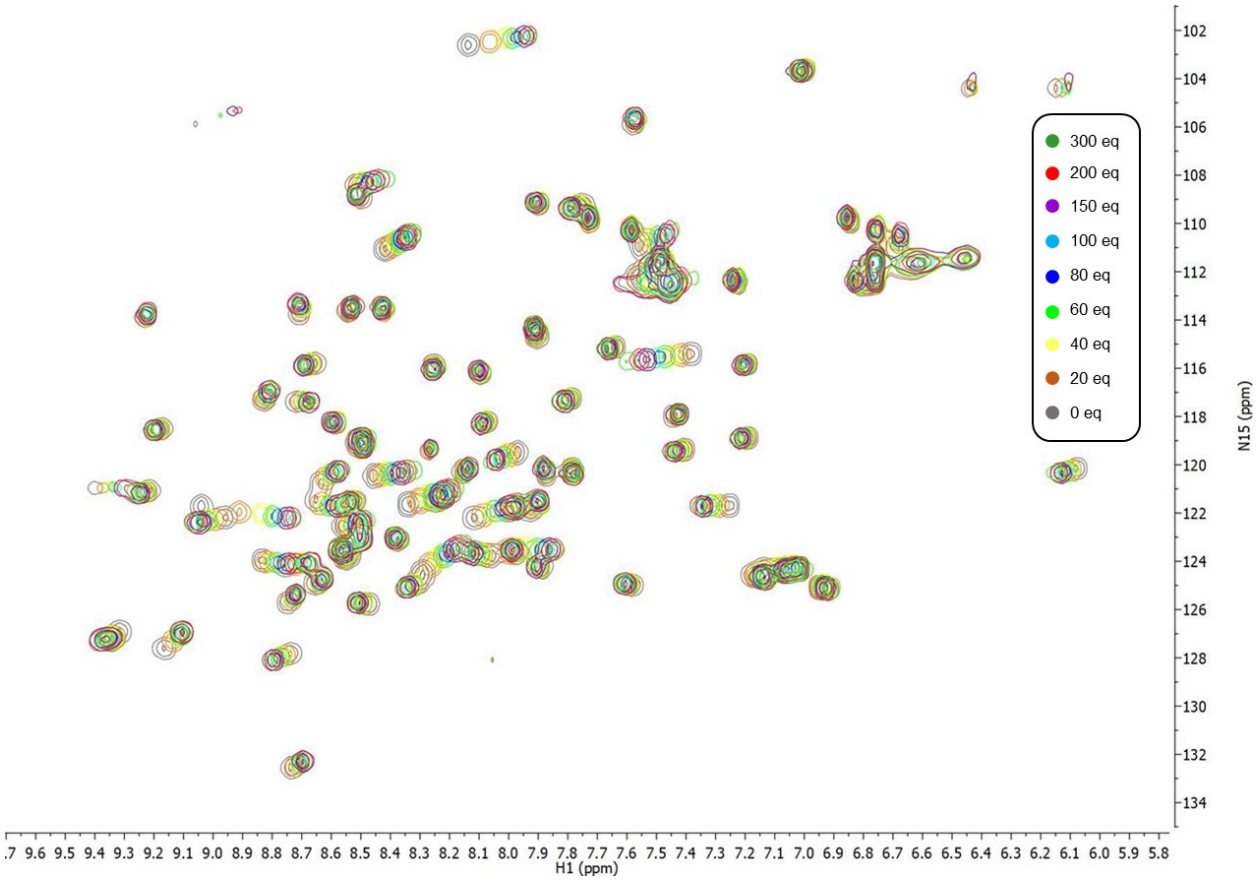


Figure S59 Stack of ^{1}H -^{15}N HSQC titration spectra of 1 mM ubiquitin with ReO_4^- (from 0 to 300 equiv.), in 50 mM phosphate buffer 90% H_2O/10% D_2O, pH = 2.8.

Table S6: ^{1}H -^{15}N HSQC chemical shift data from the titration of 1 mM ubiquitin with 2 M $NaReO_4$, pH 2.8

Amino Acid No.	0 equiv.		300 equiv.		Δδ
	1H	15 N	1H	15N	
M1					
Q2	8.83	123.94	8.70	124.09	0.152
I3	8.25	115.86	8.25	116.13	0.121
F4	8.59	118.25	8.60	118.15	0.048
V5	9.22	121.06	9.26	121.15	0.057
K6	8.74	127.82	8.81	128.11	0.147
T7	8.66	115.77	8.69	115.90	0.070
L8	9.04	121.71	8.72	122.24	0.394
T9	7.58	105.87	7.58	105.62	0.111
G10	7.75	109.21	7.80	109.40	0.094
K11	7.25	121.69	7.35	121.71	0.097
T12	8.63	120.76	8.56	120.24	0.242
I13	9.32	126.92	9.37	127.34	0.196
T14	8.65	121.43	8.55	121.64	0.138
L15	8.65	124.91	8.63	124.63	0.127
E16	8.12	122.19	7.91	121.43	0.401
V17	8.83	117.31	8.80	116.83	0.216
E18	8.52	118.99	8.48	118.91	0.054
P19					
S20	7.00	103.63	7.02	103.69	0.036
D21	7.97	123.63	8.00	123.50	0.061
T22	7.90	109.17	7.92	109.15	0.025
I23	8.52	121.52	8.55	121.64	0.057
E24					
N25	7.88	120.41	7.88	120.01	0.178
V26	8.00	121.86	7.97	121.84	0.035
K27	8.49	119.21	8.49	119.27	0.026
A28	8.06	123.76	8.15	123.60	0.120
K29	7.78	120.19	7.78	120.43	0.107
I30	8.25	121.45	8.22	121.28	0.082
Q31	8.55	123.79	8.57	123.47	0.145
D32	7.97	119.52	8.06	119.90	0.193
K33	7.39	115.40	7.59	115.74	0.257
E34	8.71	113.75	8.71	113.23	0.233
G35	8.50	108.90	8.51	108.82	0.040
I36	6.08	120.14	6.15	120.33	0.108
P37					
P38					
D39	8.54	113.61	8.52	113.42	0.089
Q40	7.79	117.25	7.83	117.42	0.084

Q41	7.44	117.97	7.43	117.87	0.046
R42	8.50	122.97	8.51	123.16	0.085
L43	8.76	124.18	8.67	124.02	0.109
I44	8.96	122.18	9.06	122.41	0.145
F45	8.75	125.71	8.71	125.29	0.193
A46	8.74	132.54	8.69	132.29	0.121
G47	8.14	102.59	7.92	102.24	0.268
K48	7.93	121.84	7.92	121.53	0.140
Q49	8.56	122.53	8.48	122.34	0.118
L50	8.47	125.76	8.52	125.74	0.049
E51	8.38	123.09	8.38	122.93	0.075
D52	8.16	120.26	8.13	120.06	0.093
G53					
R54	7.41	119.37	7.45	119.45	0.058
T55	8.51	108.42	8.41	108.18	0.144
L56	8.07	118.21	8.10	118.39	0.088
S57	8.42	113.58	8.44	113.46	0.054
D58	7.88	124.24	7.92	124.14	0.058
Y59	7.20	115.83	7.21	115.89	0.032
N60	8.10	116.20	8.11	116.05	0.066
I61	7.19	118.88	7.22	118.94	0.041
Q62	7.58	124.98	7.61	124.92	0.038
K63	8.46	120.45	8.32	120.26	0.159
E64	9.23	113.87	9.22	113.70	0.075
S65	7.64	115.12	7.67	115.19	0.043
T66	8.72	117.38	8.66	117.44	0.062
L67	9.34	127.17	9.36	127.19	0.019
H68	9.17	118.50	9.20	118.60	0.055
L69	8.29	124.50	8.15	123.63	0.411
V70	9.16	127.59	9.11	126.92	0.306
L71	8.12	123.60	7.84	123.51	0.281
R72	8.55	123.79	8.57	123.47	0.145
L73	8.30	124.68	8.35	125.12	0.208
R74	8.34	121.62	8.18	121.01	0.316
G75	8.42	111.04	8.33	110.43	0.291
G76	7.90	114.65	7.91	114.23	0.187

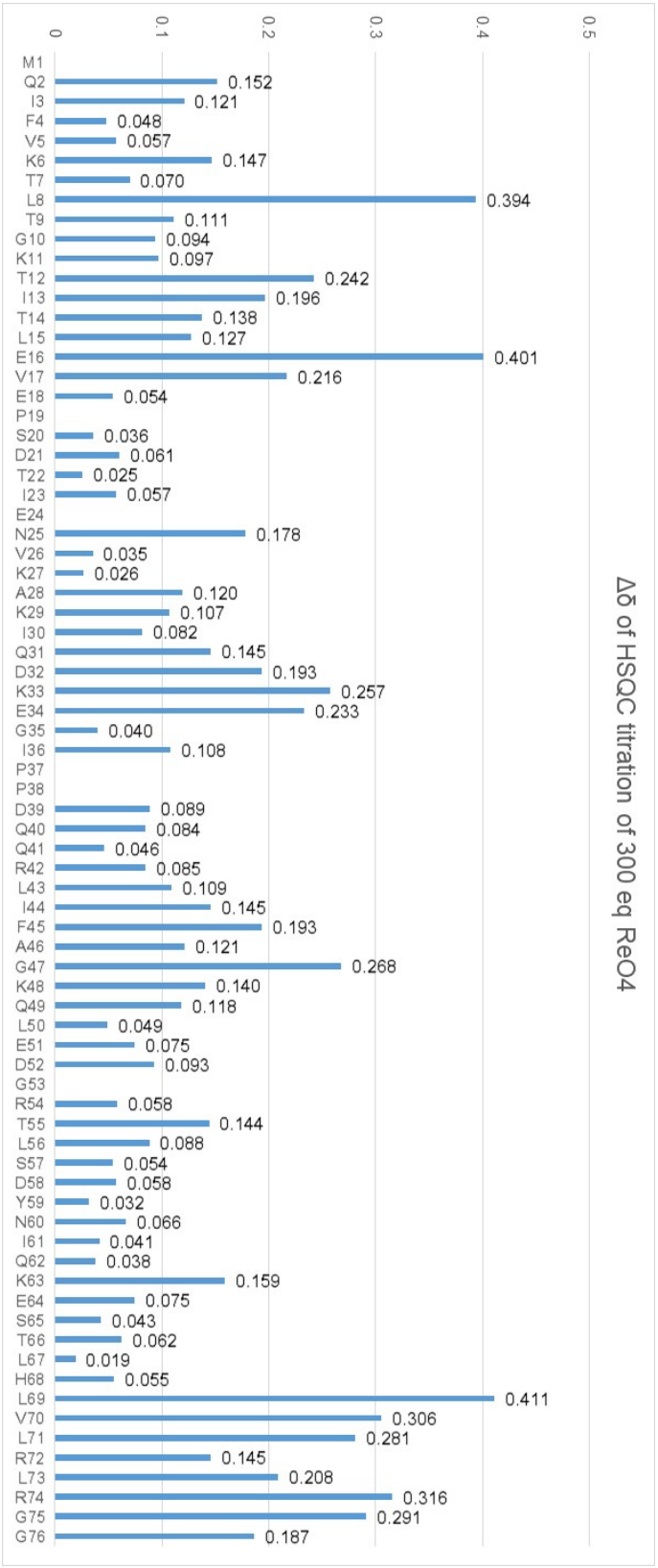


Figure S60 The Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ReO_4^- at 300 equiv. of salt, pH = 2.8.

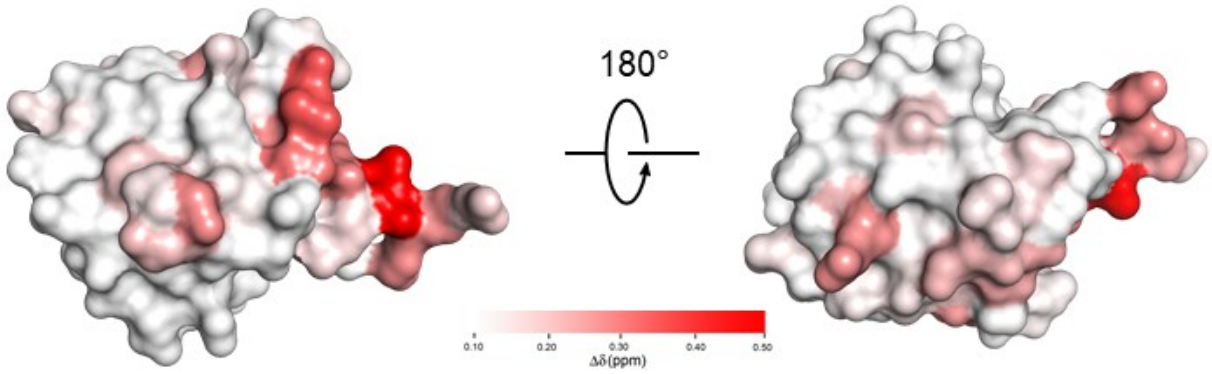


Figure S61 Visualization of the Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ReO_4^- at 300 equiv. of salt, pH = 2.8.

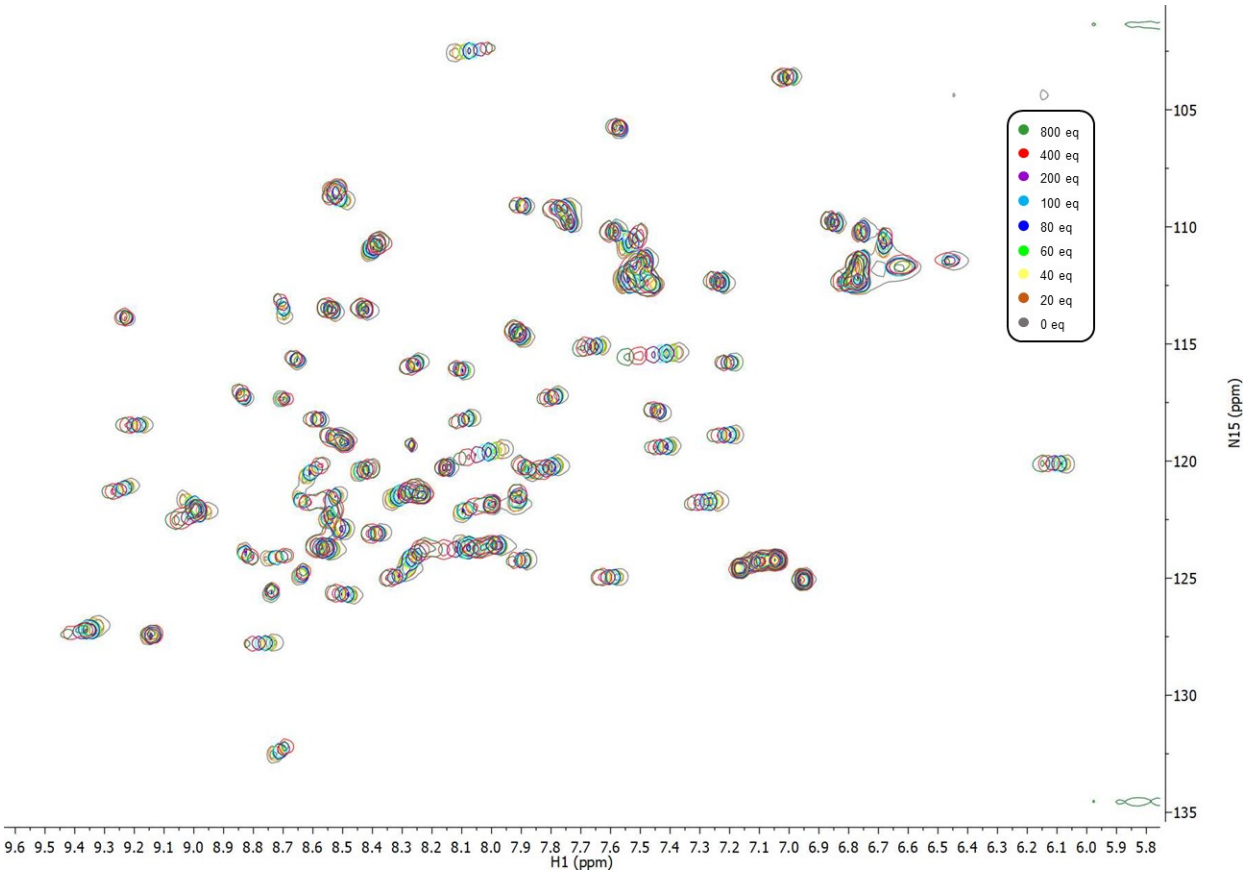


Figure S62 Stack of ^{1}H -^{15}N HSQC titration spectra of 1 mM ubiquitin with OTf^- (from 0 to 800 equiv.), in 50 mM phosphate buffer 90% H_2O/10% D_2O, pH 2.8, pH = 2.8.

Table S7:^{1}H -^{15}N HSQC chemical shift data from the titration of 1 mM ubiquitin with 2 M NaOTf, pH = 2.8

Amino Acid #.	0 equiv.		800 equiv.		Δδ
	^{1}H	^{15} N	^{1}H	^{15}N	
M1					
Q2	8.84	123.84	8.80	124.19	0.162
I3	8.25	115.80	8.28	116.02	0.104
F4	8.58	118.26	8.61	118.23	0.032
V5	9.22	121.09	9.28	121.35	0.131
K6	8.74	127.79	8.82	127.80	0.079
T7	8.66	115.76	8.67	115.60	0.073
L8	9.04	121.68	8.99	122.10	0.193
T9	7.58	105.87	7.58	105.75	0.054
G10	7.75	109.22	7.80	109.27	0.056
K11	7.24	121.74	7.33	121.83	0.094
T12	8.63	120.74	8.56	120.11	0.290
I13	9.34	127.15	9.37	127.14	0.030
T14	8.65	121.53	8.60	121.79	0.125
L15	8.65	124.95	8.63	124.65	0.133
E16	8.10	122.23	8.01	121.85	0.196
V17	8.83	117.34	8.85	117.03	0.138
E18	8.51	119.15	8.55	118.92	0.107
P19					
S20	6.99	103.61	7.03	103.66	0.042
D21	7.97	123.64	8.01	123.60	0.044
T22	7.89	109.16	7.92	109.08	0.052
I23	8.52	121.49	8.57	121.65	0.088
E24					
N25	7.87	120.47	7.90	120.12	0.162
V26	8.00	121.89	8.01	121.85	0.021
K27	8.51	119.15	8.50	119.21	0.030
A28	8.05	123.75	8.19	123.76	0.144
K29	7.78	120.21	7.84	120.47	0.132
I30	8.24	121.45	8.25	121.26	0.082
Q31	8.55	123.79	8.59	123.66	0.072
D32	7.97	119.53	8.10	119.96	0.234
K33	7.38	115.40	7.54	115.56	0.181
E34	8.70	113.82	8.72	113.08	0.333
G35	8.49	108.93	8.54	108.73	0.106
I36	6.08	120.17	6.15	120.12	0.075
P37					
P38					
D39	8.53	113.63	8.56	113.48	0.071
Q40	7.78	117.21	7.82	117.35	0.073

Q41	7.43	117.97	7.46	117.85	0.059
R42	8.50	122.96	8.54	122.98	0.041
L43	8.76	124.19	8.69	124.09	0.085
I44	8.96	122.16	9.06	122.57	0.210
F45	8.75	125.70	8.74	125.48	0.098
A46	8.74	132.55	8.69	132.20	0.166
G47	8.13	102.60	8.00	102.39	0.161
K48	7.92	121.86	7.91	121.28	0.263
Q49	8.56	122.56	8.52	122.15	0.187
L50	8.47	125.76	8.53	125.65	0.076
E51	8.37	123.10	8.41	123.09	0.039
D52	8.14	120.28	8.16	120.30	0.021
G53					
R54	7.40	119.39	7.46	119.44	0.060
T55	8.55	108.48	8.51	108.33	0.076
L56	8.07	118.18	8.12	118.35	0.091
S57	8.42	113.58	8.44	113.48	0.050
D58	7.88	124.30	7.93	124.31	0.046
Y59	7.19	115.81	7.22	115.81	0.032
N60	8.09	116.18	8.12	116.04	0.069
I61	7.19	118.89	7.25	118.93	0.058
Q62	7.58	124.98	7.63	124.99	0.056
K63	8.45	120.47	8.40	120.34	0.079
E64	9.23	113.94	9.23	113.87	0.032
S65	7.63	115.11	7.69	115.19	0.073
T66	8.71	117.37	8.69	117.41	0.030
L67	9.34	127.15	9.42	127.44	0.156
H68	9.17	118.48	9.23	118.48	0.059
L69	8.29	124.58	8.22	123.71	0.392
V70	9.16	127.51	9.14	127.41	0.050
L71	8.11	123.59	8.02	123.77	0.118
R72	8.55	123.79	8.59	123.66	0.072
L73	8.29	124.58	8.35	125.05	0.219
R74	8.34	121.68	8.25	121.26	0.207
G75	8.42	111.07	8.37	110.64	0.194
G76	7.90	114.73	7.92	114.41	0.145

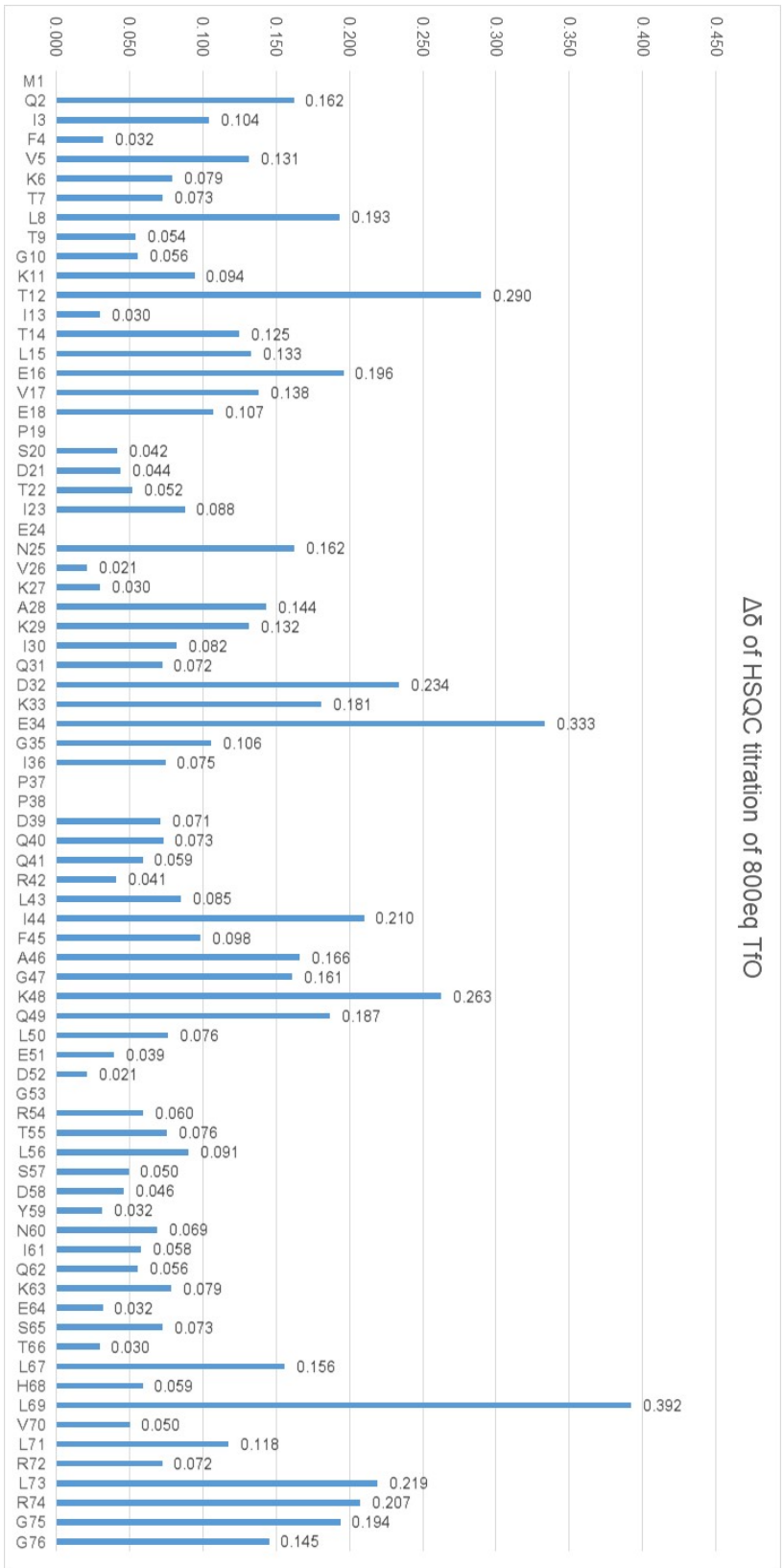


Figure S63 The Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with TfO^{-} at 800 equiv. of salt, pH = 2.8.

