Supporting Information

Electrochemical reduction of disulfide-containing proteins for hydrogen/deuterium exchange monitored by mass spectrometry

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EXPERIMENTAL SECTION

Materials. Recombinant human insulin (containing two Zn^{2+} ions per insulin hexamer) and ammonium-d_4 acetate-d_3 (99 atom% D) was purchased from Sigma-Aldrich (St. Louis, MO, USA). D_2O (99.9 atom % D) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Deuterated PBS buffer was prepared from PBS tablets (0.01 M phosphate buffer, 2.7 mM KCl and 137 mM NaCl, Sigma-Aldrich). Zinc acetate and all other chemicals were of the highest grade commercially available.

Sample Preparation. A slightly alkaline insulin stock solution (4.0 mM insulin, 50 mM ammonium acetate, pH 8.8) was freshly prepared. The stock solution was used to prepare the insulin solution (2.0 mM) for the investigation of insulin self-assembly in 50 mM ammonium acetate, 2 mM Zn^{2+}, 1 mM ammonium chloride and 30 mM phenol. Note, the value include the Zn^{2+} ions originating from the lyophilized insulin sample, which contained two Zn^{2+} ions for every six insulin molecules.

Recombinant human $uPAR^{wt}$ and $uPAR^{H47C\text{-}N259C}$ were expressed as secreted proteins (residues 1-283) by Drosophila melanogaster S2-cells and affinity purified as described previuosly.[18] Purified $uPAR^{wt}$ and $uPAR^{H47C\text{-}N259C}$ was dissolved in PBS buffer to a concentration of 33 μM.

Isotopic exchange. Isotopic exchange was initiated by diluting a 2 mM insulin solution 5-fold into 300 mM deuterated ammonium acetate buffer. The exchange was performed at 25°C in an Eppendorf Thermomixer. After dilution, the pH of the exchange solutions was 8.73 ±0.04 (uncorrected for the deuterium isotope effect on the pH glass electrode). After 10, 100 and 1000 seconds, aliquots were withdrawn and isotopic exchange quenched by 400-fold dilution into iced 1% formic acid solution and snap frozen in liquid nitrogen.

Fully exchanged insulin controls were prepared by incubation for 24 h in 50 mM ammonium acetate and 0.7 mM Zn^{2+}. Isotopic exchange was expected to be complete, as no additional deuterium incorporation could be observed after 48 hours of exchange. Chemically reduced insulin chains were prepared by incubating insulin (0.2 mM) at pH 2.5 in 25 mM TCEP for 3 h at 30°C. The reduced chains were equilibrium labeled with 80 atom % D by a 5-fold dilution into D_2O followed by 3 h exchange at 25°C.

For chemical reduction measurements, isotopic exchange of $uPAR^{wt}$ and $uPAR^{H47C\text{-}N259C}$ was initiated by 20-fold dilution into deuterated PBS (95%D, pD 7.8) at 25°C in an Eppendorf

Thermomixer. After 10, 100 and 1000 seconds aliquots were withdrawn and isotopic exchange quenched by adding FA to a final concentration of 0.5%, followed by snap freezing in liquid nitrogen. Samples were thawed, diluted 1:1 with 800 mM TCEP (pH 2.5) and incubated on ice for 2 minutes prior to injection.

For electrochemical reduction measurements, isotopic exchange of $uPAR^{wt}$ and $uPAR^{H47C-N259C}$ was initiated by 5-fold dilution into deuterated PBS (80%D, pD 7.8) at 25°C in an Eppendorf Thermomixer. After 10, 100 and 1000 seconds aliquots were withdrawn and isotopic exchange quenched by 10-fold dilution into iced 1% formic acid solution, followed by snap freezing in liquid nitrogen.

Liquid chromatography and mass spectrometry (extended). The desalting flow was delivered by an Agilent 1260 Infinity Quaternary pump (Agilent Technologies, Santa Clara, CA, USA). A Zorbax Stablebond Guard Column (1.0 x 17 mm, Agilent Technologies) was used for trapping and desalting samples. The desalting time was 4 min for the analysis of the intact chains (i.e., without the pepsin column), while it was 6 min for the local analysis with the pepsin column present. Peptides were separated along a 1.0 x 100 mm ACQUITY UPLC BEH 1.7 µm C18 column (Waters) with a linear acetonitrile gradient (5 – 50% over 6 min for insulin, 5 – 50% over 12 min for uPAR) with 0.23% (v/v) formic acid at 40 µL/min provided by a nanoAcquity UPLC Binary Solvent Manager (Waters). MS of intact uPAR was performed using a MCP detector voltage of 1700 V, while MS of insulin and peptides was performed using a voltage of 1950 V.