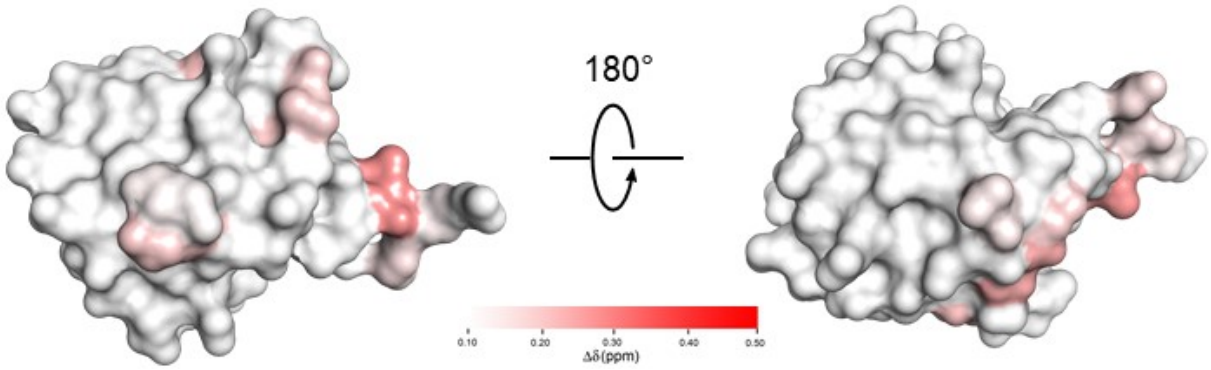


Figure S64 Visualization of the Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with TfO^{-} at 800 equiv. of salt , pH = 2.8.

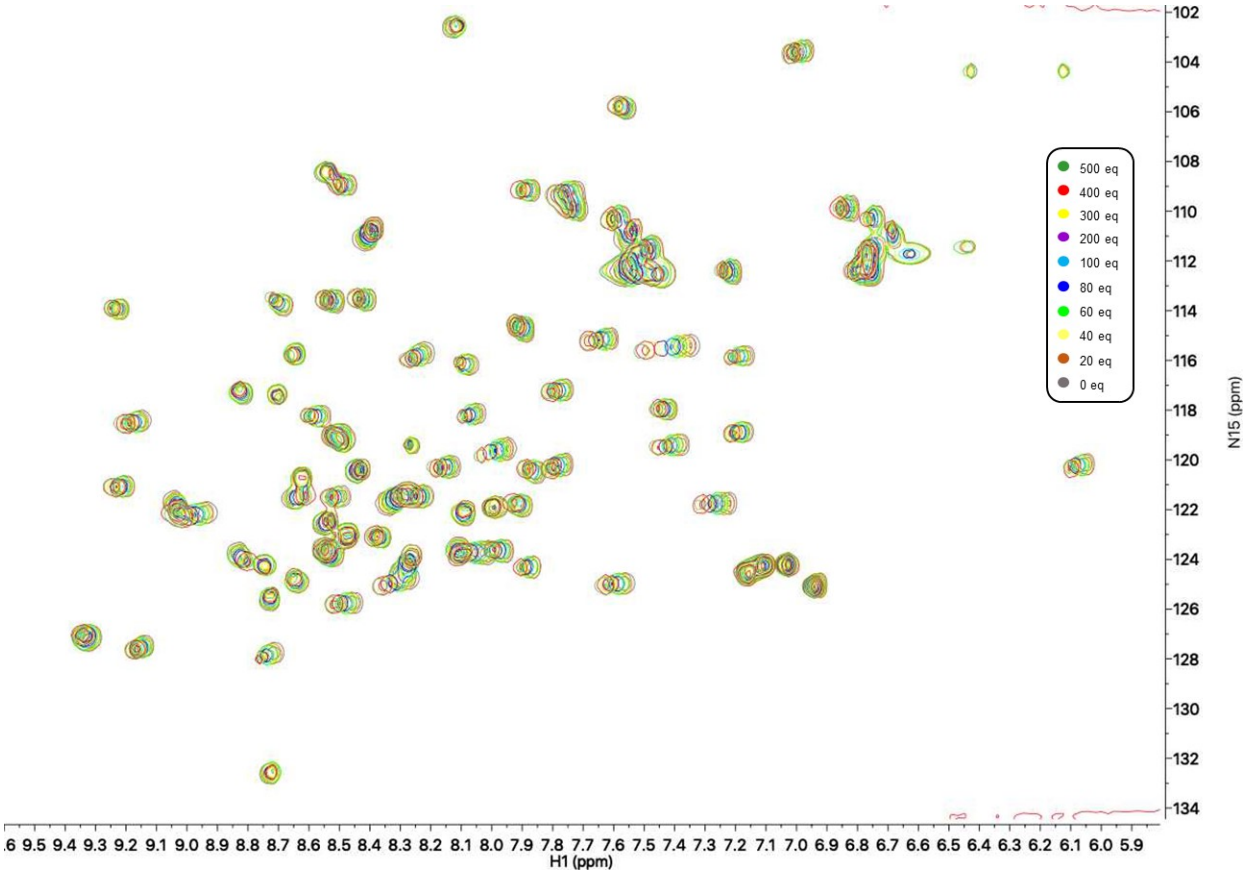


Figure S65 Stack of ^{1}H -^{15}N HSQC titration spectra of 1 mM ubiquitin with NO_3^- (from 0 to 500 equiv.), in 50 mM phosphate buffer 90% H_2O/10% D_2O, pH = 2.8.

Table S8:^{1}H -^{15}N HSQC chemical shift data from the titration of 1 mM ubiquitin with 2 M $NaNO_3$, pH 2.8

Amino Acid #.	0 equiv.		500 equiv.		Δδ
	^{1}H	^{15}N	^{1}H	^{15}N	
M1					
Q2	8.83	123.76	8.80	124.10	0.151
I3	8.22	115.69	8.27	115.97	0.133
F4	8.56	118.23	8.61	118.21	0.053
V5	9.21	121.05	9.24	121.06	0.040
K6	8.72	127.75	8.78	128.01	0.129
T7	8.64	115.74	8.67	115.79	0.032
L8	9.04	121.68	9.03	122.19	0.228
T9	7.56	105.87	7.59	105.77	0.053
G10	7.73	109.24	7.79	109.35	0.076
K11	7.22	121.75	7.32	121.81	0.100
T12	8.62	120.73	8.63	120.72	0.004
I13	9.32	127.16	9.35	127.06	0.053
T14	8.65	121.55	8.61	121.46	0.058
L15	8.64	124.92	8.65	124.73	0.088
E16	8.09	122.21	8.09	121.93	0.127
V17	8.82	117.34	8.83	117.10	0.105
E18	8.49	119.14	8.52	119.08	0.044
P19					
S20	6.97	103.55	7.02	103.66	0.071
D21	7.96	123.66	8.01	123.60	0.056
T22	7.87	109.16	7.91	109.12	0.041
I23	8.50	121.47	8.54	121.46	0.043
E24					
N25	7.86	120.52	7.89	120.32	0.093
V26	7.99	121.90	8.00	121.96	0.029
K27	8.49	119.14	8.50	119.25	0.054
A28	8.01	123.72	8.10	123.81	0.096
K29	7.76	120.18	7.81	120.37	0.101
I30	8.22	121.45	8.27	121.37	0.060
Q31	8.53	123.78	8.55	123.61	0.078
D32	7.95	119.55	8.04	119.85	0.162
K33	7.35	115.39	7.51	115.63	0.192
E34	8.68	113.80	8.72	113.48	0.148
G35	8.47	108.95	8.51	108.98	0.037
I36	6.05	120.18	6.11	120.29	0.077
P37					
P38					
D39	8.51	113.63	8.55	113.57	0.047
Q40	7.76	117.18	7.81	117.24	0.059
Q41	7.42	117.97	7.46	117.97	0.039
R42	8.47	122.99	8.48	123.16	0.076
L43	8.75	124.20	8.76	124.40	0.093

I44	8.94	122.11	9.03	122.19	0.102
F45	8.73	125.64	8.73	125.41	0.103
A46	8.73	132.61	8.72	132.52	0.043
G47	8.13	102.60	8.12	102.48	0.054
K48	7.90	121.87	7.93	121.73	0.070
Q49	8.55	122.57	8.53	122.47	0.052
L50	8.46	125.76	8.53	125.81	0.072
E51	8.36	123.12	8.39	123.07	0.031
D52	8.14	120.27	8.19	120.32	0.056
G53					
R54	7.38	119.40	7.46	119.48	0.082
T55	8.55	108.43	8.54	108.54	0.050
L56	8.05	118.14	8.10	118.24	0.069
S57	8.41	113.57	8.44	113.50	0.046
D58	7.86	124.32	7.91	124.29	0.048
Y59	7.17	115.81	7.22	115.88	0.060
N60	8.08	116.18	8.11	115.97	0.099
I61	7.17	118.88	7.22	118.98	0.061
Q62	7.56	124.97	7.63	125.06	0.077
K63	8.44	120.45	8.42	120.36	0.047
E64	9.22	113.94	9.25	113.88	0.042
S65	7.61	115.12	7.69	115.26	0.101
T66	8.70	117.36	8.70	117.51	0.068
L67	9.32	127.16	9.35	127.06	0.053
H68	9.15	118.42	9.21	118.56	0.087
L69	8.29	124.58	8.26	123.80	0.350
V70	9.14	127.48	9.17	127.64	0.081
L71	8.11	123.57	8.10	123.81	0.108
R72	8.53	123.78	8.55	123.61	0.078
L73	8.29	124.58	8.37	125.08	0.238
R74	8.34	121.74	8.27	121.37	0.177
G75	8.42	111.11	8.39	110.66	0.202
G76	7.89	114.76	7.93	114.55	0.102

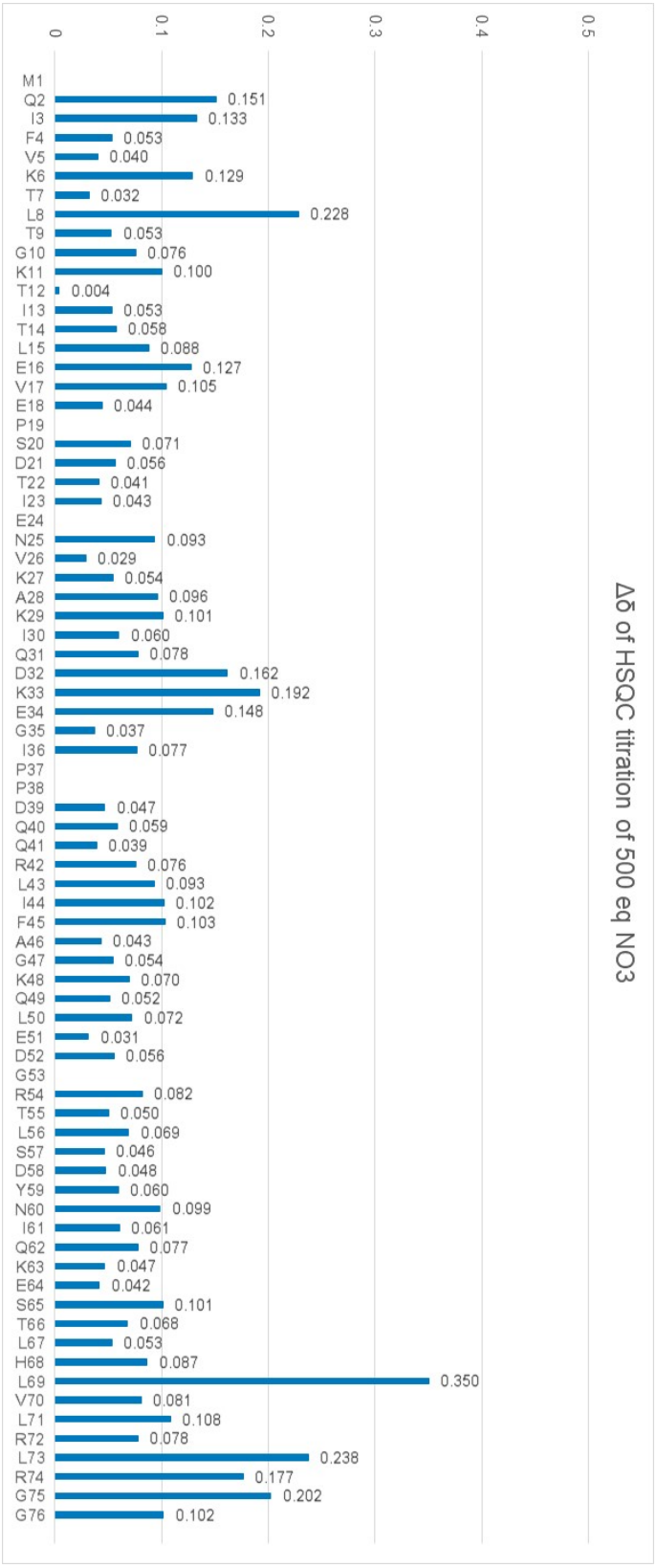


Figure S66 The Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with NO_3^- at 800 equiv. of salt, pH = 2.8.

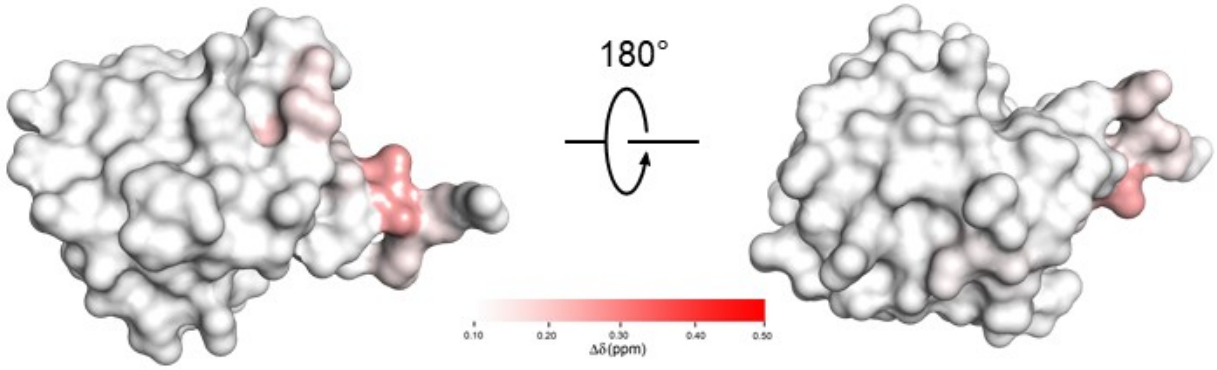


Figure S67 Visualization of the Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with NO_3^- at 800 equiv. of salt, pH = 2.8.

5.3. Binding sites.

To identify the binding sites we compared the chemical shifts (Δδ) in the presence of 200 equiv. of the six salts examined. A gamma distribution was applied for each anion titration results to provide a direct comparison. The result and analysis are as follows:

Table S9:^{1}H -^{15}N HSQC chemical shift from titration of 1 mM ubiquitin with different anions at 200 equiv., pH = 2.8

Amino Acid #.	Δδ of 200 equiv.					
	TfO^-	Cl^-	ClO_4^-	NO_3^-	ReO_4^-	PF_6^-
M1						
Q2	0.086	0.013	0.128	0.098	0.122	0.199
I3	0.062	0.066	0.064	0.099	0.091	0.128
F4	0.029	0.010	0.013	0.033	0.017	0.038
V5	0.092	0.018	0.013	0.028	0.059	0.028
K6	0.054	0.007	0.113	0.086	0.136	0.118
T7	0.044	0.011	0.097	0.017	0.053	0.062
L8	0.163	0.078	0.299	0.142	0.364	0.385
T9	0.032	0.025	0.111	0.044	0.117	0.213
G10	0.040	0.022	0.046	0.031	0.079	0.064
K11	0.064	0.033	0.071	0.062	0.094	0.106
T12	0.186	0.011	0.144	0.003	0.245	0.263
I13	0.066	0.118	0.108	0.042	0.162	0.024
T14	0.088	0.101	0.118	0.038	0.132	0.129
L15	0.072	0.010	0.088	0.056	0.114	0.134
E16	0.093	0.040	0.356	0.074	0.261	0.619
V17	0.064	0.007	0.168	0.060	0.194	0.252
E18	0.061	0.006	0.059	0.046	0.036	0.044
P19						
S20	0.028	0.049	0.011	0.043	0.021	0.034
D21	0.048	0.024	0.017	0.037	0.060	0.187
T22	0.029	0.031	0.061	0.018	0.039	0.048
I23	0.041	0.012	0.032	0.035	0.027	0.068
E24						
N25	0.091	0.059	0.117	0.065	0.156	0.247
V26	0.022	0.009	0.041	0.012	0.074	0.107
K27	0.043	0.010	0.015	0.050	0.071	0.067
A28	0.084	0.033	0.130	0.061	0.104	0.117
K29	0.066	0.025	0.135	0.055	0.092	0.119
I30	0.015	0.015	0.069	0.026	0.090	0.091
Q31	0.042	0.008	0.075	0.049	0.147	0.076
D32	0.128	0.030	0.154	0.088	0.140	0.136
K33	0.098	0.051	0.176	0.107	0.204	0.240
E34	0.220	0.103	0.192	0.097	0.217	0.309
G35	0.165	0.030	0.051	0.030	0.057	0.126
I36	0.056	0.033	0.073	0.046	0.091	0.067
P37						
P38						
D39	0.052	0.035	0.059	0.038	0.083	0.097
Q40	0.049	0.062	0.049	0.041	0.053	0.154

Q41	0.056	0.021	0.030	0.024	0.043	0.062
R42	0.039	0.011	0.060	0.033	0.050	0.095
L43	0.073	0.013	0.080	0.052	0.106	0.190
I44	0.088	0.046	0.125	0.091	0.123	0.193
F45	0.065	0.012	0.174	0.070	0.168	0.156
A46	0.116	0.064	0.133	0.038	0.133	0.217
G47	0.108	0.080	0.340	0.019	0.273	0.441
K48	0.175	0.034	0.113	0.061	0.189	0.240
Q49	0.093	0.012	0.076	0.037	0.084	0.213
L50	0.051	0.036	0.046	0.044	0.056	0.070
E51	0.029	0.03	0.024	0.018	0.055	0.057
D52	0.019	0.036	0.031	0.031	0.083	0.077
G53						
R54	0.038	0.027	0.042	0.057	0.046	0.033
T55	0.046	0.102	0.058	0.015	0.133	0.230
L56	0.043	0.027	0.064	0.048	0.049	0.084
S57	0.035	0.018	0.036	0.027	0.050	0.043
D58	0.030	0.055	0.030	0.029	0.039	0.114
Y59	0.026	0.032	0.030	0.040	0.020	0.042
N60	0.047	0.030	0.040	0.041	0.056	0.050
I61	0.036	0.013	0.026	0.024	0.027	0.030
Q62	0.040	0.036	0.011	0.044	0.039	0.048
K63	0.044	0.014	0.083	0.025	0.133	0.108
E64	0.030	0.012	0.046	0.024	0.086	0.129
S65	0.044	0.032	0.045	0.052	0.028	0.063
T66	0.011	0.011	0.041	0.025	0.049	0.038
L67	0.078	0.050	0.012	0.042	0.020	0.074
H68	0.041	0.012	0.048	0.055	0.037	0.054
L69	0.176	0.074	0.326	0.184	0.266	0.402
V70	0.025	0.117	0.189	0.059	0.311	0.128
L71	0.080	0.152	0.247	0.115	0.276	0.308
R72	0.042	0.018	0.065	0.049	0.147	0.086
L73	0.265	0.180	0.166	0.240	0.171	0.202
R74	0.142	0.046	0.194	0.132	0.270	0.091
G75	0.120	0.063	0.180	0.129	0.261	0.206
G76	0.084	0.095	0.091	0.066	0.157	0.264
Avg.	0.072	0.040	0.097	0.056	0.115	0.142

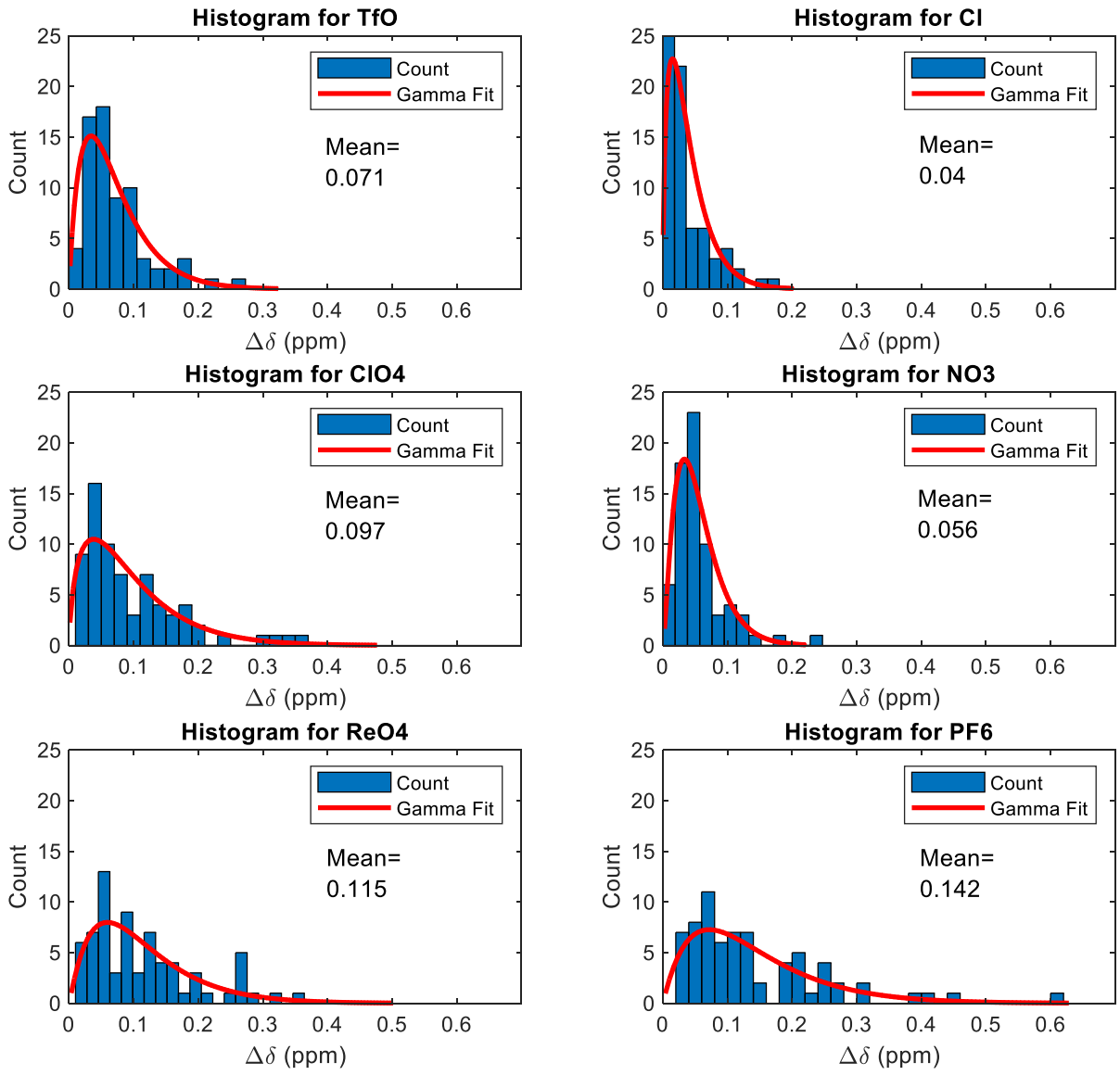


Figure S68 The histgram bar graphs and gamma distribution fittings of six salts at 200 equiv. The chemical shift (Δδ) values are from ^{1}H -^{15}N HSQC titration of 1 mM ubiquitin at pH = 2.8.

Using the data for perchlorate binding as a reference, a Δδ value of 0.18 was selected to define a binding site (top 20% of chemical shifts). However, if some adjacent residues underwent signal shifts greater than Δδ = 0.18 in the presence of other anions, these were also included in the defined binding site. Figure S69 shows the combined bar-graphs for the obtained Δδ values for the different anions studied, and the corresponding six binding sites highlighted in red, orange, yellow, aquamarine, blue and purple.

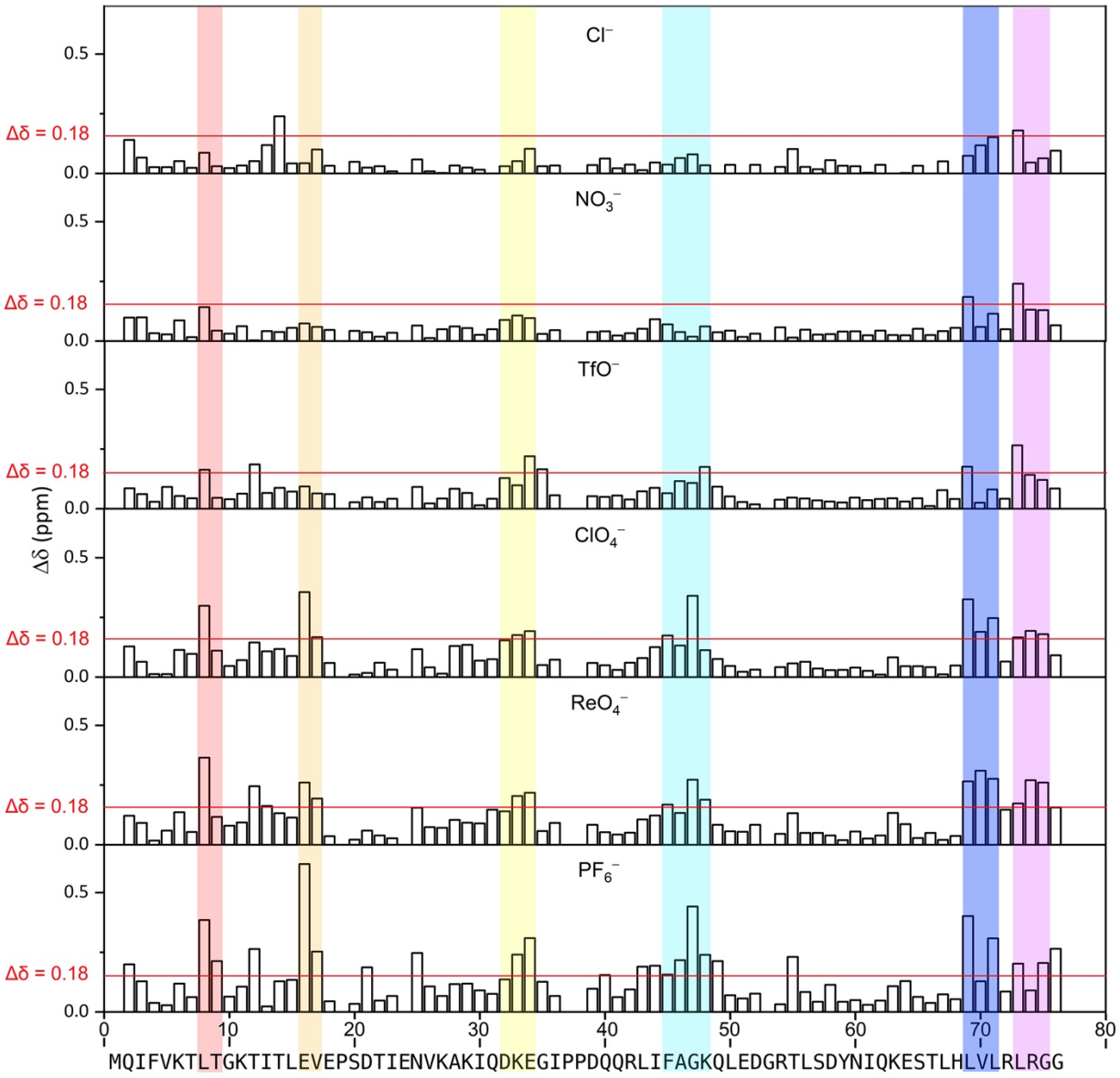


Figure S69 Combined bar-graphs for the Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from ^{1}H-^{15}N HSQC titrations with different salts at 200 equiv.; all pH values were 2.8. The corresponding six binding sites highlighted in red, organge, yellow, aquamarine, blue and purple.

Figure S70 to Figure S75 show the six individual anion binding sites of ubiquitin. Representations are based on the reported crystal structure of ubiquitin 87% of its N–H or C=O amide groups are H-bonded.[7,8] Detail of the binding site follow.

The X-ray structure reveals that one third of Ub is comprised of a five-strand, mixed β-sheet (main text, Figure 4),[9] and the first anion binding site in the primary sequence, Site 1 (Figure S70), is centered on the NH of L8, located at turn 1, a Type I β-turn. This turn protrudes from the bulk of Ub and includes a β-bulge involving residues T7, G10, and K11. This point, and the weak hydrogen bond (HB) between the NH of G10 and the C=O of T7,[9] results in four free NH groups (L8–K11) available to form a Nest motif.[10] Alternatively, considering that $C_\alpha H$ methine/methylenes can also act as HD donors for anions, the L8–T9–G10 array can be viewed as a $C^\alpha NN$ motif.[11] Hence this site is a hybrid Nest/$C^\alpha NN$ motif. Using the more significant shifts of L8 and T9 gave an average affinity of $\langle K_a \rangle = 29\ M^{-1}$ (main text, Table 1 and Table S10). Binding is likely promoted by the hydroxy sidechains of T7 and T9 acting as HB donors, the lack of sidechain in G10 providing access, and the strongly positive electrostatic potential field (EPF) of the flanking ammonium groups of K6 and K11 (main text, Figure 4).

Site 2 (Figure S71) is located at the C-terminal end of strand 2 and involves the NH of E16 and V17. The E16 NH group is on the frayed edge of the β-sheet and is free to bind to anions, whilst in the crystal structure the NH of V17 is turned 'inward' to form a HB to the C=O of residue M1. However, the sizable shift in the NH signal of V17 suggests that this HB to the frayed *N*-terminus is weak. Fitting using both residues gave $\langle K_a \rangle = 9\ M^{-1}$. At low pH, the carboxylic acid sidechain of E16 is assumed to promote binding as an HB donor, but the biggest contributor is likely the positive EPF of the *N*-terminal ammonium (main text, Figure 4).

The *N*-terminus of an α-helix can act as an anion binding site, but in the case of Ub this site is blocked because the N–H groups of I23 and E24 are hydrogen bonded to the C=O groups of E51 and R54. Rather, site 3 (Figure S72) is located at the C-terminal of the primary α-helix and involves residues D32, K33 and E34. In the crystal structure the backbone NHs at the site point towards the N-terminus and the center of the protein, hydrogen bonding to the C=O groups of A28, K29 and I30. Hence the observed NH signal shifts are likely a secondary effect arising from anions interacting with the $C_\alpha H$ methines of D32–E34 that constitute a triangular array of donors on the surface of the protein. Using the three NH signals, $\langle K_a \rangle$ was calculated to be $14\ M^{-1}$. The EPF of K33 (main text, Figure 4) likely plays a key role in compensating for the helix dipole, as may the more distant K29. The two carboxylic acid sidechains of D32 and E34 may also play roles as HB donors.

Site 4 (Figure S73) is located at another protuberance, the Type III β-turn involving residues F45 to K48. In the crystal structure the NH groups A46 and G47 are free, but all NH groups of residues F45 to K48 undergo appreciable shifts upon binding. The largest δΔ shift for G47 suggests binding is centered at this residue, but models reveal that ClO_4^- is large enough to interact with all of the N–H groups and all of the α-carbon methines of these four residues. Akin to Site 1, this site can be viewed as an polydentate hybrid of a Nest[10] and a $C^\alpha NN$ motif.[11] The average affinity value for ClO_4^- was $\langle K_a \rangle = 21\ M^{-1}$; an affinity augmented by the lack of sidechain in residue G47, and the EPF of K48 (main text, Figure 4).

Site 5 (Figure S74) involves residues L69, V70 and L71, located at a frayed edge of the main β-sheet at the end of the central (5^{th}) strand (main text, Figure 4). The largest δΔ shift is seen for residue L69, and the surface around this is a mixture of positively charged (K6, R42, and H68) and non-polar residues (L8, I44 and V70). This area is therefore relatively hydrophobic, but possesses a strongly positive EPF. Being located at the end of the strand, L71 also possesses a NH signal that undergoes a large shift. Fitting of the δΔ shifts for L69– L71 gave $\langle K_a \rangle = 20\ M^{-1}$.

Finally, Site 6 is located at the relatively flexible, C-terminus of Ub (Figure S75). Based on the NH δΔ shift values, ClO_4^- binds to residues L73, R74 and G75. The NH of the L73 residue is sandwiched (main text, Figure 4) between the positively charged R72 and R74. Indeed, this

is the strongest affinity determined from a *single* NH group; K_a for ClO_4^- = 66 M^{-1}. Interestingly, the K_a values for R74 and G75 were calculated to be only 14, and 13 M^{-1} respectively (overall $\langle K_a \rangle$ = 31 M^{-1}), suggesting that the flexibility of the C-terminus means that its donor groups can only help chelate anion binding at L73 to a limited extent. Overall, from R72 and G76 there are four NH and seven $C_\alpha H$ groups to act as HB donors.

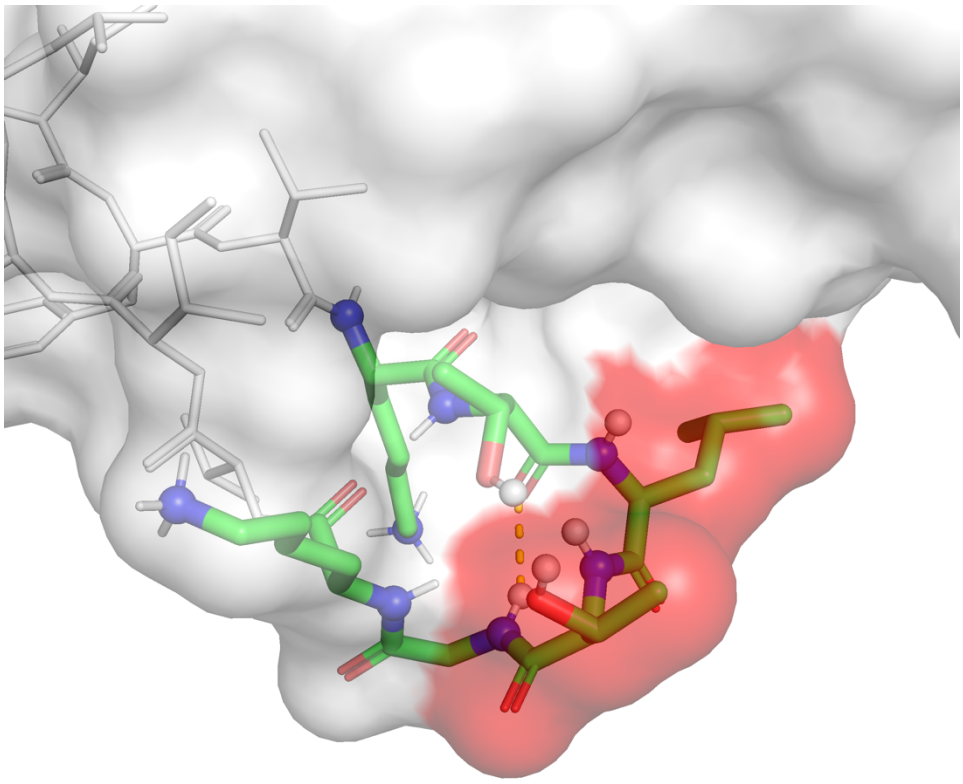


Figure S70 Anion binding site 1.

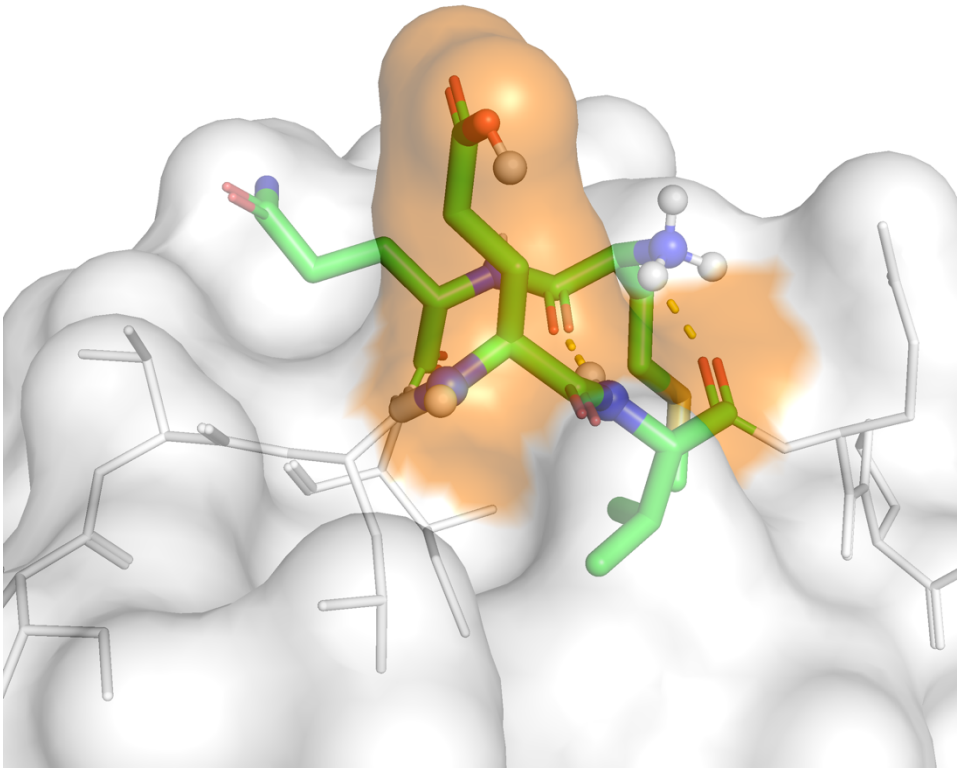


Figure S71 Anion binding site 2.

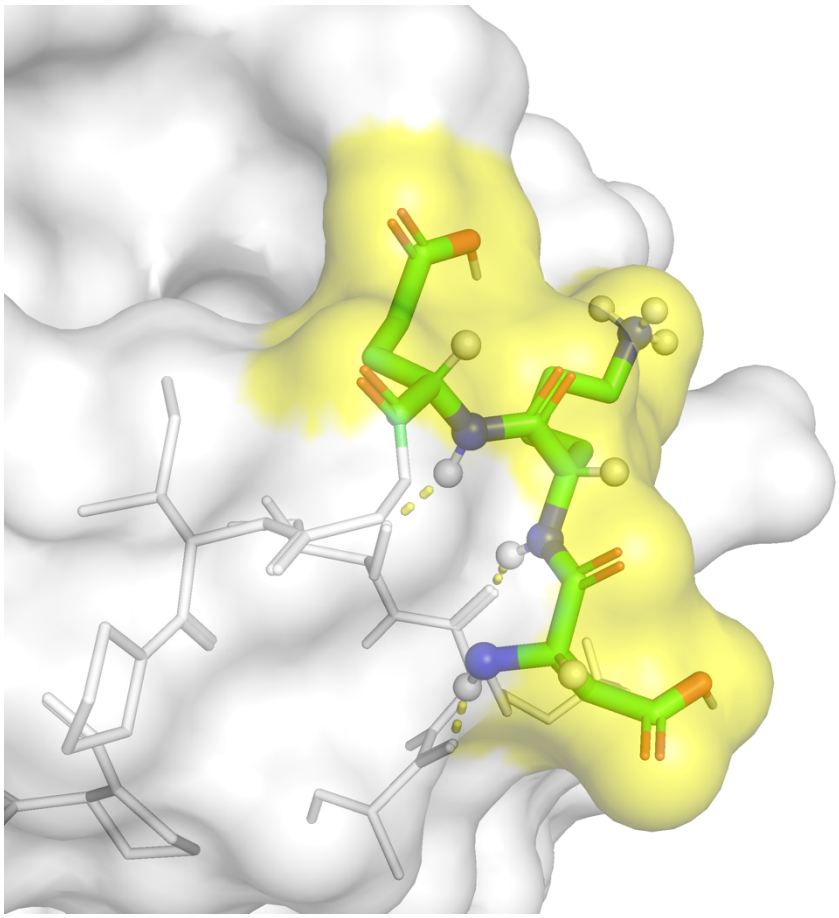


Figure S72 Anion binding site 3.

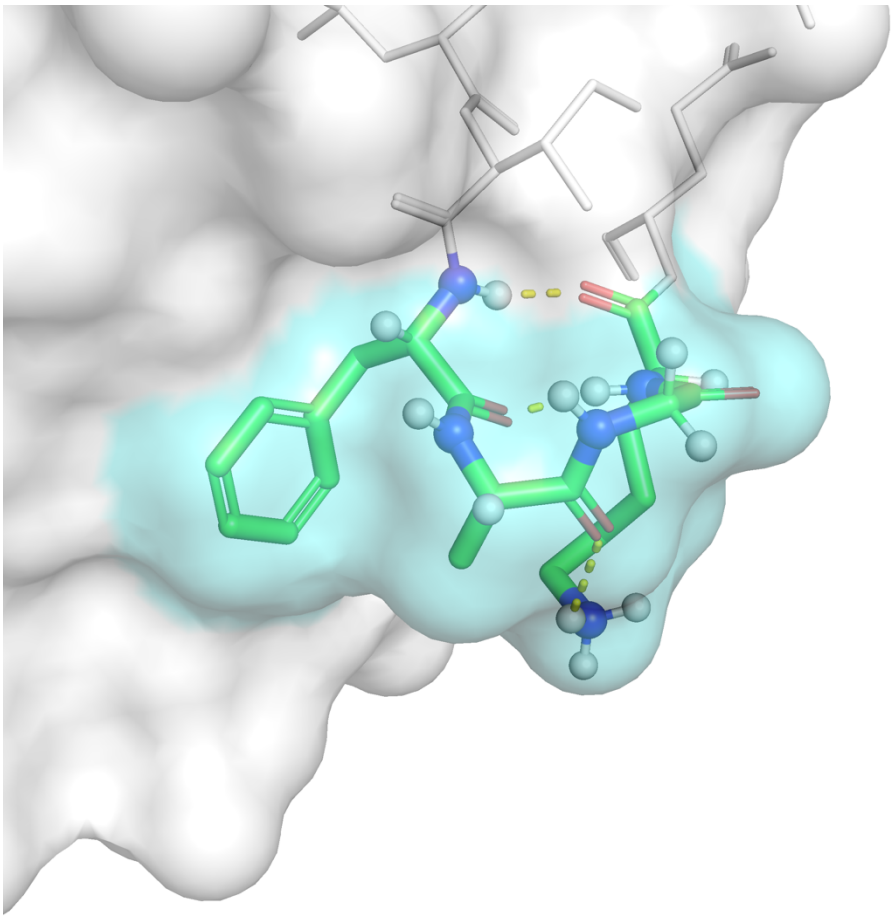


Figure S73 Anion binding site 4.

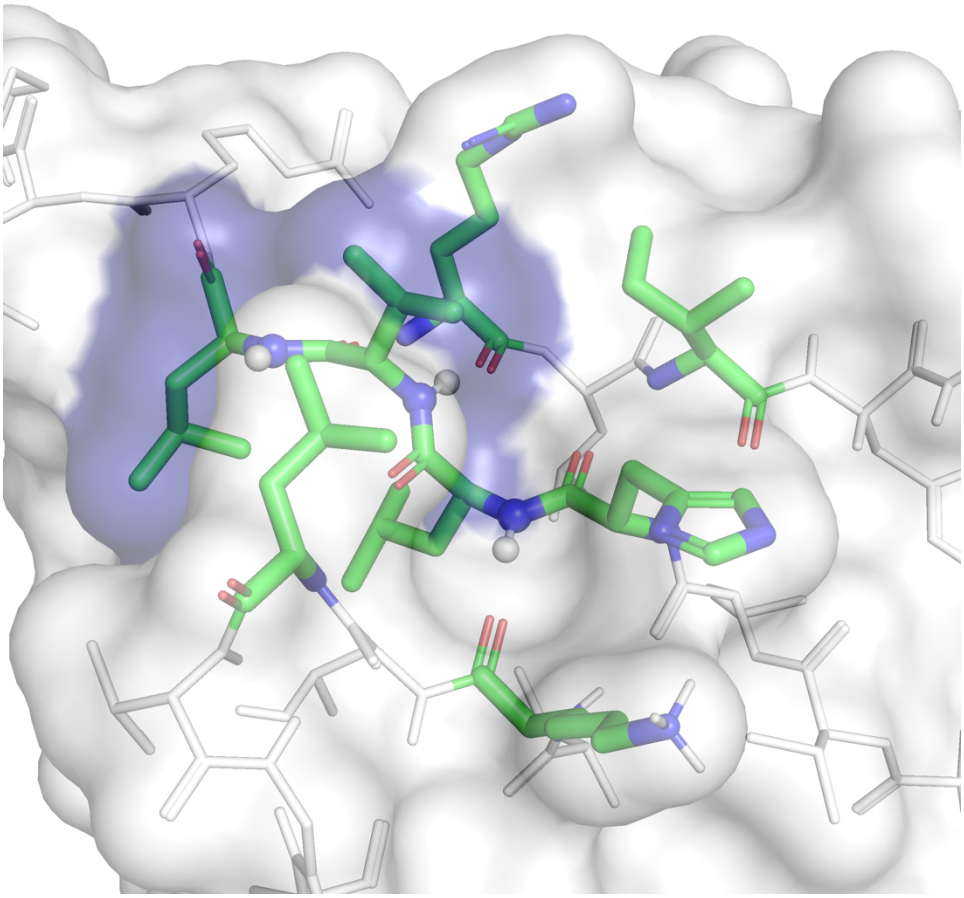


Figure S74 Anion binding site 5.

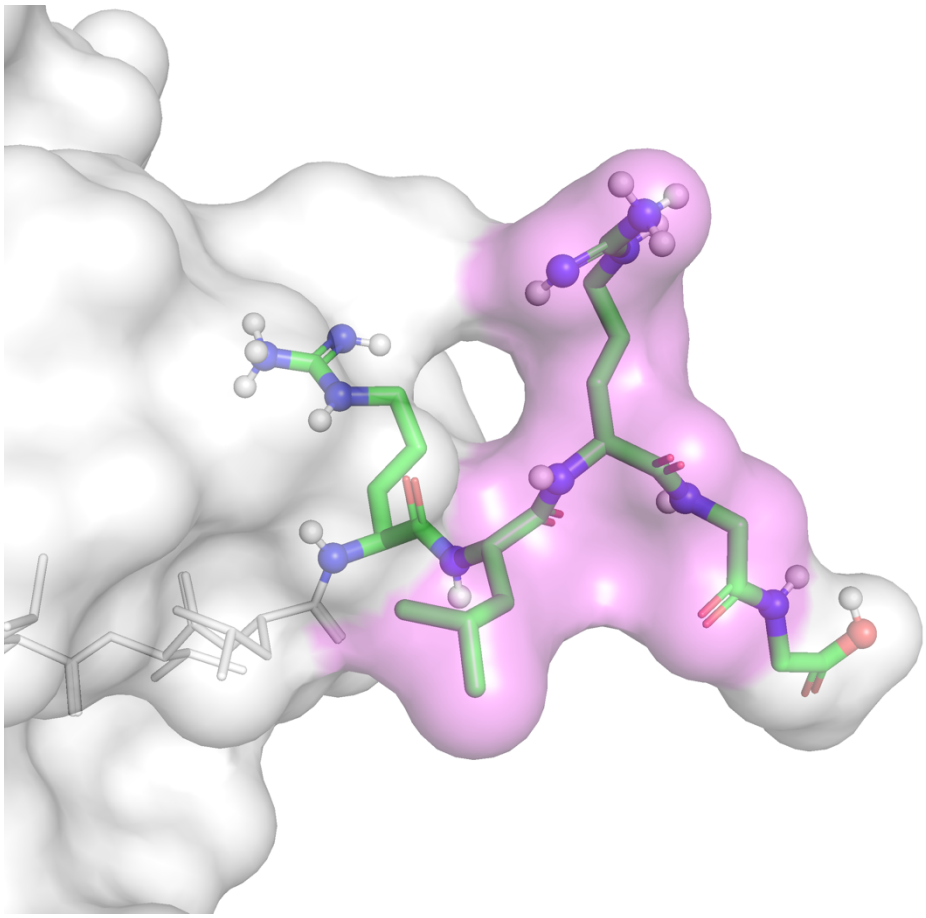


Figure S75 Anion binding site 6.

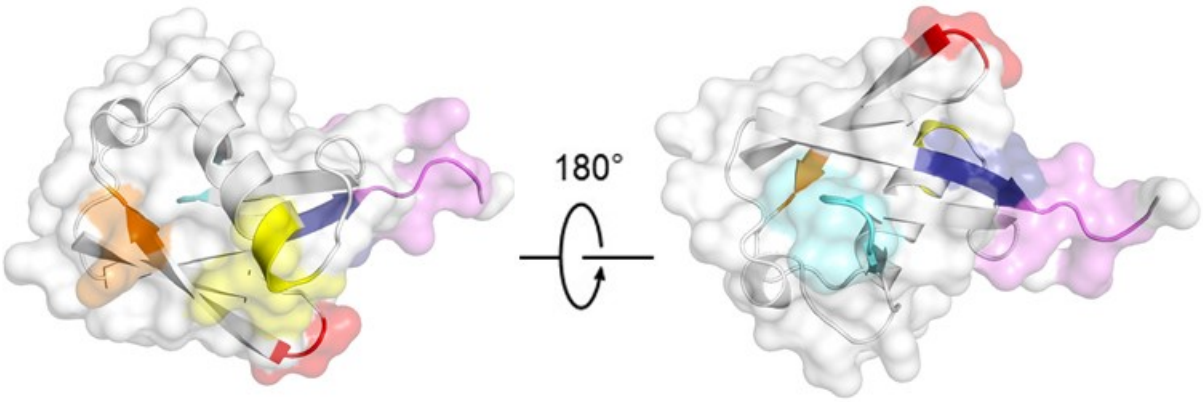


Figure S76. The six anion binding sites on Ub. The two images differ by a 180˚ rotation around the in-plane, horizontal axis. In both images, tracing the outline of the protein from Site 1 (red) to Site 6 (purple), by moving clockwise in the left-hand image and anti-clockwise in the right-hand image, highlights a wide 270˚ belt where anion binding is not observed.