Data analysis. Peptides from peptic digests were identified from DDA MS/MS runs using ProteinLynx Global Server v2.4 (Waters) and MassAI v1.05 (MassAI Bioinformatics, Stenstrup, DK, http://www.massai.dk). Deuterium incorporation for intact proteins and peptides was quantified using DynamX V1.0 (Waters). The insulin hexamer (R state) visualizations (PDB ID 1EV6) were created using VMD 1.9.1 (University of Illinois, http://www.ks.uiuc.edu)[24] and rendered using POV-Ray 3.6 (Persistence of Vision Pty. Ltd., Williamstown, Victoria, AU, http://www.povray.org).

Back-exchange measurements using Human Growth Hormone. Recombinant human growth hormone was obtained from Novo Nordisk (Bagsværd, Denmark). Deuterated human growth hormone was prepared by dissolving lyophilized recombinant human growth hormone in

deuterated PBS buffer to a concentration of 50 μM. The solution was incubated at 37°C for 24 hours and then stored at 4°C for several days before analysis. Back-exchange measurements of human growth hormone were performed on a similar setup using Waters HDX nanoAcquity UPLC system including the HD-x PAL auto sampler (LEAP Technologies Inc., Carrboro, NC, USA) for automated sample preparation coupled to a Waters Synapt G2 mass spectrometer. The desalting flow rate over the cell onto the trap-column was 50 μL/min and the protein was trapped for 5 min. The experiments were performed without any pepsin digestion and the cell was either placed within the refrigerated chamber of the HDX-unit or outside the chamber (Fig. 1b).

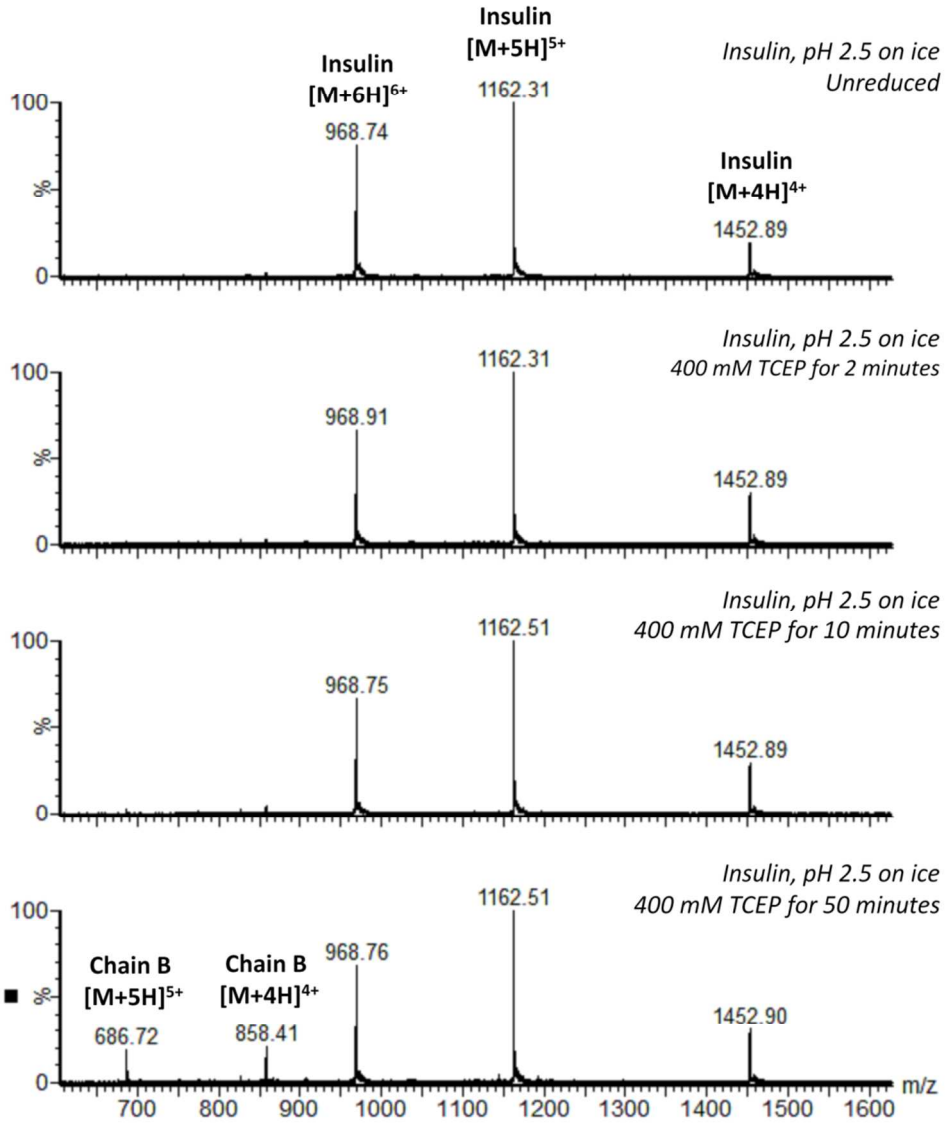

Figure S1. Chemical reduction of insulin using TCEP under quench conditions, pH 2.5, 0°C. Insulin samples were incubated with 400 mM TCEP for 2, 10 and 50 minutes and analyzed using ESI-MS.