5.4. Additional Binding Sites on Ub?

There is little evidence of additional binding sites to Ub. The strongest such examples involve strongest binding ReO_4^- and PF_6^-. Specifically, the $\langle K_a \rangle$ of ReO_4^- binding to Site 1 was measured at 54 M^{-1}, but the adjacent NH of T12 also undergoes a sizable δΔ shift (K_a = 33 M^{-1}) The proximity and similar affinity suggest it is best to view this as an extension of Site 1. Similarly, δΔ shift data for PF_6^- suggests a new potential site involving spatially adjacent residues D21 and T55, respectively positioned at the end of turn 2 and beginning of turn 6. However, the K_a value of PF_6^- for D21 and T55 were only 1 and 3 M^{-1}. A contributor to this weak binding is likely that only positively charged R54 is in the vicinity of this site. Regardless, considering the small sizer and weak affinity, we do not classify this as a significant binding site. It is also observed in the presence of PF_6^- that the third residue in the α-helix, N25, undergoes a sizable δΔ shift. That noted, the NH residue of V26 does not undergo a sizable δΔ shift and the signal from E24 is not evident by ^1H ^{15}N HSQC NMR spectroscopy. Thus, we have little evidence of a significant binding site, and the calculated K_a value of 6 M^{-1} for N25 make us reticent to classify this as a significant binding site. Finally, residues L43, I44, and Q49 were also shifted above our 0.18 ppm threshold in the presence of excess PF_6^-. Considering the location of these residues, we see these shifts as an indication of an extension of Site 4 (F45 – K48), located between strands 3 and 4. Interestingly, if the binding constants for all seven residues affected by PF_6^- are determined, it is evident that L43 and Q49 at the extremes of the binding site have the weakest recorded affinities (K_a = 12 and 3 M^{-1} respectively), whilst the strongest binding constant is seen for G47 at the central of the site (K_a = 23). This extension to Site 4 likely arises because strand 4 is only two residues in length (K48 and Q49) and has only one HB to strand 3: the NH of F45 to the C=O of K48. Moreover, of the two extension residues in longer strand 3 (L43 and I44), only the N–H of I44 has a HB to the C=O of H68 on strand 5; the NH of L43 does not form a HB to strand 5. Additionally, the fact that L43 and I44 are sandwiched between positively charged residues K48 and H68 undoubtedly promotes anion binding.

5.5. 1:1 Binding constant determinations

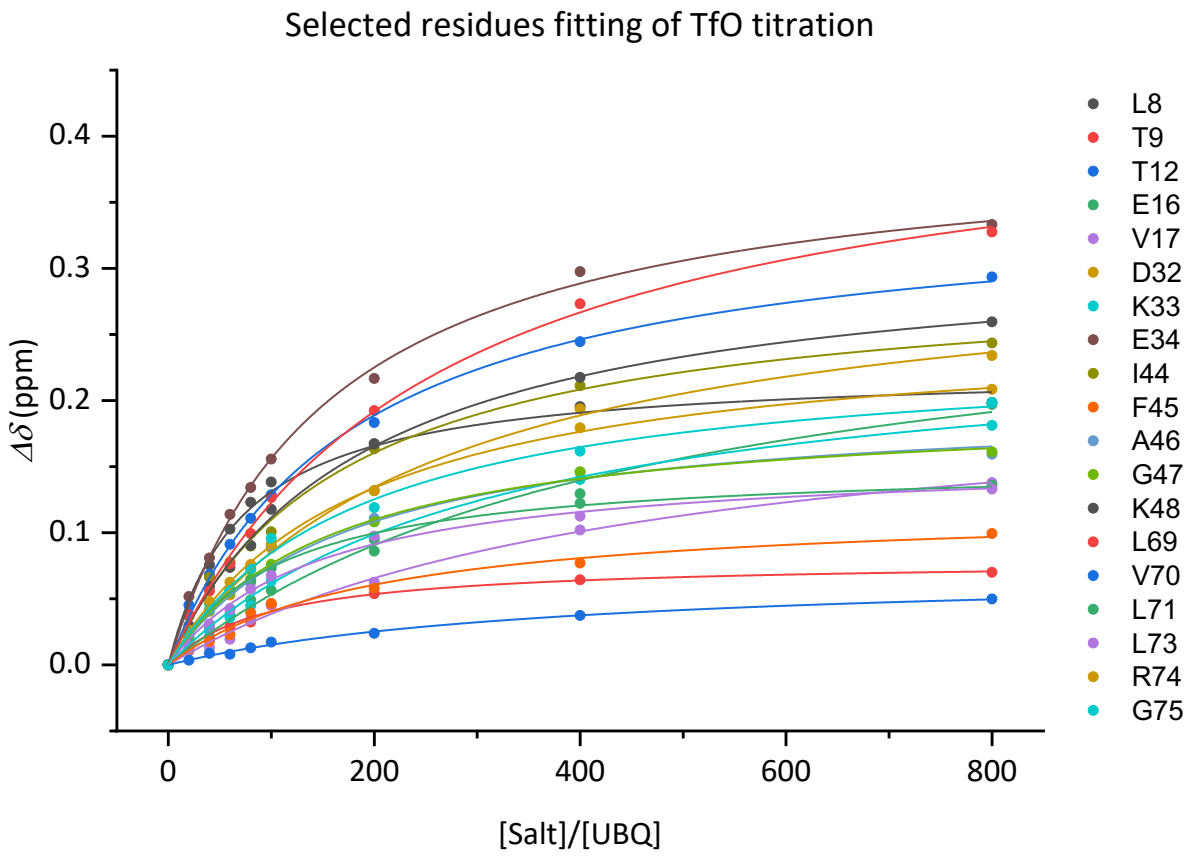
We determined the K_a values for anion binding to each site. The adjusted chemical shift Δδ was plotted against the anion concentration and fitted to the a 1:1 binding isotherm ($\Delta\delta = \left(\frac{\Delta\delta_{max}}{2[H]_t}\right) * \{\left([H]_t + [G]_t + \frac{1}{K_a}\right) - \sqrt{\left([H]_t + [G]_t + \frac{1}{K_a}\right) + 4[H]_t[G]_t}\}$ Eq. S3):

Here, $\Delta\delta_{max}$ is the maximum adjusted chemical shift at saturation, $[H]_t$ is the total protein concentration, K_a is the equilibrium dissociation constant, and $[G]_t$ is the total salt concentration. Notably, as is routine in these kinds of fitting, there are two major assumption of this process: a). a large excess amount of salt was added to protein solution, which should fully saturate each binding site, and b). Each binding site is thermodynamically ideal and independent. The residues chosen for fitting were based on if their chemical shift perturbation Δδ was larger than a threshold value 0.18, which is an arbitrary value. The binding sites are listed in Table S10. The individual binding isotherms are shown in Figure S77 to Figure S82)

Table S10:The binding constant of six anions to selected residues from the titration of 1 mM ubiquitin with different anions at pH = 2.8.

Binding site #	ClO_4^-		PF_6^-		ReO_4^-		NO_3^-		TfO^-		Cl^-	
	Residue	K_a (M^{-1})	Residue	K_a (M^{-1})	Residue	K_a (M^{-1})	Residue	K_a (M^{-1})	Residue	K_a (M^{-1})	Residue	K_a (M^{-1})
1 (L8+T9)	L8	25	L8	31	L8	72	L8	9	L8	28	L8	7
	T9	33	T9	21	T9	36	T9	12	T9	21	T9	*
2 (E16-V17)	E16	10	E16	4	E16	26	E16	4	E16	4	E16	2
	V17	8	V17	2	V17	11	V17	4	V17	4	V17	10
3 (D32-E34)	D32	12	D32	20	D32	21	D32	5	D32	7	D32	6
	K33	7	K33	4	K33	10	K33	6	K33	6	K33	15
	E34	23	E34	18	E34	33	E34	9	E34	13	E34	10
4 (F45-K48)	F45	26	F45	17	F45	34	F45	10	F45	10	F45	*
	A46	20	A46	15	A46	35	A46	*	A46	11	A46	*
	G47	24	G47	23	G47	53	G47	16	G47	13	G47	9
	K48	15	K48	16	K48	20	K48	14	K48	11	K48	*
5 (L69-L71)	L69	16	L69	7	L69	41	L69	11	L69	8	L69	10
	V70	22	V70	27	V70	70	V70	18	V70	5	V70	7
	L71	22	L71	27	L71	73	L71	22	L71	18	L71	5
6 (L73-G75)	L73	66	L73	44	L73	48	L73	8	L73	14	L73	15
	R74	14	R74	*	R74	31	R74	14	R74	11	R74	*
	G75	13	G75	5	G75	29	G75	11	G75	11	G75	11

Note: * bad fitting.



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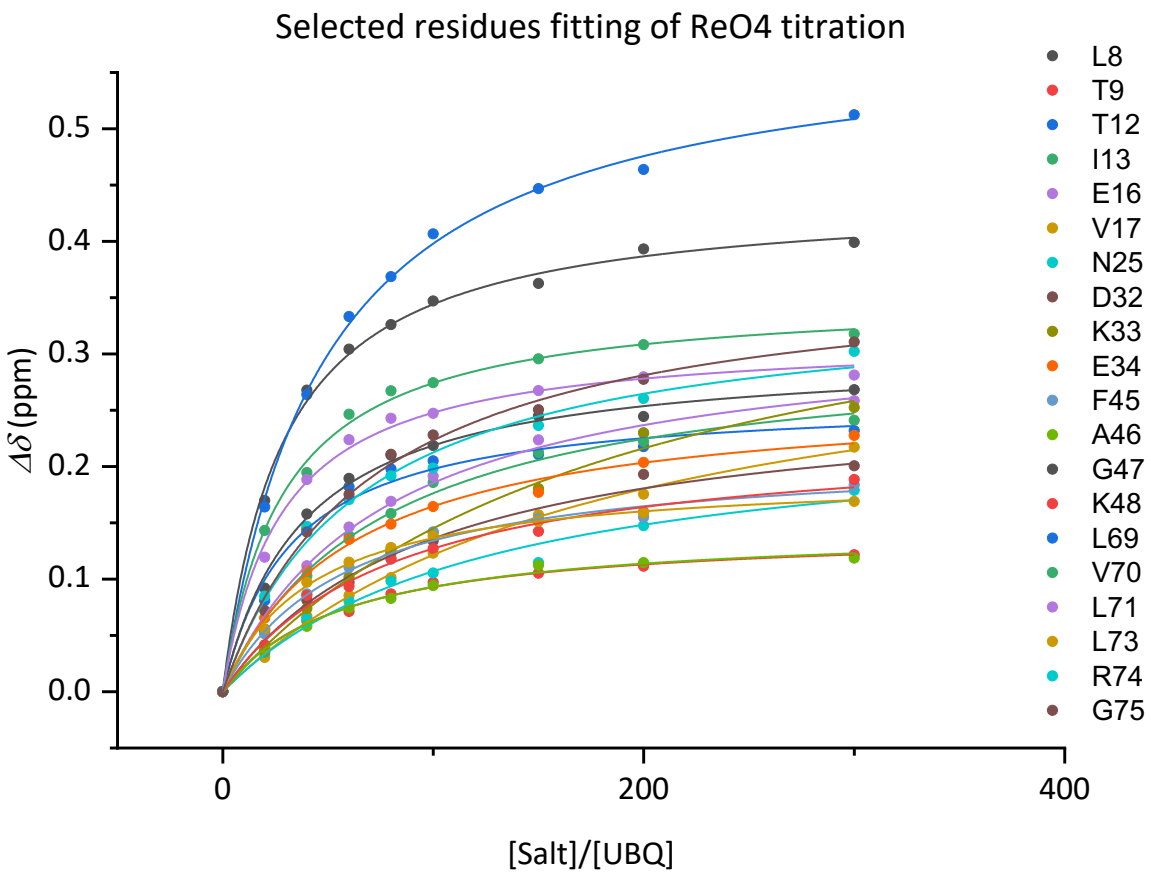
Equa	0.5*a*((1+x+b*((x*0.0005+5)/0.0025))-sqrt((1+x+b*((x*0.0005+5)/0.0025))^2-4*x))																		
Plot	L8	T9	T12	E16	V17	D32	K33	E34	I44	F45	A46	G47	K48	L69	V70	L71	L73	R74	G75
a	0.226	0.079	0.359	0.318	0.229	0.324	0.261	0.408	0.302	0.123	0.203	0.199	0.326	0.449	0.075	0.154	0.160	0.263	0.244
b	0.035	0.048	0.088	0.246	0.243	0.138	0.160	0.079	0.086	0.100	0.085	0.079	0.095	0.131	0.197	0.053	0.073	0.094	0.093
Redu	4.722	7.915	1.855	4.100	1.667	1.003	2.832	2.984	3.664	1.715	7.768	6.170	1.386	2.635	1.809	8.350	1.603	3.541	2.676
R-Sq	0.991	0.987	0.998	0.990	0.993	0.998	0.993	0.997	0.995	0.985	0.979	0.998	0.998	0.998	0.994	0.996	0.993	0.999	0.994
Adj.	0.990	0.985	0.997	0.989	0.992	0.998	0.992	0.997	0.994	0.983	0.976	0.997	0.998	0.997	0.993	0.995	0.992	0.999	0.993

Figure S77 1:1 Fitting of the signal shifts for selected N–H signals in the ^{1}H -^{15}N HSQC spectra from titrating 1 mM ubiquitin with TfO^{-} in 50 mM phosphate buffer, pH = 2.8.



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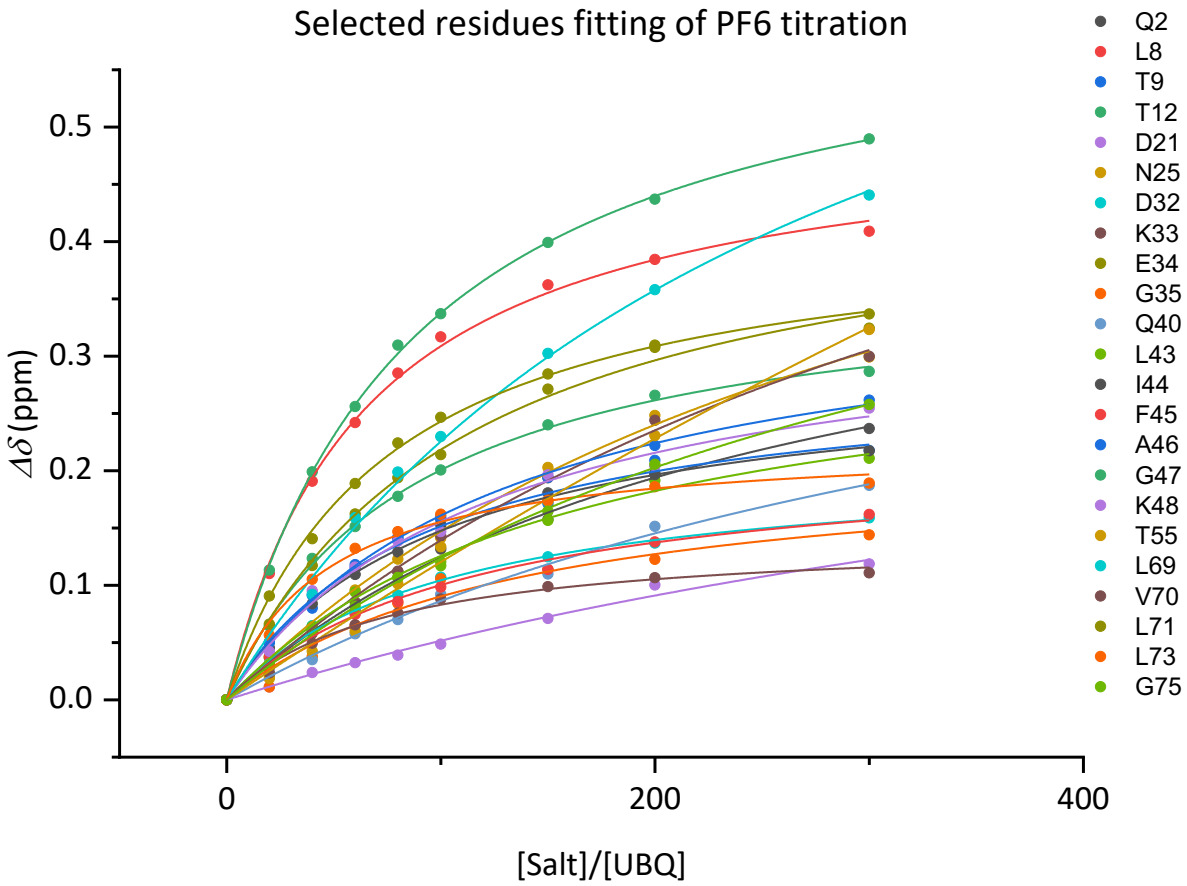
Equa	0.5*a*((1+x+b*((x*0.0005+5)/0.0025))-sqrt((1+x+b*((x*0.0005+5)/0.0025))^2-4*x))																				
Plot	L8	T9	T12	I13	E16	V17	N25	D32	K33	E34	F45	A46	G47	K48	L69	V70	L71	L73	R74	G75	
a	0.4420	0.144	0.261	0.311	0.329	0.354	0.244	0.270	0.431	0.267	0.214	0.146	0.302	0.232	0.593	0.3535	0.317	0.194	0.352	0.381	
b	0.0139	0.027	0.015	0.037	0.038	0.094	0.063	0.048	0.097	0.030	0.029	0.028	0.018	0.040	0.024	0.0142	0.013	0.021	0.032	0.034	
Redu	5.7748	6.755	1.110	4.145	4.484	1.236	6.173	5.185	4.113	3.057	3.180	9.975	4.313	3.645	4.959	3.5129	4.945	8.126	9.610	4.967	
R-Sq	0.9969	0.996	0.983	0.994	0.994	0.997	0.981	0.989	0.994	0.994	0.991	0.994	0.994	0.990	0.998	0.9970	0.994	0.997	0.990	0.995	
Adj.	0.9964	0.995	0.981	0.993	0.993	0.997	0.979	0.987	0.994	0.993	0.990	0.993	0.994	0.989	0.998	0.9966	0.994	0.997	0.988	0.995	

Figure S78 1:1 Fitting of the signal shifts for selected N–H signals in the ^{1}H -^{15}N HSQC spectra from titrating 1 mM ubiquitin with ReO_4^- in 50 mM phosphate buffer, pH = 2.8.



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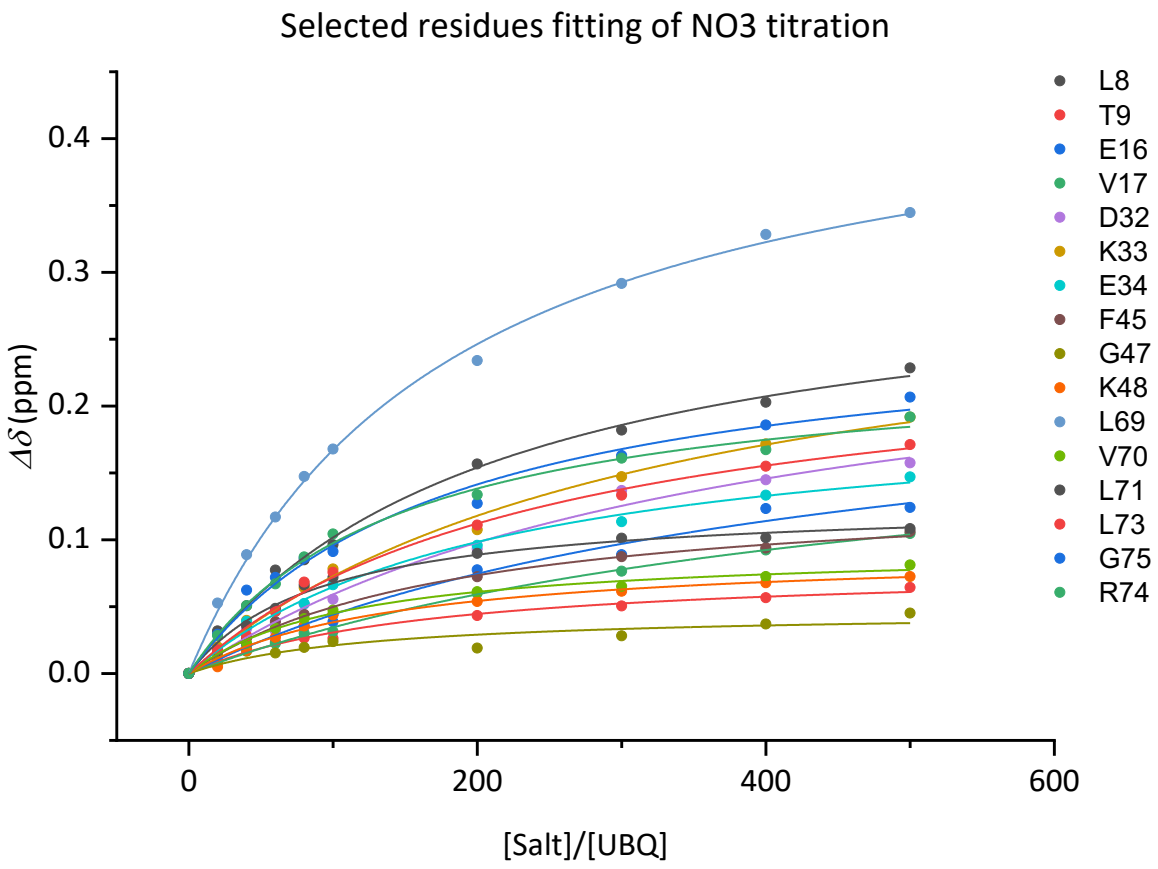
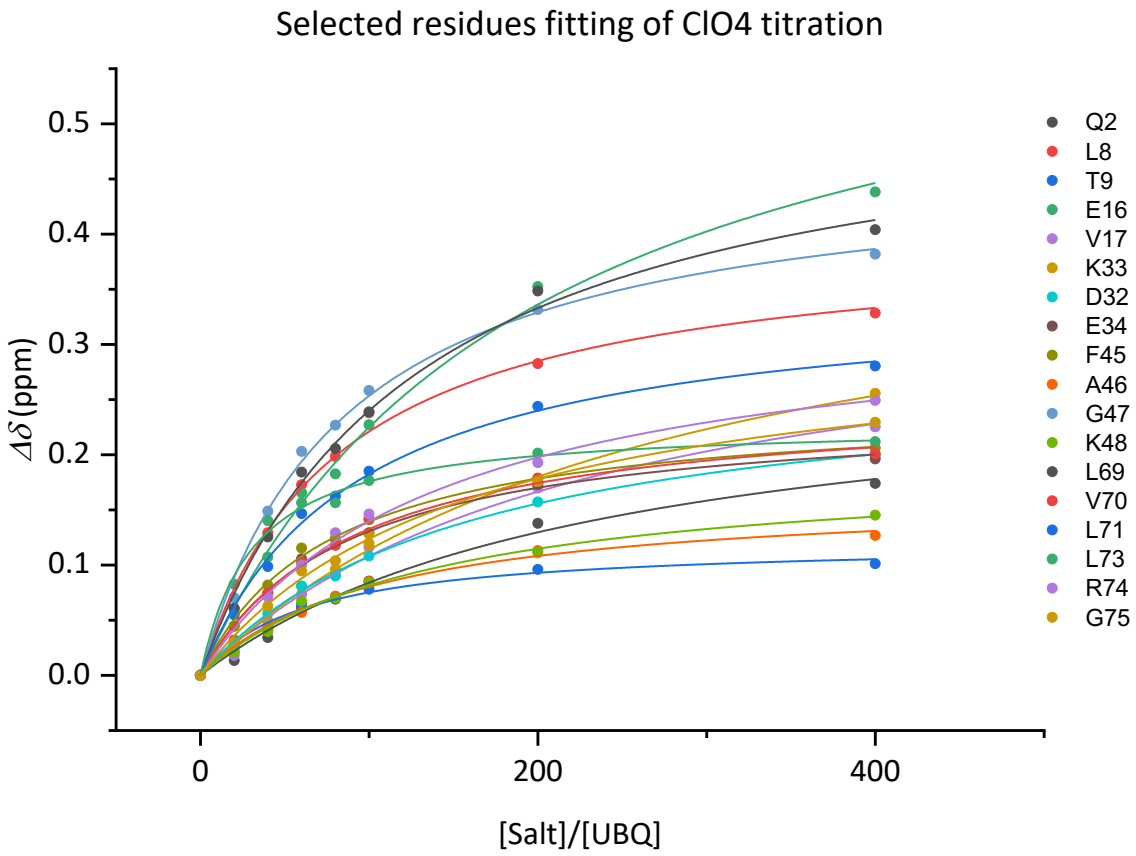
Equ	0.5*a*((1+x+b*((x*0.0005+5)/0.0025))-sqrt((1+x+b*((x*0.0005+5)/0.0025))^2-4*x))																						
Plot	Q2	L8	T9	T12	D21	N25	D32	K33	E34	G35	Q40	L43	I44	F45	A46	G47	K48	T55	L69	V70	L71	L73	G75
a	0.45	0.51	0.29	0.37	0.41	0.67	0.21	0.79	0.46	0.21	0.47	0.33	0.29	0.22	0.37	0.63	0.35	2.62	0.88	0.14	0.42	0.22	0.58
b	0.13	0.03	0.04	0.04	0.34	0.17	0.05	0.23	0.05	0.06	0.22	0.08	0.04	0.05	0.06	0.04	0.06	1.02	0.14	0.03	0.03	0.02	0.18
Red	4.18	4.58	4.89	1.60	1.69	2.34	9.77	2.99	6.02	9.43	2.42	2.30	1.06	2.51	7.79	1.18	6.36	7.12	1.50	1.57	1.40	3.86	1.34
R-S	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.96	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Adj.	0.99	0.99	0.99	0.99	0.98	0.99	0.99	0.99	0.99	0.96	0.99	0.99	0.99	0.98	0.99	0.99	0.99	0.99	0.99	0.98	0.99	0.99	0.99

Figure S79 1:1 Fitting of the signal shifts for selected N–H signals in the ^{1}H -^{15}N HSQC spectra from titrating 1 mM ubiquitin with PF_6^- in 50 mM phosphate buffer, pH = 2.8.



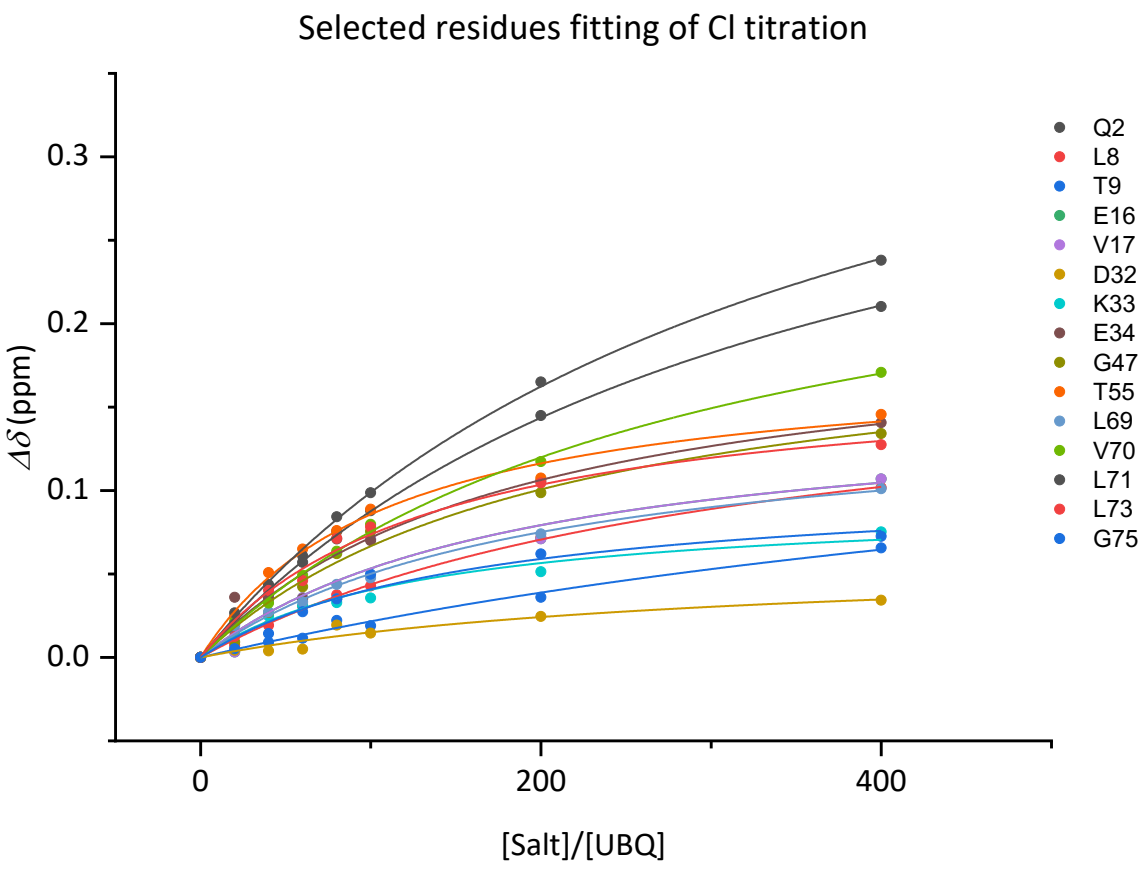
Model	NewFunction2 (User)															
Equati	0.5*a*((1+x+b*((x*0.0005+5)/0.0025))-sqrt((1+x+b*((x*0.0005+5)/0.0025))^2-4*x))															
Plot	L8	T9	E16	V17	D32	K33	E34	F45	G47	K48	L69	V70	L71	L73	G75	R74
a	0.3229	0.0822	0.2528	0.2200	0.2956	0.3216	0.2086	0.1422	0.0476	0.0938	0.4752	0.0954	0.1301	0.2595	0.2726	0.2407
b	0.1072	0.0828	0.2337	0.2657	0.1974	0.1689	0.1096	0.0914	0.0624	0.0714	0.0908	0.0551	0.0454	0.1285	0.0907	0.0724
Reduc	2.2505	9.1919	3.0412	3.3073	3.5420	2.5725	1.4849	9.6208	2.8003	1.1079	3.0907	5.9375	1.6285	1.7721	6.9632	2.3750
R-Squ	0.9966	0.9809	0.9865	0.9977	0.9904	0.9946	0.9946	0.9931	0.8561	0.9852	0.9980	0.9924	0.9898	0.9955	0.9863	0.9948
Adj. R	0.9962	0.9785	0.9848	0.9975	0.9892	0.9940	0.9939	0.9922	0.8382	0.9834	0.9978	0.9915	0.9885	0.9950	0.9846	0.9941

Figure S80 1:1 Fitting of the signal shifts for selected N–H signals in the ^{1}H -^{15}N HSQC spectra from titrating 1 mM ubiquitin with NO_3^- in 50 mM phosphate buffer, pH = 2.8.



Model	NewFunction2 (User)																	
Equat	0.5*a*((1+x+b*((x*0.0005+5)/0.0025))-sqrt((1+x+b*((x*0.0005+5)/0.0025))^2-4*x))																	
Plot	Q2	L8	T9	E16	V17	K33	D32	E34	F45	A46	G47	K48	L69	V70	L71	L73	R74	G75
a	0.290	0.403	0.122	0.676	0.370	0.440	0.284	0.245	0.249	0.167	0.472	0.195	0.549	0.256	0.352	0.229	0.342	0.323
b	0.121	0.040	0.030	0.098	0.119	0.141	0.081	0.043	0.038	0.053	0.042	0.068	0.063	0.045	0.046	0.015	0.071	0.079
Redu	3.753	1.141	1.617	1.408	4.141	1.953	8.724	5.372	1.768	1.579	8.135	2.073	1.062	5.533	3.713	4.751	4.998	2.113
R-Sq	0.991	0.992	0.988	0.994	0.993	0.997	0.998	0.989	0.996	0.992	0.995	0.992	0.995	0.990	0.996	0.991	0.993	0.996
Adj. R	0.989	0.990	0.986	0.993	0.992	0.997	0.997	0.987	0.996	0.991	0.995	0.990	0.994	0.988	0.995	0.990	0.992	0.996

Figure S81 1:1 Fitting of the signal shifts for selected N–H signals in the ^{1}H -^{15}N HSQC spectra from titrating 1 mM ubiquitin with ClO_4^- in 50 mM phosphate buffer, pH = 2.8.



Model	NewFunction2 (User)														
Equati	0.5*a*((1+x+b*((x*0.0005+5)/0.0025))-sqrt((1+x+b*((x*0.0005+5)/0.0025))^2-4*x))														
Plot	Q2	L8	T9	E16	V17	D32	K33	E34	G47	T55	L69	V70	L71	L73	G75
a	0.4111	0.1907	0.2079	0.1570	0.1570	0.0634	0.0948	0.2085	0.2093	0.1823	0.1525	0.3010	0.4693	0.1760	0.1078
b	0.1822	0.1663	0.4263	0.0959	0.0959	0.1586	0.0662	0.0939	0.1055	0.0555	0.1007	0.1477	0.1850	0.0685	0.0807
Reduc	3.534E	3.1966	2.6574	1.1605	1.1605	1.3350	3.8072	9.5646	3.9805	2.0660	7.5005	7.2312	6.5028	3.6739	3.2717
R-Squ	0.9993	0.9976	0.9451	0.9245	0.9245	0.9215	0.9318	0.9596	0.9837	0.9916	0.9936	0.9979	0.9990	0.9835	0.9602
Adj. R-	0.9992	0.9972	0.9360	0.9119	0.9119	0.9084	0.9205	0.9529	0.9810	0.9902	0.9926	0.9976	0.9989	0.9808	0.9536

Figure S82 1:1 Fitting of the signal shifts for selected N–H signals in the ^{1}H -^{15}N HSQC spectra from titrating 1 mM ubiquitin with Cl^- in 50 mM phosphate buffer, pH = 2.8.

5.6. pH study of ubiquitin titrations with $NaClO_4$

To study the pH effect on anion binding with ubiquitin four titration experiments with ClO_4^- were carried out at pH = 3.8, 4.8, 5.8, and 7.3. Combined with the ClO_4^- data at pH 2.8 (see above), this covers the pH range covered by the SLS and DSC studies. According to the Protein Calculator (http://protcalc.sourceforge.net/) we have the following charges (in parenthesis) at the following pH values: 2.3 (12.8), 2.8 (12.4), 3.8 (10.0), 4.8 (4.1), 5.8 (1.3), 7.3 (0.0).

For each titration data set the following is shown: 1) A stack of (eight to ten) HSQC spectra, which showed the raw data from the titration of each salt; 2) A table of the chemical shift data (^1H and ^{15}N) highlighting signal positions at both the beginning (0 equiv.) and the end of the titration (300 – 800 equiv. depending on the anion), and the adjusted chemical perturbation value Δδ); 3) the bar graph from Δδ value of each titration; 4) A representation of the ubiquitin surface showing (on the structure of PDB ID: 1UBQ[7]) the location of anion binding. A color scale of white to dark red corresponds to a shift of 0 to 0.5 ppm was applied for all representation because most of the chemical shift perturbation values falls in the range from 0.1 to 0.5 ppm, and 5) The individual binding isotherms for each experiment (Figure S98 to Figure S101).

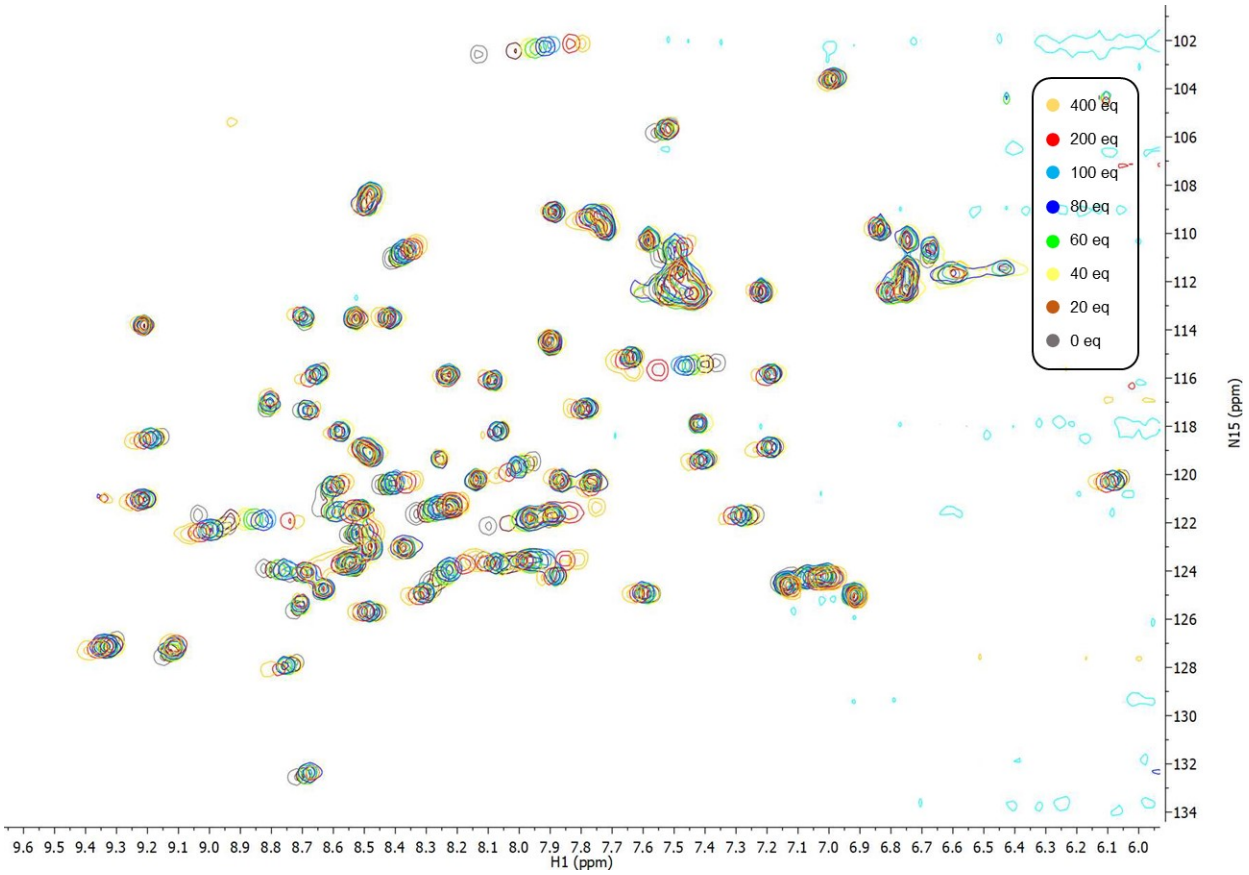


Figure S83 Stack of ^{1}H -^{15}N HSQC titration spectra of 1 mM ubiquitin with ClO_4^- (from 0 to 400 equiv.), in 50 mM phosphate buffer 90% H_2O/10% D_2O, pH = 3.8.

Table S11: ^{1}H -^{15}N HSQC chemical shift data from the titration of 1 mM ubiquitin with ClO_4^- at pH 3.8.

Amino Acid #.	0 equiv.		400 equiv.		Δδ
	^{1}H	^{15}N	^{1}H	^{15}N	
M1					
Q2	8.82	123.89	8.65	123.93	0.171
I3	8.23	115.78	8.25	115.93	0.070
F4	8.57	118.19	8.61	118.28	0.057
V5	9.2	121.01	9.26	121.15	0.087
K6	8.72	127.78	8.81	128.09	0.165
T7	8.64	115.74	8.71	116.05	0.155
L8	9.04	121.67	8.71	121.97	0.356
T9	7.56	105.84	7.52	105.62	0.106
G10	7.73	109.2	7.81	109.46	0.141
K11	7.24	121.67	7.34	121.83	0.123
T12	8.63	120.74	8.56	120.31	0.205
I13	9.3	126.89	9.39	127.36	0.229
T14	8.64	121.42	8.51	121.58	0.148
L15	8.64	124.87	8.65	124.57	0.135
E16	8.1	122.13	7.75	121.37	0.488
V17	8.82	117.25	8.81	116.78	0.210
E18	8.49	119.08	8.52	118.97	0.058
P19					
S20	6.98	103.54	7.01	103.67	0.065
D21	7.96	123.58	8.01	123.58	0.050
T22	7.89	109.14	7.91	109.07	0.037
I23	8.51	121.44	8.54	121.48	0.035
E24					
N25	7.86	120.38	7.89	120.13	0.116
V26	7.99	121.83	7.97	121.82	0.020
K27	8.49	119.08	8.49	119.2	0.054
A28	8.04	123.74	8.13	123.56	0.121
K29	7.77	120.17	7.79	120.54	0.167
I30	8.23	121.45	8.22	121.19	0.117
Q31	8.54	123.77	8.59	123.63	0.080
D32	7.95	119.46	8.07	120.05	0.290
K33	7.36	115.37	7.63	115.73	0.314
E34	8.69	113.7	8.73	113.3	0.183
G35	8.48	108.84	8.52	108.89	0.046
I36	6.06	120.13	6.13	120.29	0.100
P37					
P38					
D39	8.53	113.59	8.52	113.48	0.050
Q40	7.77	117.23	7.83	117.3	0.068
Q41	7.43	117.94	7.44	117.91	0.017

R42	8.49	122.96	8.51	123.19	0.105
L43	8.74	124.15	8.66	124.07	0.088
I44	8.94	122.12	9.06	122.45	0.190
F45	8.73	125.65	8.72	125.17	0.215
A46	8.72	132.54	8.67	132.33	0.106
G47	8.13	102.58	7.8	102.13	0.387
K48	7.91	121.82	7.91	121.52	0.134
Q49	8.55	122.5	8.47	122.31	0.117
L50	8.46	125.71	8.53	125.68	0.071
E51	8.37	123.06	8.39	123.09	0.024
D52	8.14	120.23	8.13	120.14	0.041
G53					
R54	7.39	119.35	7.45	119.45	0.075
T55	8.5	108.38	8.48	108.31	0.037
L56	8.05	118.17	8.12	118.36	0.110
S57	8.42	113.56	8.44	113.48	0.041
D58	7.87	124.22	7.93	124.27	0.064
Y59	7.18	115.8	7.23	115.84	0.053
N60	8.08	116.16	8.12	115.92	0.115
I61	7.18	118.86	7.24	118.97	0.078
Q62	7.57	124.94	7.63	124.96	0.061
K63	8.44	120.41	8.34	120.26	0.120
E64	9.22	113.83	9.22	113.82	0.004
S65	7.62	115.1	7.68	115.25	0.090
T66	8.7	117.33	8.66	117.27	0.048
L67	9.33	127.22	9.37	127.15	0.051
H68	9.16	118.43	9.24	118.58	0.104
L69	8.29	124.6	8.35	125.06	0.214
V70	9.15	127.56	9.12	127.05	0.230
L71	8.12	123.6	7.81	123.51	0.313
R72	8.54	123.77	8.57	123.41	0.164
L73	8.29	124.6	8.15	123.57	0.481
R74	8.33	121.63	8.19	121.15	0.256
G75	8.41	111.04	8.33	110.51	0.250
G76	7.9	114.63	7.91	114.41	0.099

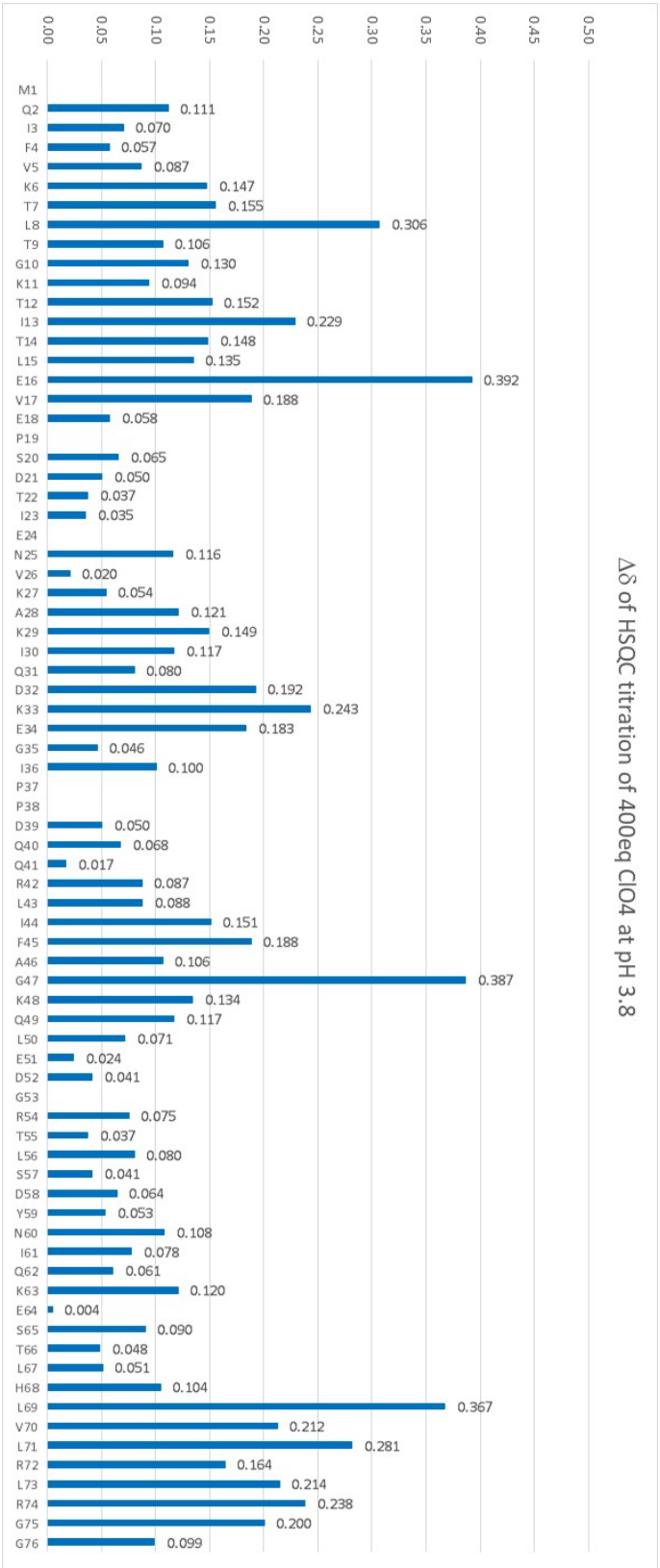


Figure S84 Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ClO_4^- at 400 equiv. of salt., pH = 3.8.

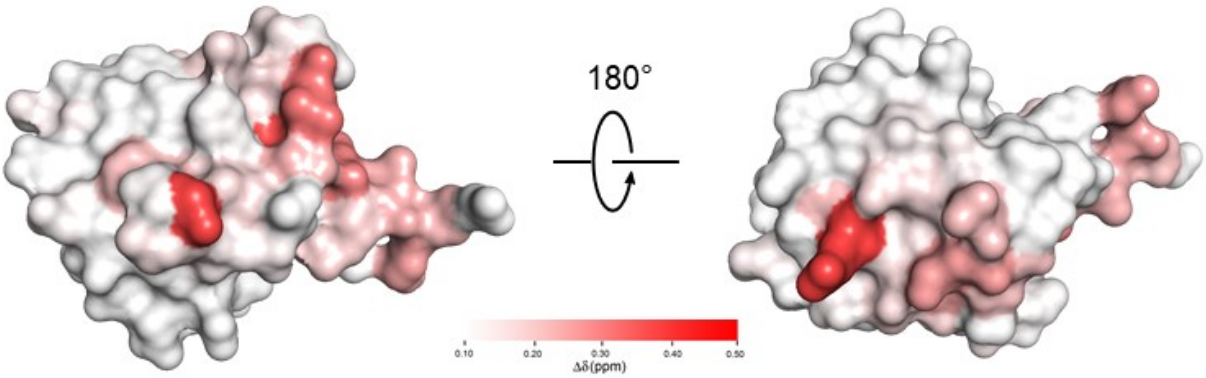


Figure S85 Visualization of the $\Delta\delta_{max}$ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ClO_4^- at 400 equiv. of salt, pH = 3.8.

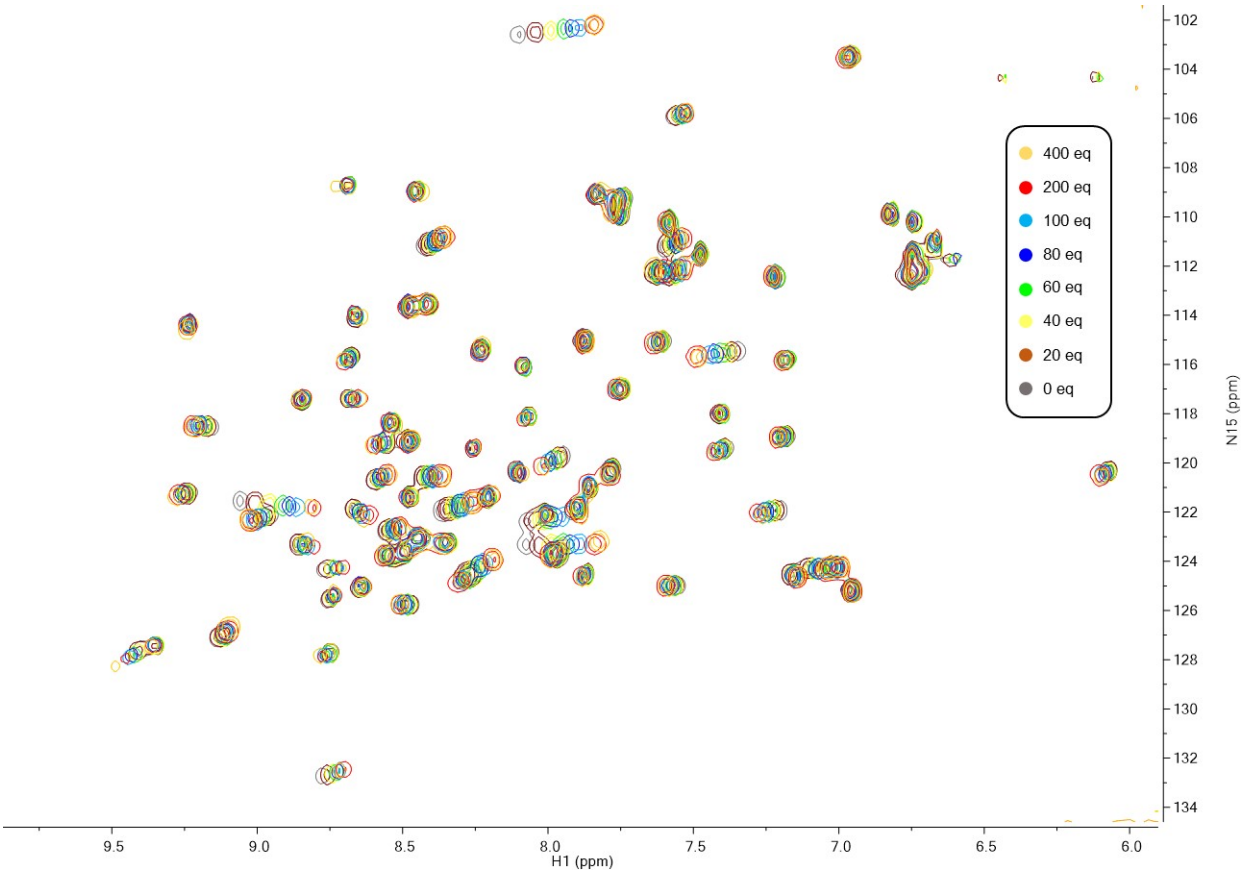


Figure S86 Stack of ^{1}H -^{15}N HSQC titration spectra of 1 mM ubiquitin with ClO_4^- (from 0 to 400 equiv.), in 50 mM phosphate buffer 90% H_2O/10% D_2O, pH 4.8.