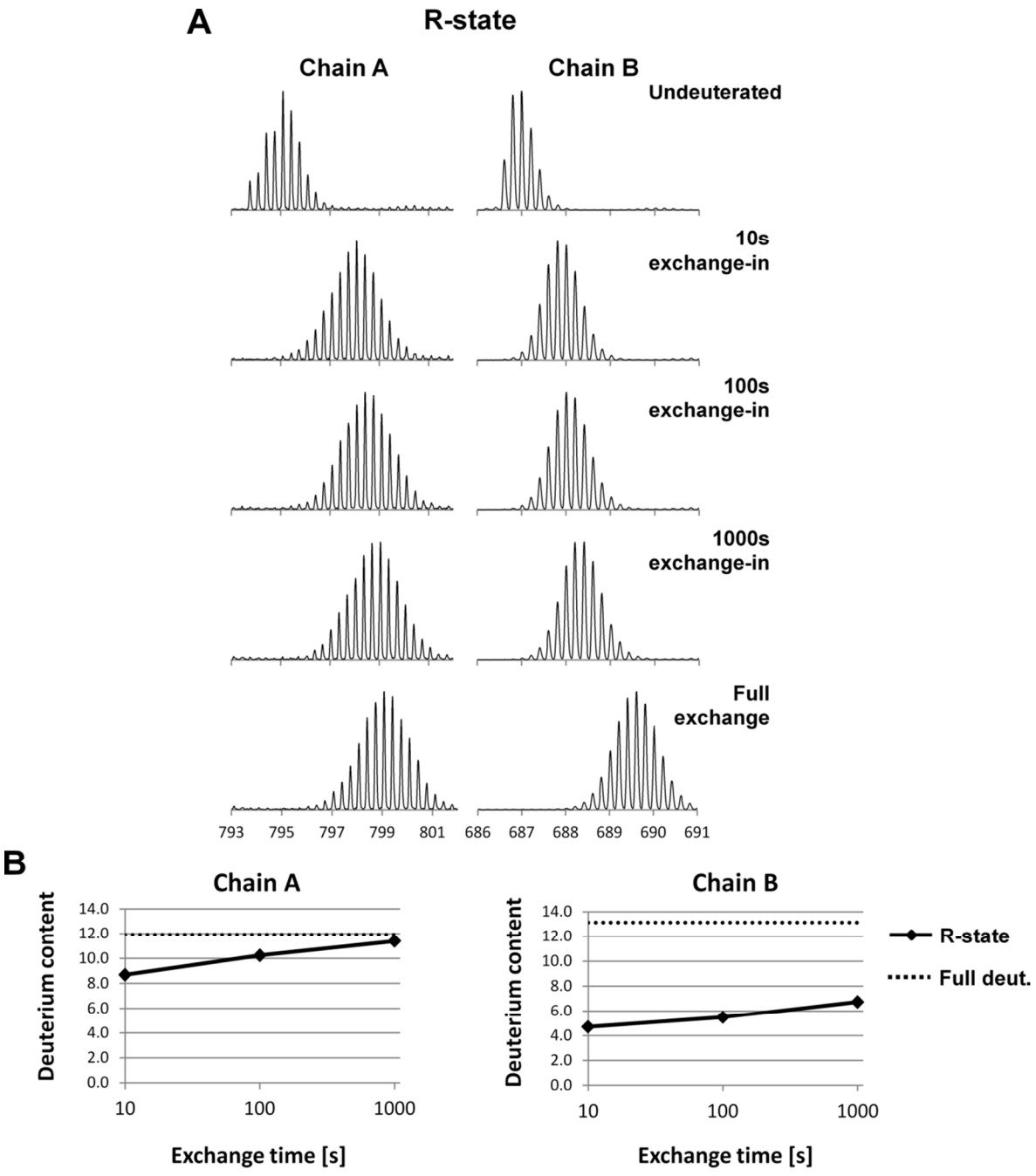

Figure S2. (A) Mass spectra depicting deuterium uptake in chain A and B derived from Insulin forming R-state hexamers after 10, 100 and 1000 seconds of deuteration. (B) Deuterium uptake plots representing the average deuterium content of the chains after 10, 100 and 1000 seconds. References depicting full deuteration were obtained by deuteration for 24 hours.