Table S12: ^{1}H -^{15}N HSQC chemical shift data from the titration of 1 mM ubiquitin with ClO_4^- at pH = 4.8.

Amino Acid #	0 equiv.		400 equiv.		Δδ
	^{1}H	^{15}N	^{1}H	^{15}N	
M1					
Q2	8.86	123.3	8.83	123.18	0.061
I3	8.23	115.38	8.23	115.24	0.063
F4	8.54	118.37	8.53	118.42	0.024
V5	9.23	121.25	9.27	121.54	0.136
K6	8.74	127.73	8.78	127.84	0.063
T7	8.67	115.7	8.7	115.78	0.047
L8	9.06	121.58	8.82	121.79	0.258
T9	7.57	105.95	7.52	105.76	0.099
G10	7.74	109.26	7.77	109.34	0.047
K11	7.2	121.96	7.26	122.1	0.087
T12	8.6	120.75	8.55	120.49	0.127
I13	9.43	127.63	9.49	128.25	0.284
T14	8.67	121.85	8.63	122.29	0.201
L15	8.65	125.11	8.63	125.08	0.024
E16	8.07	122.39	7.89	122.15	0.210
V17	8.85	117.53	8.85	117.41	0.054
E18	8.55	119.19	8.6	119.28	0.064
P19					
S20	6.96	103.53	6.96	103.46	0.031
D21	7.97	123.71	7.95	123.53	0.083
T22	7.84	109.13	7.81	109	0.065
I23	8.47	121.39	8.47	121.4	0.004
E24					
N25	7.86	121.08	7.86	121.16	0.036
V26	8.01	122.1	8.01	122.17	0.031
K27	8.49	119.1	8.47	119.08	0.022
A28	7.97	123.71	7.99	123.91	0.092
K29	7.78	120.24	7.79	120.47	0.103
I30	8.21	121.41	8.19	121.29	0.057
Q31	8.52	123.83	8.56	123.72	0.063
D32	7.95	119.75	8.01	120.17	0.197
K33	7.35	115.49	7.48	115.69	0.158
E34	8.67	114.18	8.64	114.07	0.058
G35	8.45	108.94	8.44	108.97	0.017
I36	6.07	120.32	6.1	120.56	0.111
P37					
P38					
D39	8.48	113.71	8.46	113.65	0.033
Q40	7.75	117.04	7.75	116.92	0.054

Q41	7.41	118.08	7.39	117.99	0.045
R42	8.45	123.05	8.44	123.18	0.059
L43	8.78	124.38	8.71	124.27	0.086
I44	8.96	122.14	9.02	122.32	0.100
F45	8.76	125.59	8.74	125.23	0.162
A46	8.78	132.76	8.72	132.51	0.127
G47	8.1	102.62	7.84	102.17	0.329
K48	7.91	121.98	7.89	121.84	0.066
Q49	8.56	122.79	8.51	122.69	0.067
L50	8.47	125.8	8.51	125.73	0.051
E51	8.34	123.23	8.35	123.31	0.037
D52	8.11	120.39	8.09	120.44	0.030
G53					
R54	7.39	119.44	7.42	119.54	0.054
T55	8.69	108.72	8.73	108.77	0.046
L56	8.06	118.13	8.08	118.19	0.033
S57	8.41	113.62	8.42	113.51	0.050
D58	7.86	124.54	7.88	124.65	0.053
Y59	7.18	115.85	7.19	115.83	0.013
N60	8.08	116.16	8.08	115.99	0.076
I61	7.18	118.95	7.2	118.96	0.020
Q62	7.56	125.02	7.58	125	0.022
K63	8.44	120.61	8.36	120.55	0.084
E64	9.23	114.34	9.24	114.49	0.068
S65	7.6	115.08	7.62	115.06	0.022
T66	8.69	117.42	8.65	117.38	0.044
L67	9.4	127.59	9.36	127.45	0.074
H68	9.16	118.58	9.21	118.54	0.053
L69	8.35	124.97	8.29	124.7	0.135
V70	9.13	127.14	9.09	126.7	0.201
L71	8.08	123.36	7.82	123.17	0.274
R72	8.52	123.83	8.47	123.57	0.127
L73	8.21	124.43	8.18	123.97	0.208
R74	8.36	121.97	8.26	121.63	0.182
G75	8.42	111.19	8.36	110.82	0.176
G76	7.88	115.06	7.87	115.06	0.010

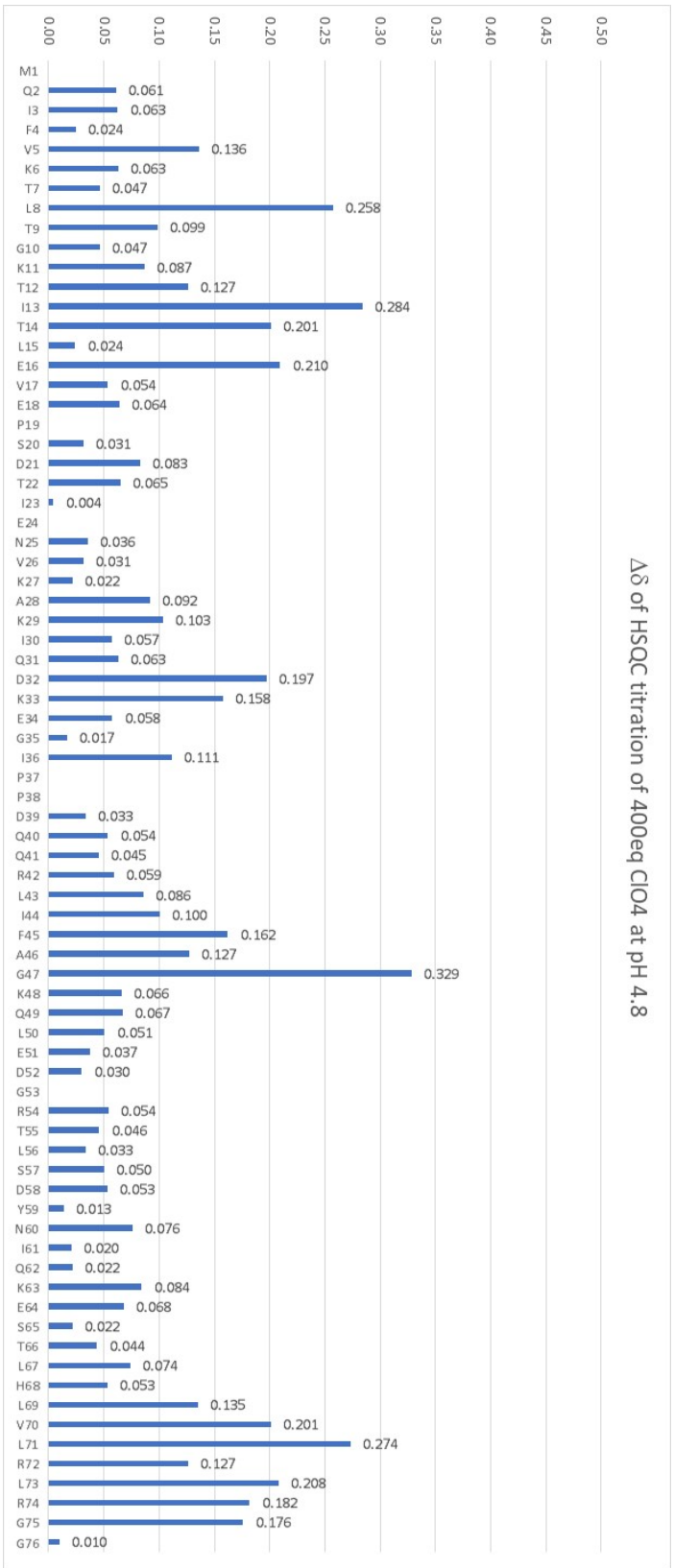


Figure S87 Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ClO_4^- at 400 equiv. of salt., pH = 4.8.

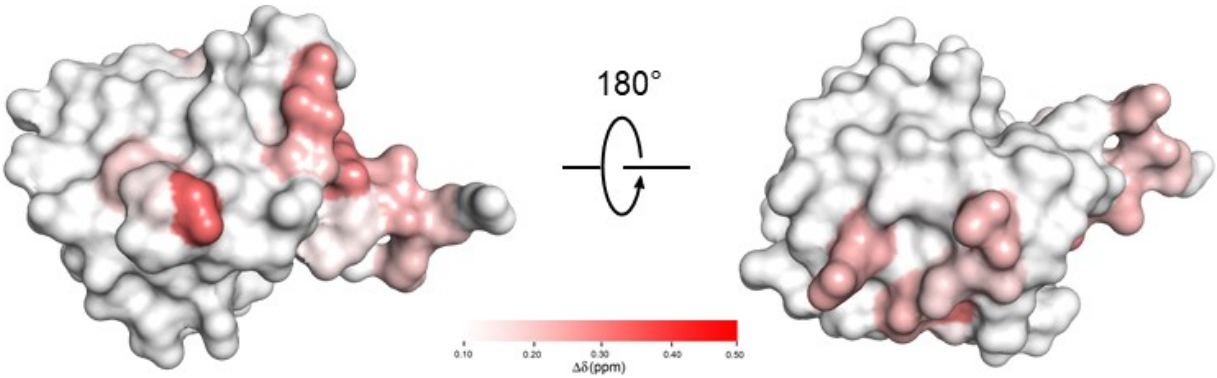


Figure S88 Visualization of the $\Delta\delta_{max}$ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ClO_4^- at 400 equiv. of salt, pH 4.8.

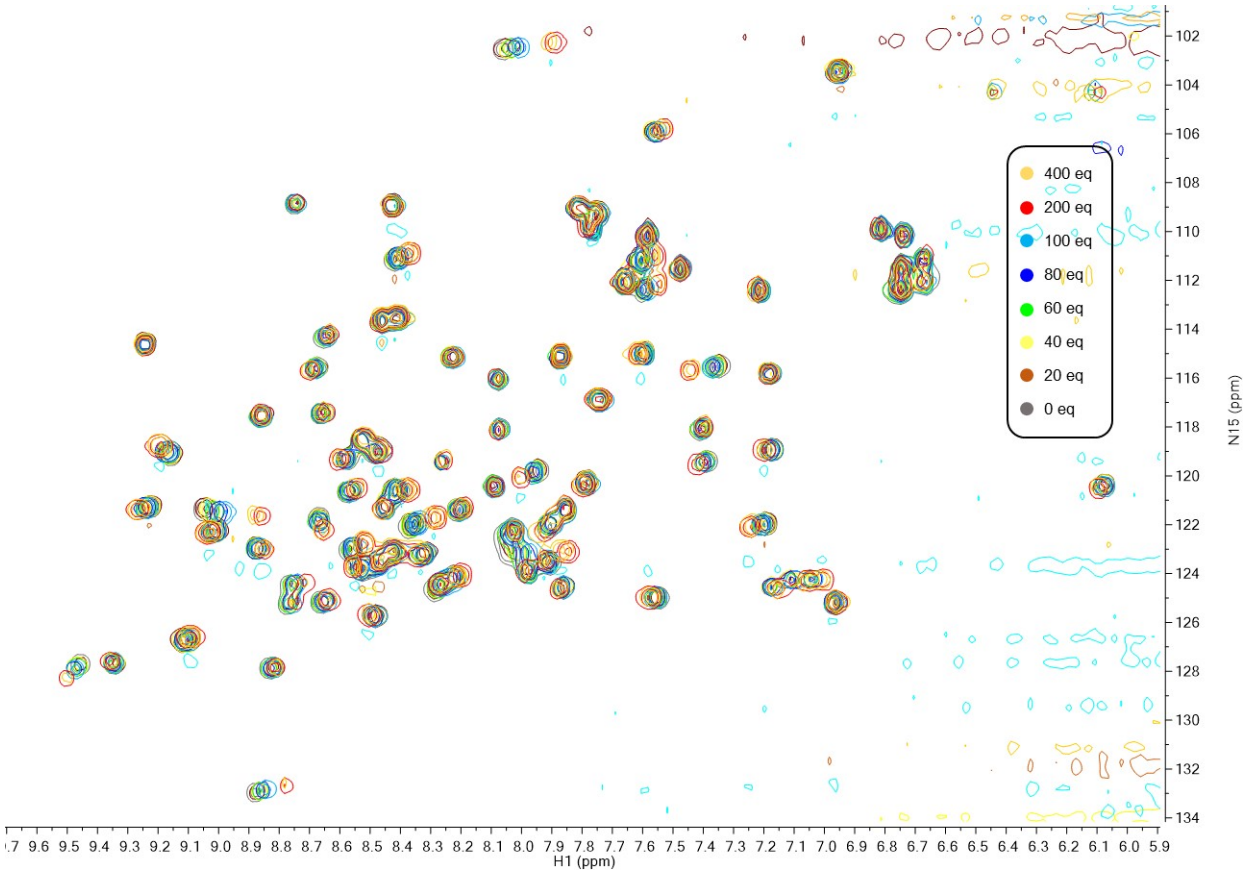


Figure S89 Stack of ^{1}H -^{15}N HSQC titration spectra of 1 mM ubiquitin with ClO_4^- (from 0 to 400 equiv.), in 50 mM phosphate buffer 90% H_2O/10% D_2O, pH = 5.8.

Table S13: ^{1}H -^{15}N HSQC chemical shift data from the titration of 1 mM ubiquitin with ClO_4^- at pH 5.8.

Amino Acid #.	0 equiv.		400 equiv.		Δδ
	^{1}H	^{15}N	^{1}H	^{15}N	
M1					
Q2	8.87	122.95	8.85	122.97	0.023
I3	8.23	115.15	8.23	115.12	0.013
F4	8.53	118.57	8.52	118.43	0.063
V5	9.23	121.29	9.27	121.35	0.047
K6	8.83	127.87	8.81	127.82	0.029
T7	8.68	115.57	8.70	115.65	0.044
L8	9.05	121.38	8.88	121.61	0.200
T9	7.56	105.94	7.54	105.79	0.072
G10	7.75	109.29	7.77	109.26	0.025
K11	7.19	121.97	7.24	122.04	0.059
T12	8.57	120.66	8.55	120.50	0.075
I13	9.46	127.72	9.50	128.29	0.260
T14	8.67	121.73	8.66	122.19	0.208
L15	8.66	125.17	8.64	125.07	0.049
E16	8.05	122.53	7.95	122.30	0.144
V17	8.86	117.58	8.86	117.55	0.014
E18	8.57	119.32	8.60	119.26	0.039
P19					
S20	6.96	103.47	6.96	103.43	0.016
D21	7.97	123.94	7.99	123.92	0.018
T22	7.81	109.07	7.81	108.96	0.050
I23	8.44	121.19	8.46	121.36	0.079
E24					
N25	7.86	121.43	7.86	121.30	0.058
V26	8.04	122.30	8.02	122.24	0.028
K27	8.48	119.02	8.47	118.98	0.022
A28	7.91	123.55	7.92	123.35	0.092
K29	7.78	120.27	7.80	120.34	0.033
I30	8.21	121.40	8.20	121.36	0.017
Q31	8.48	123.65	8.46	123.38	0.126
D32	7.95	119.79	8.00	120.02	0.118
K33	7.35	115.50	7.44	115.64	0.108
E34	8.65	114.36	8.64	114.16	0.092
G35	8.43	108.95	8.43	108.94	0.003
I36	6.07	120.36	6.09	120.43	0.037
P37					
P38					
D39	8.46	113.68	8.46	113.63	0.025
Q40	7.74	116.93	7.74	116.83	0.042
Q41	7.41	118.11	7.40	117.94	0.079
R42	8.43	123.13	8.42	123.07	0.028
L43	8.76	124.47	8.72	124.35	0.067
I44	9.01	122.27	9.03	122.30	0.024

F45	8.77	125.24	8.76	125.17	0.038
A46	8.88	132.96	8.78	132.86	0.106
G47	8.06	102.54	7.91	102.30	0.189
K48	7.91	122.06	7.89	121.66	0.182
Q49	8.57	123.04	8.53	122.74	0.141
L50	8.48	125.76	8.50	125.67	0.045
E51	8.32	123.19	8.34	123.13	0.035
D52	8.09	120.42	8.09	120.46	0.020
G53					
R54	7.39	119.40	7.41	119.47	0.038
T55	8.75	108.87	8.74	108.83	0.022
L56	8.07	118.08	8.08	118.12	0.019
S57	8.41	113.58	8.42	113.50	0.036
D58	7.86	124.58	7.88	124.62	0.027
Y59	7.18	115.82	7.19	115.77	0.023
N60	8.08	116.07	8.08	115.96	0.049
I61	7.17	118.95	7.20	118.93	0.027
Q62	7.55	125.00	7.57	124.98	0.030
K63	8.42	120.65	8.38	120.54	0.064
E64	9.24	114.62	9.25	114.64	0.011
S65	7.59	115.01	7.61	115.01	0.022
T66	8.66	117.45	8.65	117.38	0.033
L67	9.34	127.71	9.36	127.55	0.072
H68	9.16	119.15	9.19	118.76	0.176
L69	8.22	124.12	8.27	124.41	0.141
V70	9.11	126.78	9.10	126.62	0.075
L71	8.04	123.09	7.92	123.38	0.173
R72	8.52	123.83	8.55	123.71	0.065
L73	8.29	124.63	8.21	124.13	0.236
R74	8.37	122.06	8.29	121.73	0.167
G75	8.42	111.17	8.38	110.88	0.140
G76	7.87	115.13	7.87	115.07	0.026

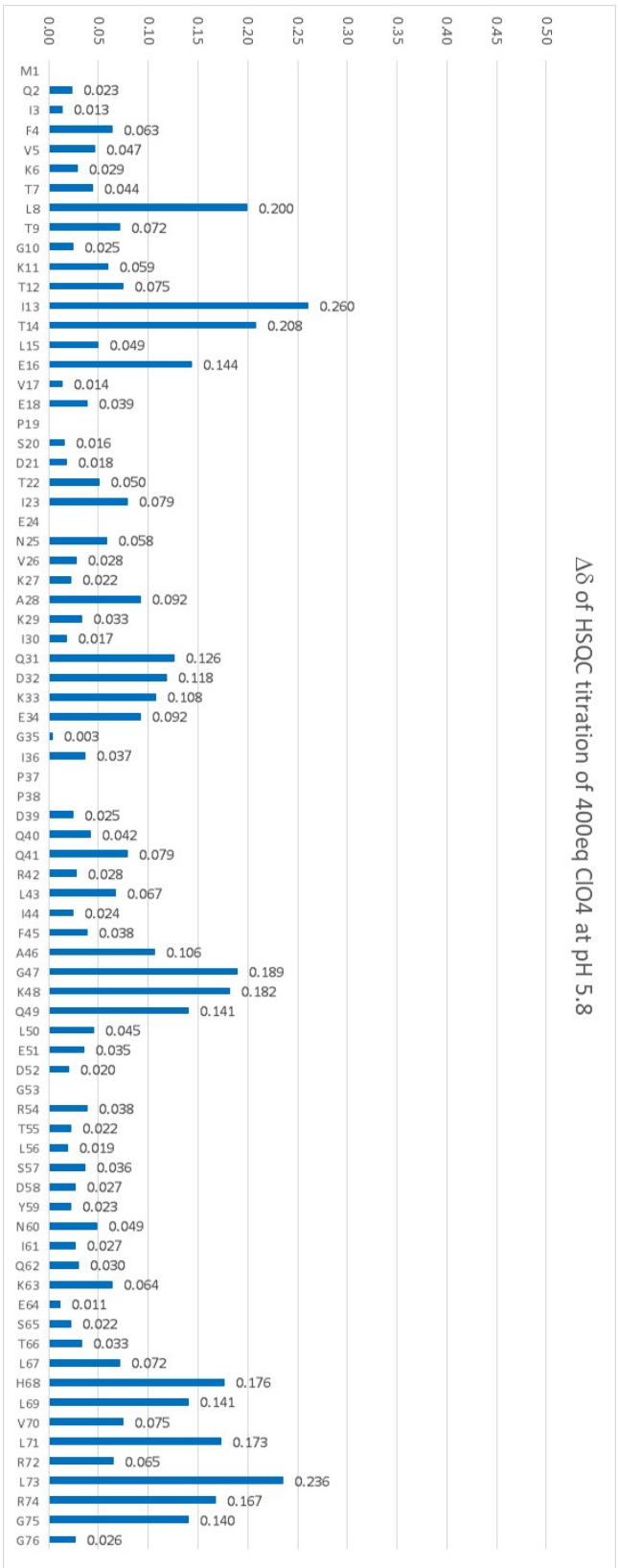


Figure S90 Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ClO_4^- at 400 equiv. of salt., pH 5.8.

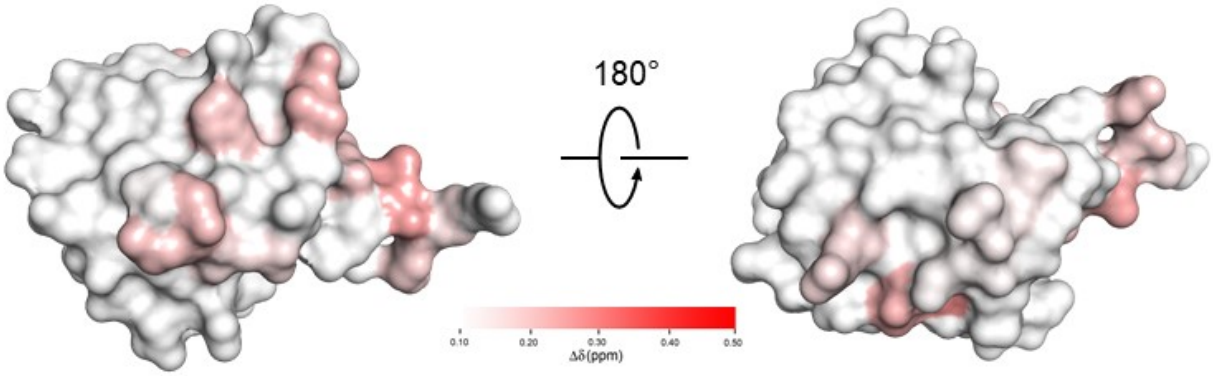


Figure S91 Visualization of the $\Delta\delta_{max}$ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ClO_4^- at 400 equiv. of salt, pH = 5.8.

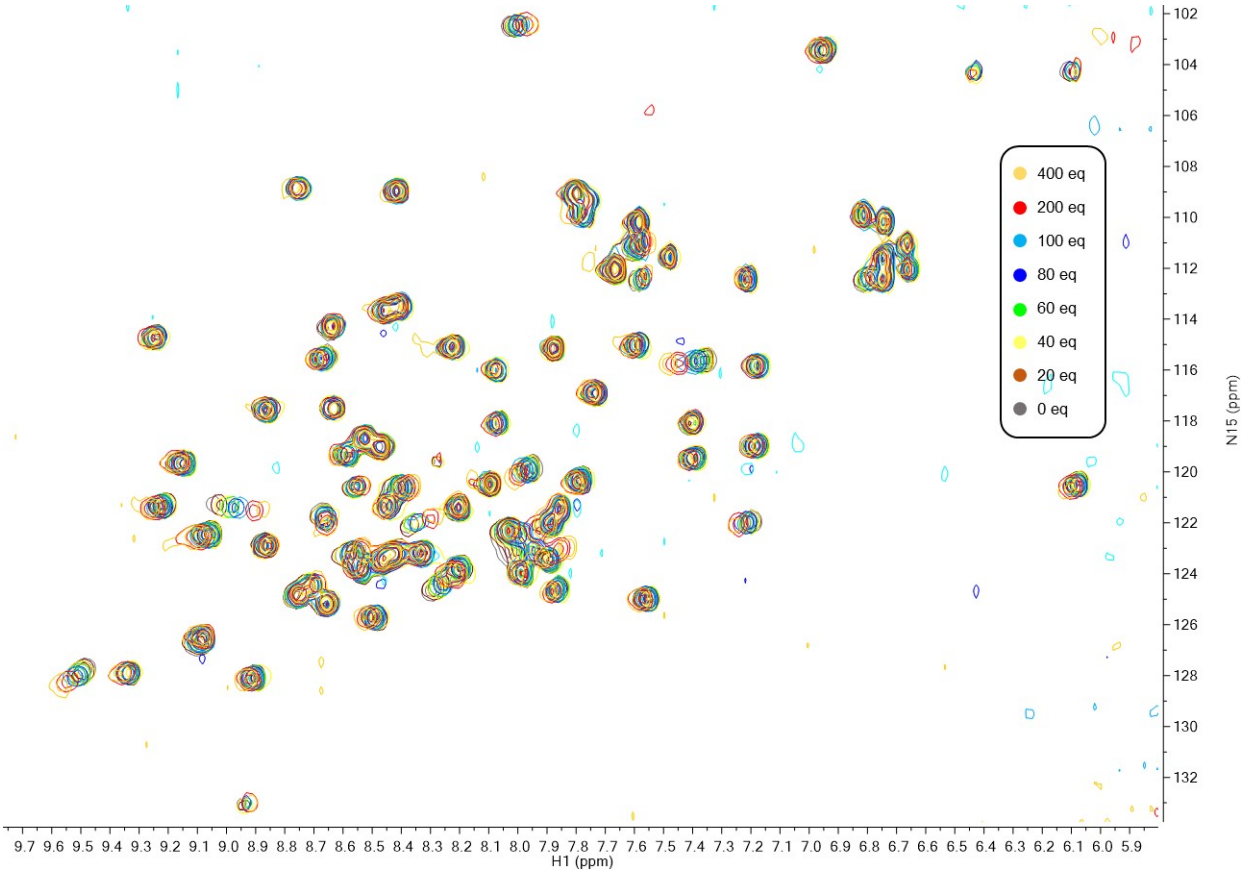


Figure S92 Stack of ^{1}H -^{15}N HSQC titration spectra of 1 mM ubiquitin with ClO_4^- (from 0 to 400 equiv.), in 50 mM phosphate buffer 90% H_2O/10% D_2O, pH = 7.3.

Table S14: ^{1}H -^{15}N HSQC chemical shift data from the titration of 1 mM ubiquitin with ClO_4^- at pH 7.3.

Amino Acid #.	0 equiv.		400 equiv.		Δ
	^{1}H	^{15}N	^{1}H	^{15}N	
M1					
Q2	8.87	122.89	8.85	122.90	0.021
I3	8.23	115.10	8.23	115.10	0.006
F4	8.53	118.70	8.53	118.71	0.004
V5	9.21	121.30	9.26	121.44	0.079
K6	8.91	128.09	8.93	128.17	0.044
T7	8.66	115.47	8.70	115.58	0.063
L8	9.04	121.30	8.87	121.48	0.188
T9					
G10	7.80	109.07	7.80	109.03	0.021
K11	7.20	121.94	7.26	122.09	0.093
T12	8.57	120.63	8.54	120.55	0.049
I13	9.49	127.75	9.56	128.38	0.288
T14	8.67	121.65	8.65	122.14	0.218
L15	8.66	125.20	8.65	125.19	0.012
E16	8.04	122.37	7.90	122.12	0.179
V17	8.86	117.61	8.87	117.53	0.038
E18	8.58	119.32	8.63	119.38	0.052
P19					
S20	6.95	103.44	6.96	103.42	0.014
D21	7.98	123.97	8.01	123.98	0.032
T22	7.80	109.07	7.80	109.03	0.021
I23	8.44	121.31	8.46	121.39	0.043
E24					
N25	7.86	121.48	7.86	121.32	0.070
V26	8.04	122.37	8.03	122.33	0.018
K27	8.48	119.02	8.47	119.01	0.010
A28	7.89	123.50	7.92	123.38	0.061
K29	7.78	120.29	7.80	120.46	0.077
I30	8.21	121.44	8.20	121.29	0.069
Q31	8.47	123.59	8.46	123.35	0.107
D32	7.95	119.82	8.01	120.20	0.184
K33	7.35	115.59	7.48	115.75	0.148
E34	8.65	114.36	8.64	114.24	0.054
G35	8.42	108.95	8.42	108.99	0.019
I36	6.08	120.42	6.11	120.64	0.104
P37					
P38					
D39	8.45	113.66	8.46	113.67	0.009
Q40	7.74	116.95	7.75	116.87	0.037

Q41	7.41	118.10	7.41	118.02	0.034
R42	8.44	123.22	8.46	123.35	0.063
L43	8.74	124.58	8.68	124.43	0.087
I44	9.06	122.45	9.10	122.56	0.069
F45	8.76	124.86	8.75	124.83	0.018
A46	8.95	133.04	8.91	132.98	0.044
G47	8.03	102.48	7.95	102.49	0.072
K48	7.89	122.10	7.90	122.12	0.012
Q49	8.59	123.27	8.53	123.04	0.119
L50	8.49	125.74	8.52	125.73	0.033
E51	8.32	123.18	8.35	123.21	0.034
D52	8.10	120.48	8.09	120.47	0.006
G53					
R54	7.39	119.43	7.43	119.55	0.066
T55	8.76	108.87	8.76	108.79	0.034
L56	8.07	118.07	8.09	118.18	0.052
S57	8.40	113.53	8.42	113.47	0.034
D58	7.86	124.61	7.89	124.69	0.042
Y59	7.18	115.83	7.20	115.86	0.023
N60	8.08	116.04	8.09	115.94	0.047
I61	7.18	118.97	7.21	119.00	0.035
Q62	7.55	124.99	7.58	125.02	0.032
K63	8.42	120.64	8.36	120.57	0.063
E64	9.25	114.69	9.25	114.76	0.034
S65	7.59	115.02	7.61	115.05	0.031
T66	8.64	117.51	8.63	117.56	0.025
L67	9.33	127.90	9.37	127.91	0.033
H68	9.15	119.69	9.18	119.62	0.043
L69	8.20	123.75	8.23	124.01	0.120
V70	9.10	126.75	9.08	126.42	0.149
L71	8.03	123.09	7.83	122.99	0.210
R72	8.53	123.89	8.57	123.70	0.089
L73	8.30	124.64	8.23	124.01	0.293
R74	8.38	122.14	8.28	121.78	0.192
G75					
G76	7.88	115.15	7.88	115.12	0.013

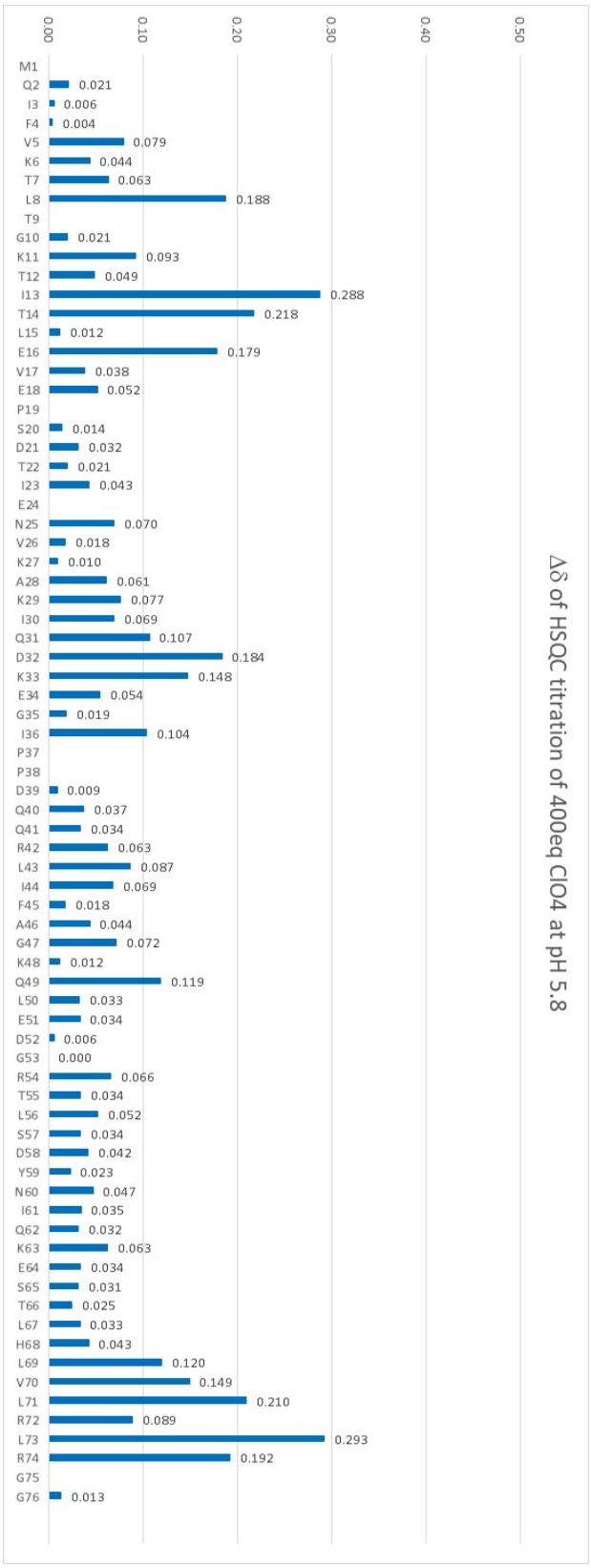


Figure S93 Δδ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ClO_4^- at 400 equiv. of salt., pH 7.3.

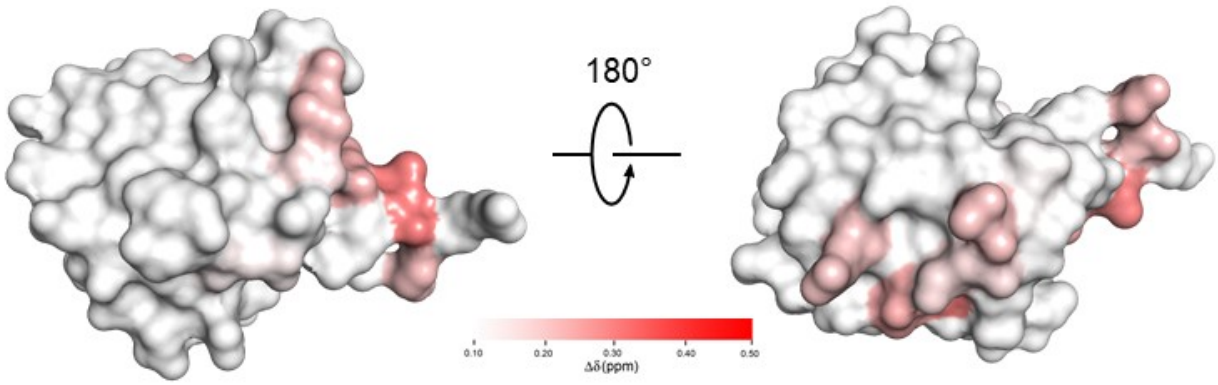


Figure S94 Visualization of the $\Delta\delta_{max}$ values for each backbone N–H of ubiquitin (1 mM) obtained from a ^{1}H-^{15}N HSQC titration with ClO_4^- at 400 equiv. of salt, pH = 7.3.

Table S15:^{1}H -^{15}N HSQC chemical shift data of 1 mM ubiquitin with zero equiv. and 400 equiv. ClO_4^- at five different pH values.

Amino Acid #	pH 2.8	pH 3.8	pH 4.8	pH 5.8	pH 7.3
M1					
Q2	0.174	0.111	0.061	0.023	0.021
I3	0.072	0.070	0.063	0.013	0.006
F4	0.026	0.057	0.024	0.063	0.004
V5	0.035	0.087	0.136	0.047	0.079
K6	0.155	0.147	0.063	0.029	0.044
T7	0.127	0.155	0.047	0.044	0.063
L8	0.323	0.306	0.258	0.200	0.188
T9	0.101	0.106	0.099	0.072	0.000
G10	0.094	0.130	0.047	0.025	0.021
K11	0.089	0.094	0.087	0.059	0.093
T12	0.179	0.152	0.127	0.075	0.049
I13	0.095	0.229	0.284	0.260	0.288
T14	0.126	0.148	0.201	0.208	0.218
L15	0.129	0.135	0.024	0.049	0.012
E16	0.438	0.392	0.210	0.144	0.179
V17	0.238	0.188	0.054	0.014	0.038
E18	0.050	0.058	0.064	0.039	0.052
P19					
S20	0.010	0.065	0.031	0.016	0.014
D21	0.048	0.050	0.083	0.018	0.032
T22	0.064	0.037	0.065	0.050	0.021
I23	0.024	0.035	0.004	0.079	0.043
E24					
N25	0.139	0.116	0.036	0.058	0.070
V26	0.043	0.020	0.031	0.028	0.018
K27	0.005	0.054	0.022	0.022	0.010
A28	0.117	0.121	0.092	0.092	0.061
K29	0.154	0.149	0.103	0.033	0.077
I30	0.138	0.117	0.057	0.017	0.069
Q31	0.129	0.080	0.063	0.126	0.107
D32	0.199	0.192	0.197	0.118	0.184
K33	0.255	0.243	0.158	0.108	0.148
E34	0.192	0.183	0.058	0.092	0.054
G35	0.026	0.046	0.017	0.003	0.019
I36	0.070	0.100	0.111	0.037	0.104
P37					
P38					
D39	0.061	0.050	0.033	0.025	0.009
Q40	0.063	0.068	0.054	0.042	0.037
Q41	0.040	0.017	0.045	0.079	0.034
R42	0.087	0.087	0.059	0.028	0.063
L43	0.115	0.088	0.086	0.067	0.087

I44	0.157	0.151	0.100	0.024	0.069
F45	0.200	0.188	0.162	0.038	0.018
A46	0.137	0.106	0.127	0.106	0.044
G47	0.398	0.387	0.329	0.189	0.072
K48	0.206	0.134	0.066	0.182	0.012
Q49	0.114	0.117	0.067	0.141	0.119
L50	0.059	0.071	0.051	0.045	0.033
E51	0.026	0.024	0.037	0.035	0.034
D52	0.076	0.041	0.030	0.020	0.006
G53					
R54	0.045	0.075	0.054	0.038	0.066
T55	0.075	0.037	0.046	0.022	0.034
L56	0.077	0.080	0.033	0.019	0.052
S57	0.036	0.041	0.050	0.036	0.034
D58	0.040	0.064	0.053	0.027	0.042
Y59	0.032	0.053	0.013	0.023	0.023
N60	0.052	0.108	0.076	0.049	0.047
I61	0.034	0.078	0.020	0.027	0.035
Q62	0.046	0.061	0.022	0.030	0.032
K63	0.120	0.120	0.084	0.064	0.063
E64	0.054	0.004	0.068	0.011	0.034
S65	0.050	0.090	0.022	0.022	0.031
T66	0.052	0.048	0.044	0.033	0.025
L67	0.014	0.051	0.074	0.072	0.033
H68	0.075	0.104	0.053	0.176	0.043
L69	0.404	0.367	0.135	0.141	0.120
V70	0.205	0.212	0.201	0.075	0.149
L71	0.280	0.281	0.274	0.173	0.210
R72	0.076	0.164	0.127	0.065	0.089
L73	0.181	0.214	0.208	0.236	0.293
R74	0.220	0.238	0.182	0.167	0.192
G75	0.232	0.200	0.176	0.140	
G76	0.135	0.099	0.01	0.026	0.013
Avg.	0.119	0.121	0.089	0.071	0.069

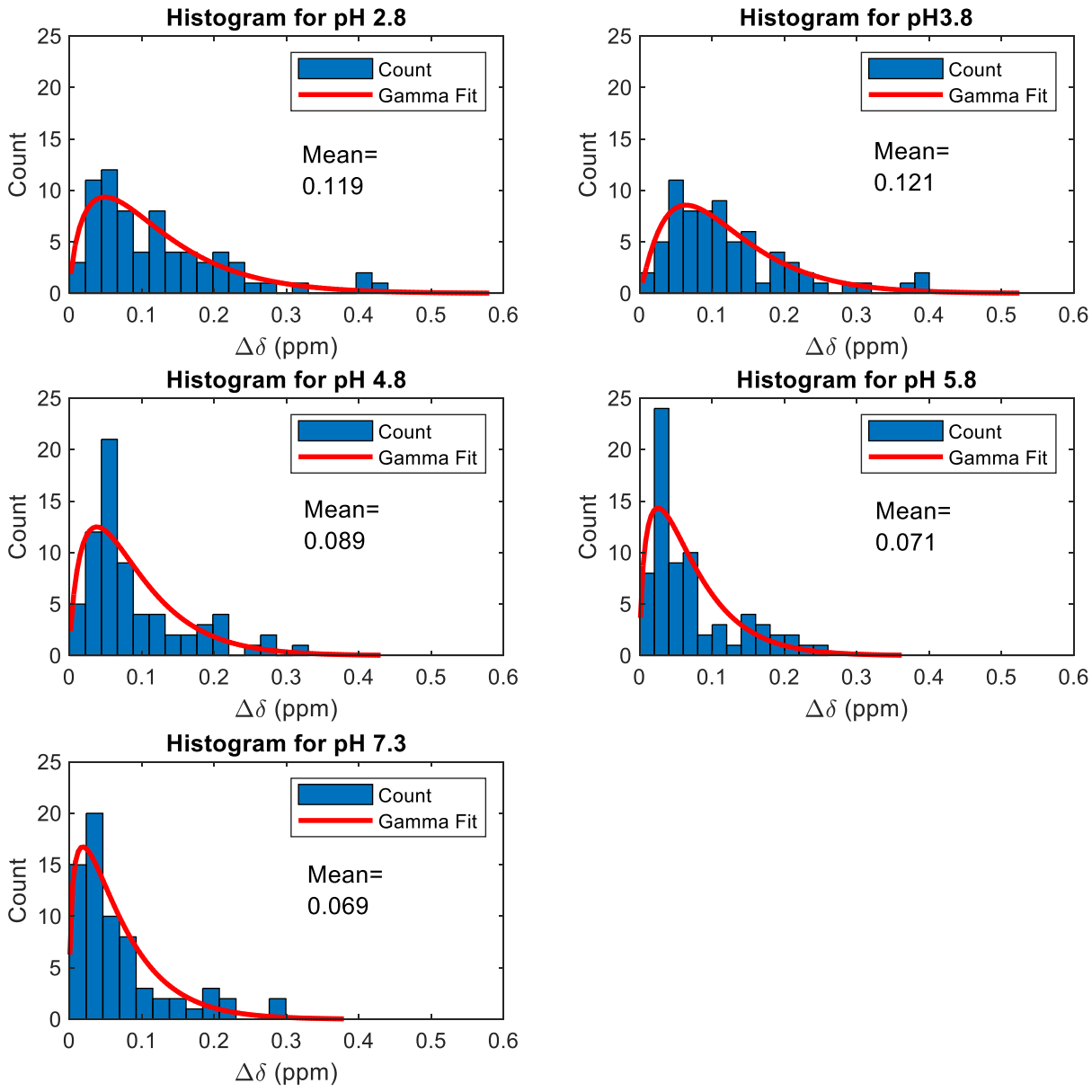


Figure S95 Histograms and gamma distribution fitting of ^{1}H -^{15}N HSQC spectra chemical shift data of 1 mM ubiquitin in the presence and absence of 400 equiv.ClO_4^-, at five different pH values.

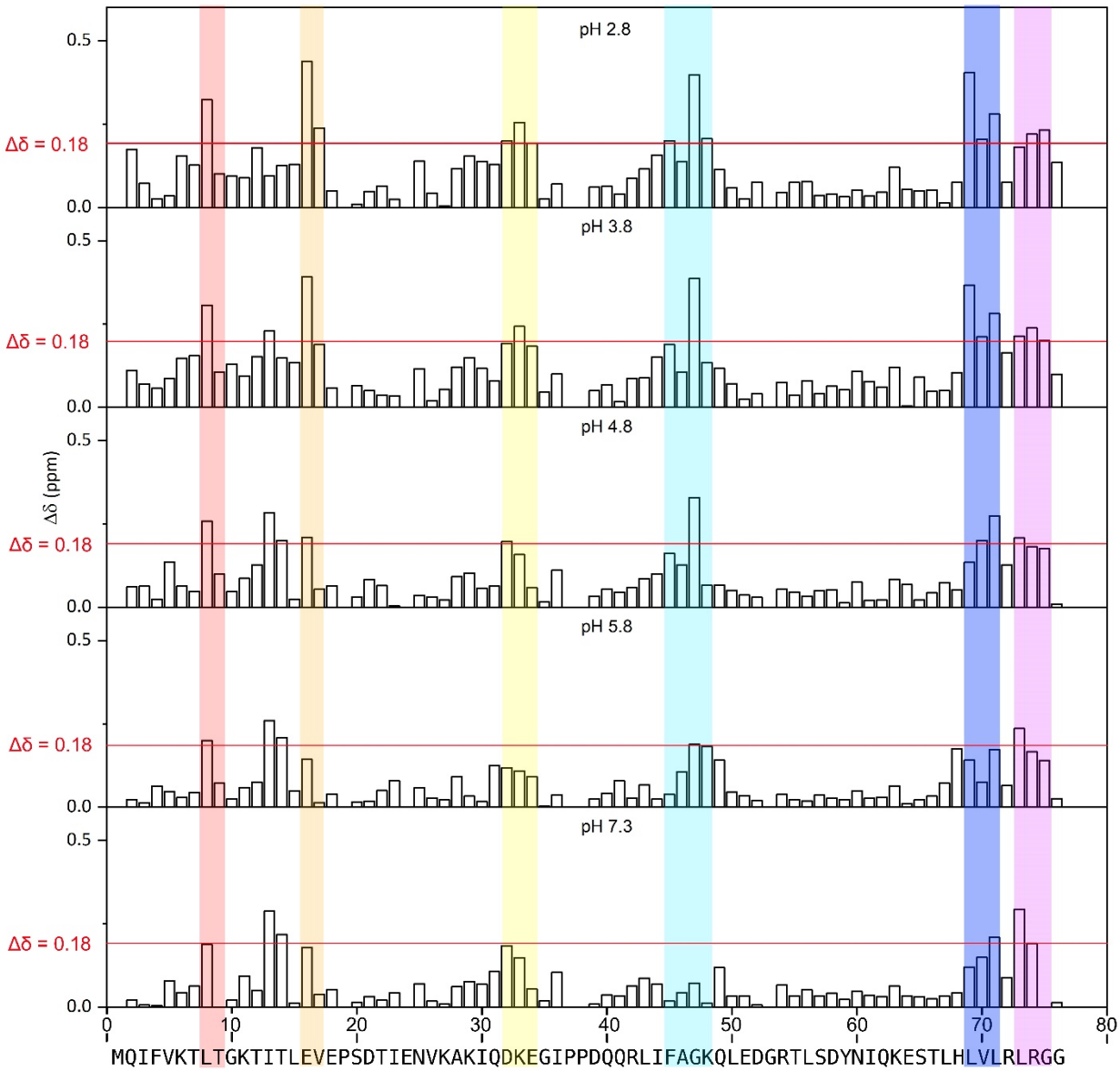


Figure S96 The six anion binding regions highlighted on the bar graphs of 400 equiv.ClO_4^-, at five different pH values.. The chemical shift (Δδ) values are from ^1H -^{15}N HSQC titration of 1 mM ubiquitin.

Table S16: The binding constant of ClO_4^- to selected residues from the titration of 1 mM ubiquitin at different pHs.

Binding site #	pH 2.8[#]		pH 3.8		pH 4.8		pH 5.8		pH 7.3	
	Residue	K_a (M^{-1})	Residue	K_a (M^{-1})	Residue	K_a (M^{-1})	Residue	K_a (M^{-1})	Residue	K_a (M^{-1})
1 (L8+T9)	L8	25	L8	33	L8	34	L8	4	L8	12
	T9	33	T9	32	T9	32	T9	*	T9	*
2 (E16-V17)	E16	10	E16	19	E16	23	E16	15	E16	2
	V17	8	V17	13	V17	*	V17	*	V17	*
3 (D32-E34)	D32	12	D32	10	D32	8	D32	4	D32	5
	K33	7	K33	7	K33	8	K33	2	K33	6
	E34	23	E34	25	E34	*	E34	2	E34	*
4 (F45-K48)	F45	26	F45	26	F45	21	F45	*	F45	*
	A46	20	A46	32	A46	40	A46	4	A46	*
	G47	24	G47	37	G47	28	G47	4	G47	15
	K48	15	K48	22	K48	*	K48	4	K48	11
5 (L69-L71)	L69	16	L69	30	L69	*	L69	9	L69	11
	V70	22	V70	35	V70	16	V70	*	V70	13
	L71	22	L71	31	L71	17	L71	5	L71	12
6 (L73-G75)	L73	66	L73	74	L73	17	L73	9	L73	6
	R74	14	R74	19	R74	30	R74	3	R74	5
	G75	13	G75	15	G75	32	G75	2	G75	*

Note: [#] data of pH 2.8 is the same data set of ClO_4^- at Table S10; [*] bad fitting

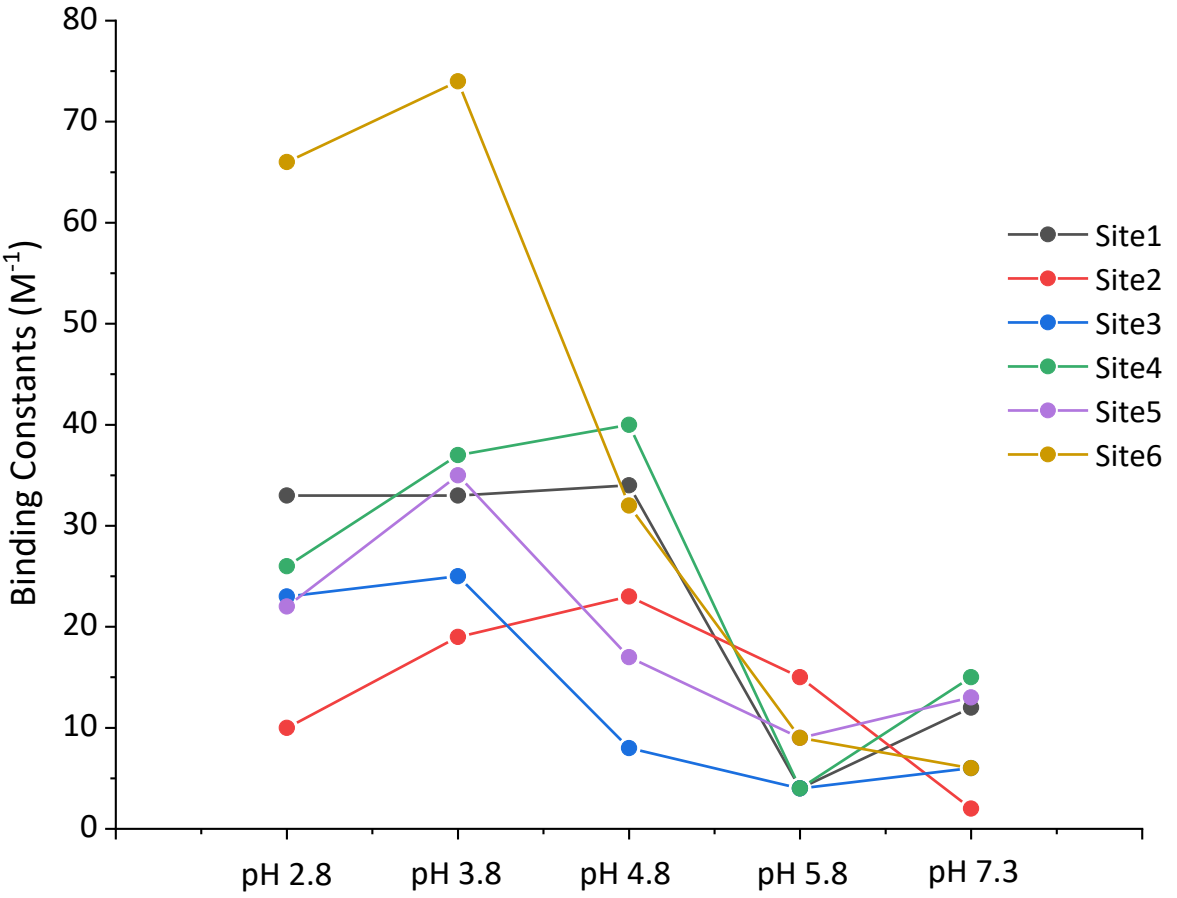
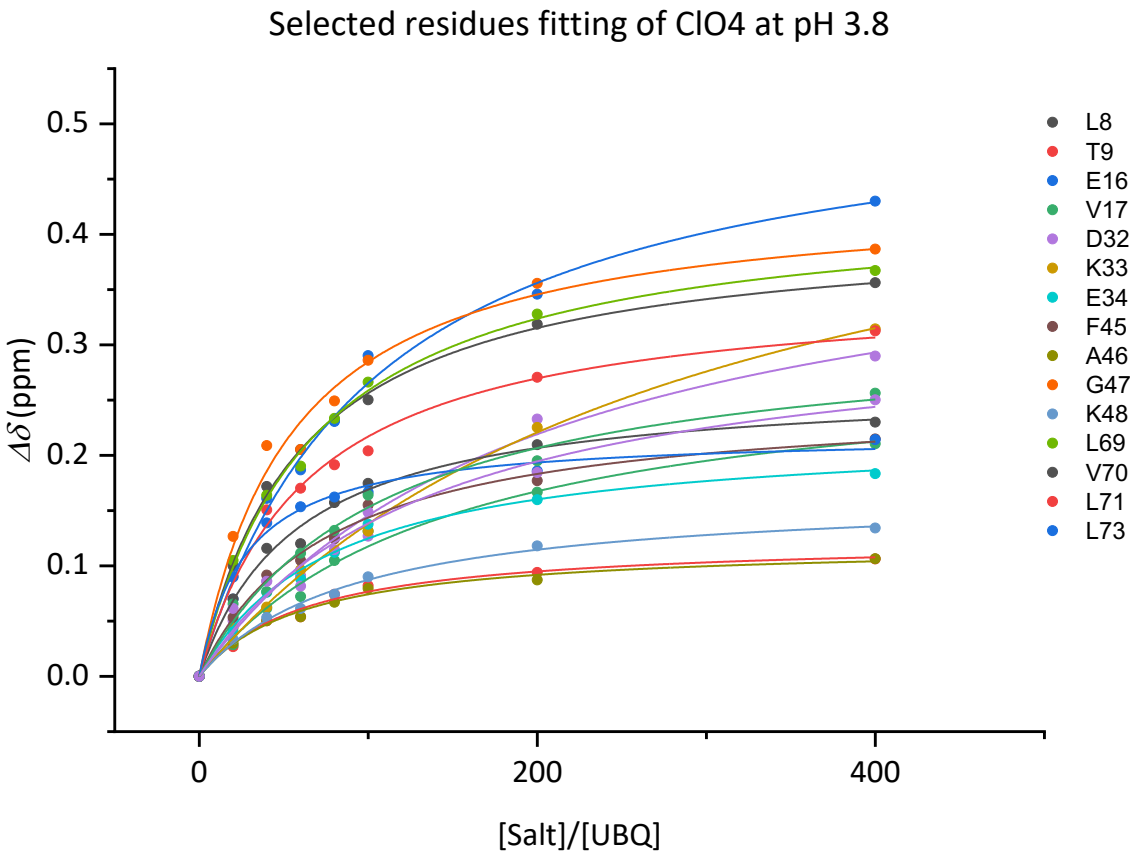
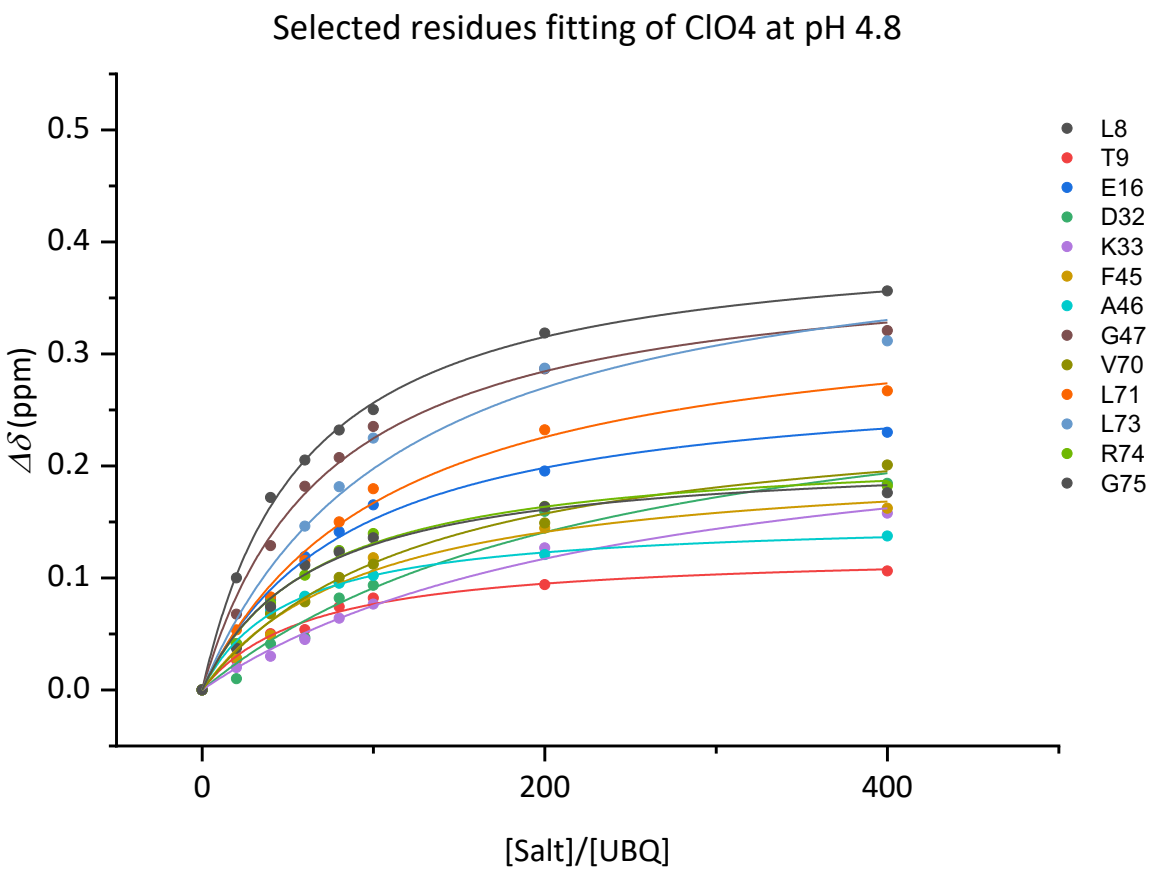


Figure S97 The strongest recorded affinities of ClO_4^- at each binding site as a function of pH (50 mM phosphate buffer).



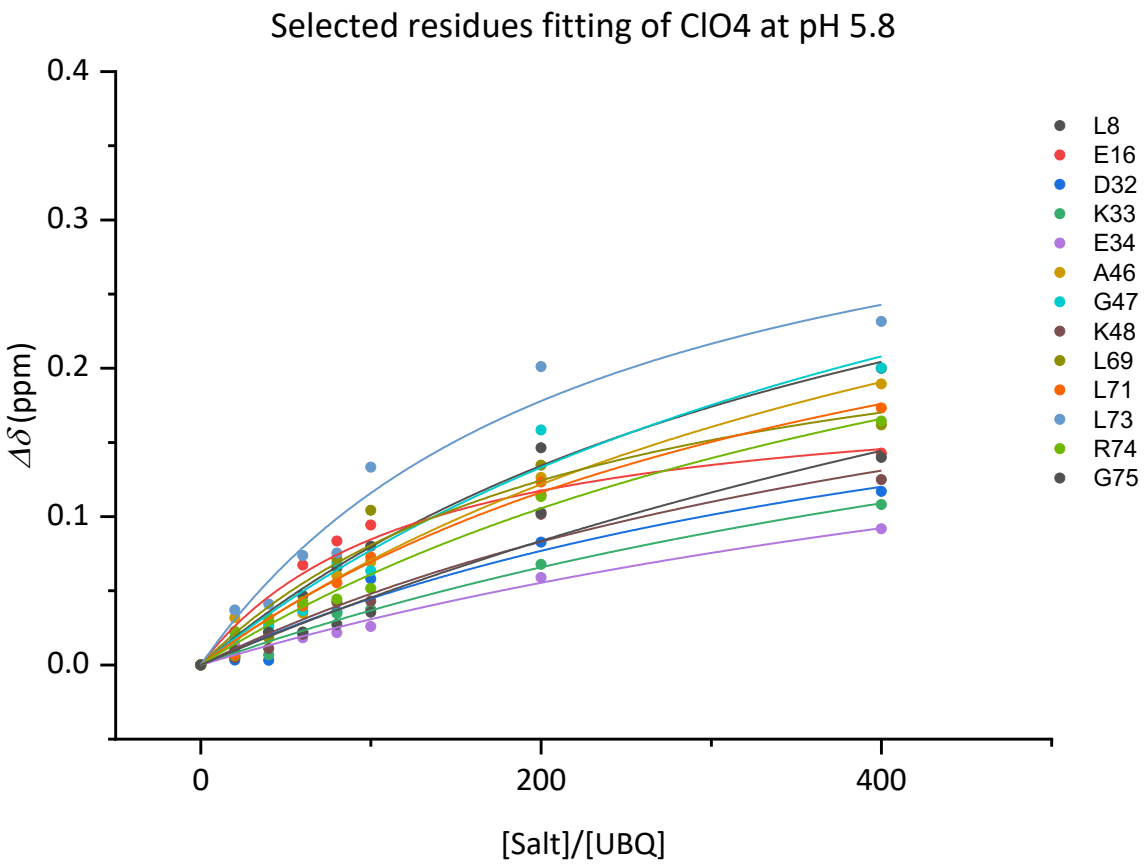
Model	NewFunction2 (User)																
Equati	0.5*a*((1+x+b*((x*0.0005+5)/0.0025))-sqrt((1+x+b*((x*0.0005+5)/0.0025))^2-4*x))																
Plot	L8	T9	E16	V17	D32	K33	E34	F45	A46	G47	K48	L69	V70	L71	L73	R74	G75
a	0.4112	0.1252	0.5453	0.2946	0.451	0.5667	0.2245	0.2543	0.1208	0.4413	0.1677	0.4345	0.2670	0.3575	0.2201	0.3214	0.3309
b	0.0297	0.0311	0.0519	0.0742	0.103	0.1533	0.0392	0.0379	0.0308	0.0270	0.0453	0.0334	0.0285	0.0318	0.0134	0.0542	0.0685
Redu	2.0288	1.9144	2.1355	6.2631	1.560	3.0176	4.4311	4.4490	1.5197	2.4879	1.2444	5.2349	5.7883	7.9616	4.1928	1.0683	1.9153
R-Squ	0.9986	0.9869	0.9908	0.9892	0.985	0.9975	0.9899	0.9919	0.9885	0.9862	0.9944	0.9968	0.9912	0.9929	0.9919	0.9858	0.9728
Adj. R	0.9984	0.9847	0.9893	0.9874	0.982	0.9971	0.9882	0.9906	0.9866	0.9839	0.9935	0.9963	0.9897	0.9917	0.9905	0.9835	0.9682

Figure S98 1:1 Fitting of the signal shifts for selected N–H signals in the ^{1}H -^{15}N HSQC spectra from titrating 1 mM ubiquitin with ClO_4^- in 50 mM phosphate buffer, pH = 3.8.



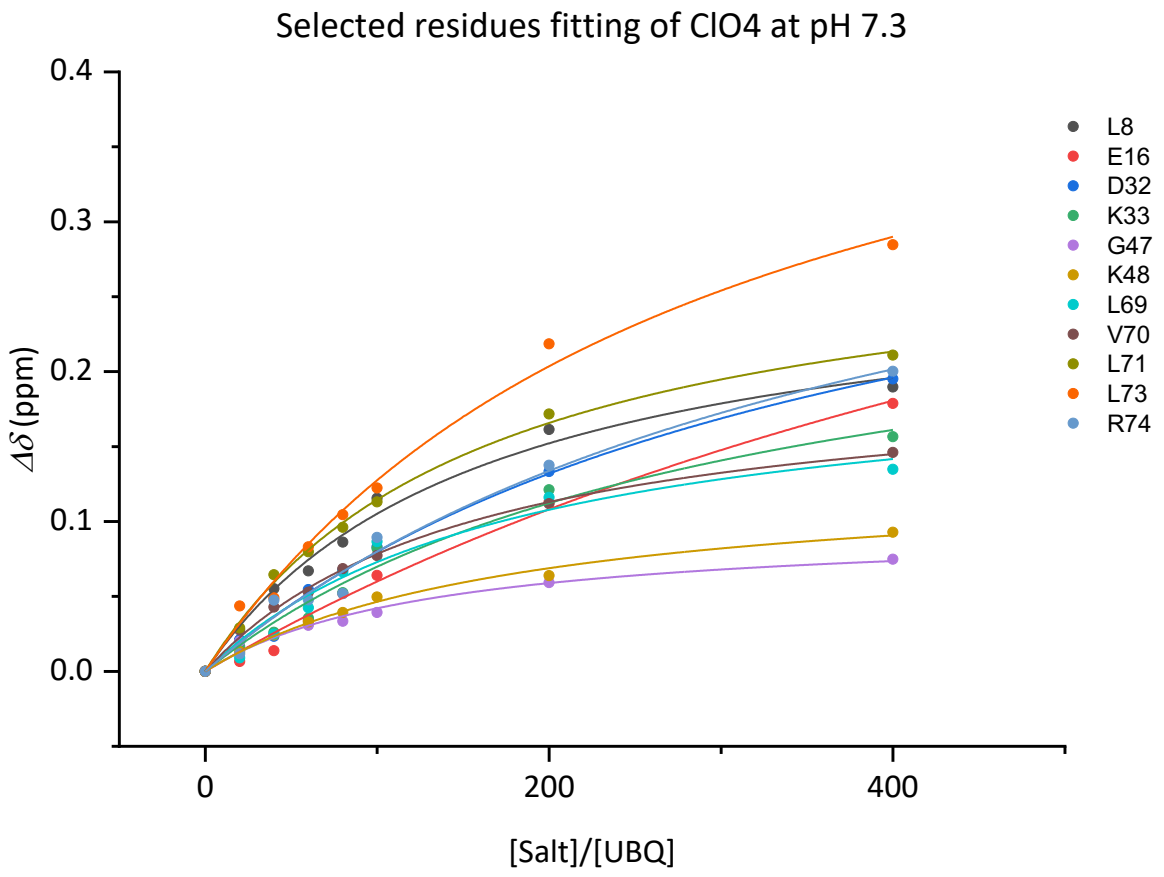
Model	NewFunction2 (User)												
Equatio	0.5*a*((1+x+b*((x*0.0005+5)/0.0025))-sqrt((1+x+b*((x*0.0005+5)/0.0025))^2-4*x))												
Plot	L8	T9	E16	D32	K33	F45	A46	G47	V70	L71	L73	R74	G75
a	0.4112 ±	0.12526	0.28625	0.31702	0.27036	0.2103 ±	0.15415	0.39001	0.26039	0.35142	0.43027	0.21901	0.21293
b	0.02972	0.03119	0.0433	0.12249	0.1279 ±	0.04799	0.02492	0.03623	0.06395	0.05441	0.05803	0.0331 ±	0.03152
Reduce	2.02885	1.91444	7.6842E	1.47627	3.20869	6.57635	3.19066	9.26127	3.34936	7.23055	5.24971	3.86077	7.06515
R-Squa	0.99869	0.98695	0.98915	0.97165	0.99059	0.9824	0.9986	0.99327	0.99264	0.99239	0.96543	0.99136	0.98388
Adj. R-	0.99847	0.98477	0.98734	0.96693	0.98902	0.97946	0.99837	0.99215	0.99142	0.99112	0.95967	0.98992	0.98119

Figure S99 1:1 Fitting of the signal shifts for selected N–H signals in the ^{1}H -^{15}N HSQC spectra from titrating 1 mM ubiquitin with ClO_4^- in 50 mM phosphate buffer, pH = 4.8.



Model	NewFunction2 (User)												
Equatio	0.5*a*((1+x+b*((x*0.0005+5)/0.0025))-sqrt((1+x+b*((x*0.0005+5)/0.0025))^2-4*x))												
Plot	L8	E16	D32	K33	E34	A46	G47	K48	L69	L71	L73	R74	G75
a	0.44604	0.19365	0.28838	0.34224	0.29882	0.46425	0.50109	0.32718	0.27562	0.37602	0.39089	0.40866	0.5901 ±
b	0.22722	0.06347	0.26911	0.41106	0.43031	0.27545	0.27055	0.28753	0.11902	0.21825	0.11706	0.28099	0.59315
Reduce	6.63025	1.14316	9.99766	1.91978	2.00409	6.5643E	1.93273	1.07906	2.6995E	3.17226	3.03197	4.78303	1.0264E
R-Squa	0.98786	0.96165	0.95291	0.98787	0.98052	0.98536	0.96728	0.95672	0.93829	0.99225	0.9616	0.98581	0.96385
Adj. R-	0.98584	0.95526	0.94506	0.98585	0.97727	0.98292	0.96183	0.94951	0.928	0.99096	0.95521	0.98345	0.95782

Figure S100 1:1 Fitting of the signal shifts for selected N–H signals in the ^{1}H -^{15}N HSQC spectra from titrating 1 mM ubiquitin with ClO_4^- in 50 mM phosphate buffer, pH = 5.8.



Model	NewFunction2 (User)										
Equation	0.5*a*((1+x+b*((x*0.0005+5)/0.0025))-sqrt((1+x+b*((x*0.0005+5)/0.0025))^2-4*x))										
Plot	L8	E16	D32	K33	G47	K48	L69	V70	L71	L73	R74
a	0.27889 ±	0.59582 ±	0.39629 ±	0.29418 ±	0.09931 ±	0.1353 ±	0.21005 ±	0.20521 ±	0.30412 ±	0.51854 ±	0.42578 ±
b	0.08141 ±	0.44165 ±	0.19608 ±	0.15857 ±	0.06701 ±	0.09438 ±	0.09268 ±	0.07974 ±	0.08158 ±	0.15134 ±	0.2136 ±
Reduced	6.59012E-	4.16712E-	3.24654E-	7.53197E-	1.27017E-	6.64781E-	1.10766E-	6.76573E-	1.60929E-	9.21004E-	8.63645E-
R-Square	0.98693	0.99047	0.9934	0.97832	0.97976	0.99342	0.96146	0.99733	0.99718	0.99127	0.9835
Adj. R-Sq	0.98475	0.98888	0.9923	0.97471	0.97638	0.99233	0.95504	0.99688	0.99671	0.98982	0.98075

Figure S101 1:1 Fitting of the signal shifts for selected N–H signals in the ^{1}H -^{15}N HSQC spectra from titrating 1 mM ubiquitin with ClO_4^- in 50 mM phosphate buffer, pH = 7.3.

6. References:

1 Kelly, S. M., Jess, T. J. & Price, N. C. How to study proteins by circular dichroism. *Biochim Biophys Acta* **1751**, 119-139, doi:10.1016/j.bbapap.2005.06.005 (2005).

2 Williamson, M. P. Using chemical shift perturbation to characterise ligand binding. *Prog Nucl Magn Reson Spectrosc* **73**, 1-16, doi:10.1016/j.pnmrs.2013.02.001 (2013).

3 Becker, W., Bhattiprolu, K. C., Gubensak, N. & Zangger, K. Investigating Protein-Ligand Interactions by Solution Nuclear Magnetic Resonance Spectroscopy. *Chemphyschem* **19**, 895-906, doi:10.1002/cphc.201701253 (2018).

4 Ziarek, J. J., Peterson, F. C., Lytle, B. L. & Volkman, B. F. Binding site identification and structure determination of protein-ligand complexes by NMR a semiautomated approach. *Methods Enzymol* **493**, 241-275, doi:10.1016/B978-0-12-381274-2.00010-8 (2011).

5 Weber, P. L., Brown, S. C. & Mueller, L. Sequential 1H NMR assignments and secondary structure identification of human ubiquitin. *Biochemistry* **26**, 7282-7290, doi:10.1021/bi00397a013 (1987).

6 Edwards, L. J., Savostyanov, D. V., Welderufael, Z. T., Lee, D. & Kuprov, I. Quantum mechanical NMR simulation algorithm for protein-size spin systems. *J Magn Reson* **243**, 107-113, doi:10.1016/j.jmr.2014.04.002 (2014).

7 Vijay-kumar, S., Bugg, C. E. & Cook, W. J. Structure of ubiquitin refined at 1.8 Å resolution. *Journal of Molecular Biology* **194**, 531-544, doi:10.1016/0022-2836(87)90679-6 (1987).

8 Cierpicki, T., Zhukov, I., Byrd, R. A. & Otlewski, J. Hydrogen bonds in human ubiquitin reflected in temperature coefficients of amide protons. *J Magn Reson* **157**, 178-180, doi:10.1006/jmre.2002.2597 (2002).

9 Vijay-Kumar, S., Bugg, C. E. & Cook, W. J. Structure of Ubiquitin Refined at the 1.8 Å Resolution. *Mol. Biol.* **194**, 531-544 (1987).

10 Watson, J. D. & Milner-White, E. J. A Novel Main-chain Anion-binding Site in Proteins: The Nest. A Particular Combination of f,c Values in Successive Residues Gives Rise to Anion-binding Sites That Occur Commonly And Are Found Often at Functionally Important Regions. *J. Mol. Biol.* **315**, 171-182 (2002).

11 Denessiouk, K. A., Johnson, M. S. & Denesyuk, A. I. Novel $C^{\alpha}NN$ structural motif for protein recognition of phosphate ions. *J. Mol. Biol.* **345**, 611-629, doi:10.1016/j.jmb.2004.10.058 (2005).