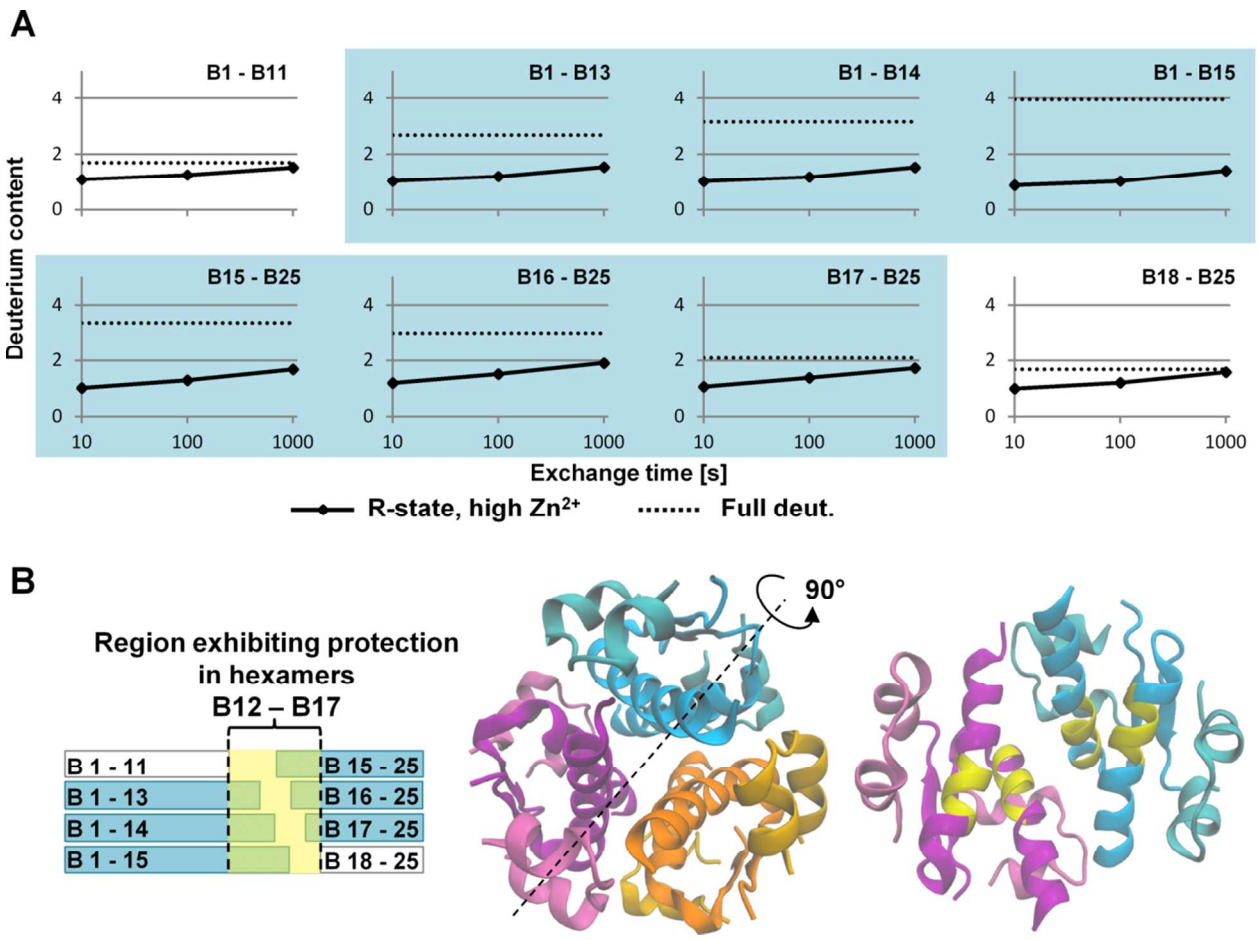

Figure S3. HDX-MS analysis of peptic peptides derived from Insulin forming R-state hexamers. The deuterium content of fully exchanged insulin is shown as dotted lines. (A) Peptide deuterium uptake plots for peptides emanating from Chain B. Plots shaded in blue represent peptides which are highlighted in figure S3B. (B) Visualization of the region where the greatest changes in HDX could be observed when comparing the different hexamers (12-17, labeled in yellow), using the R-state hexamer crystal structure, PDB ID 1EV6. The middle representation shows the intact hexamer as a trimer of dimers (blue, purple and orange). The orange dimer was removed for clarity in the right representation, in order to better visualize the 12-17 region.

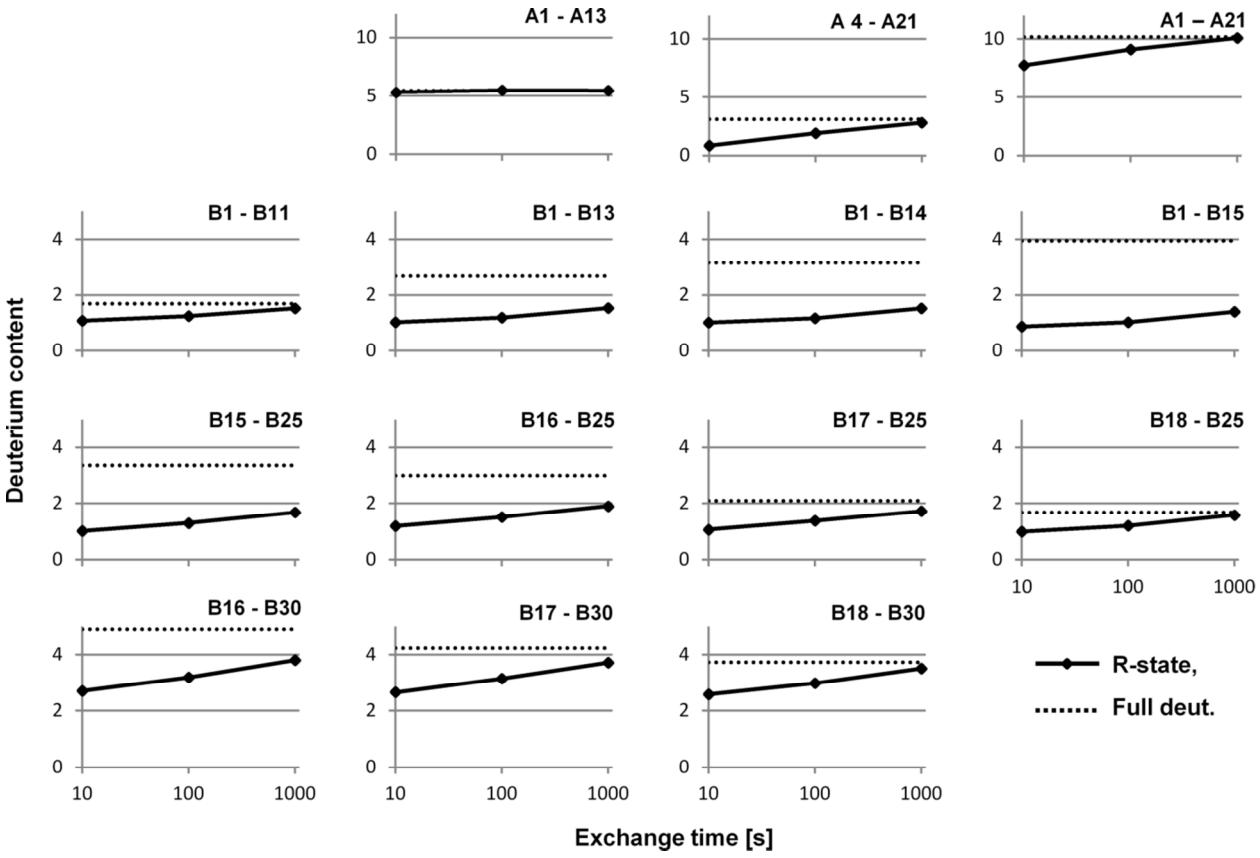

Figure S4. Deuterium uptake plots for peptic peptides derived from Insulin forming R-state hexamers. The deuterium content of fully exchanged insulin is shown as dotted lines. Peptide deuterium uptake plots for peptides emanating from Chain A and B are shown.

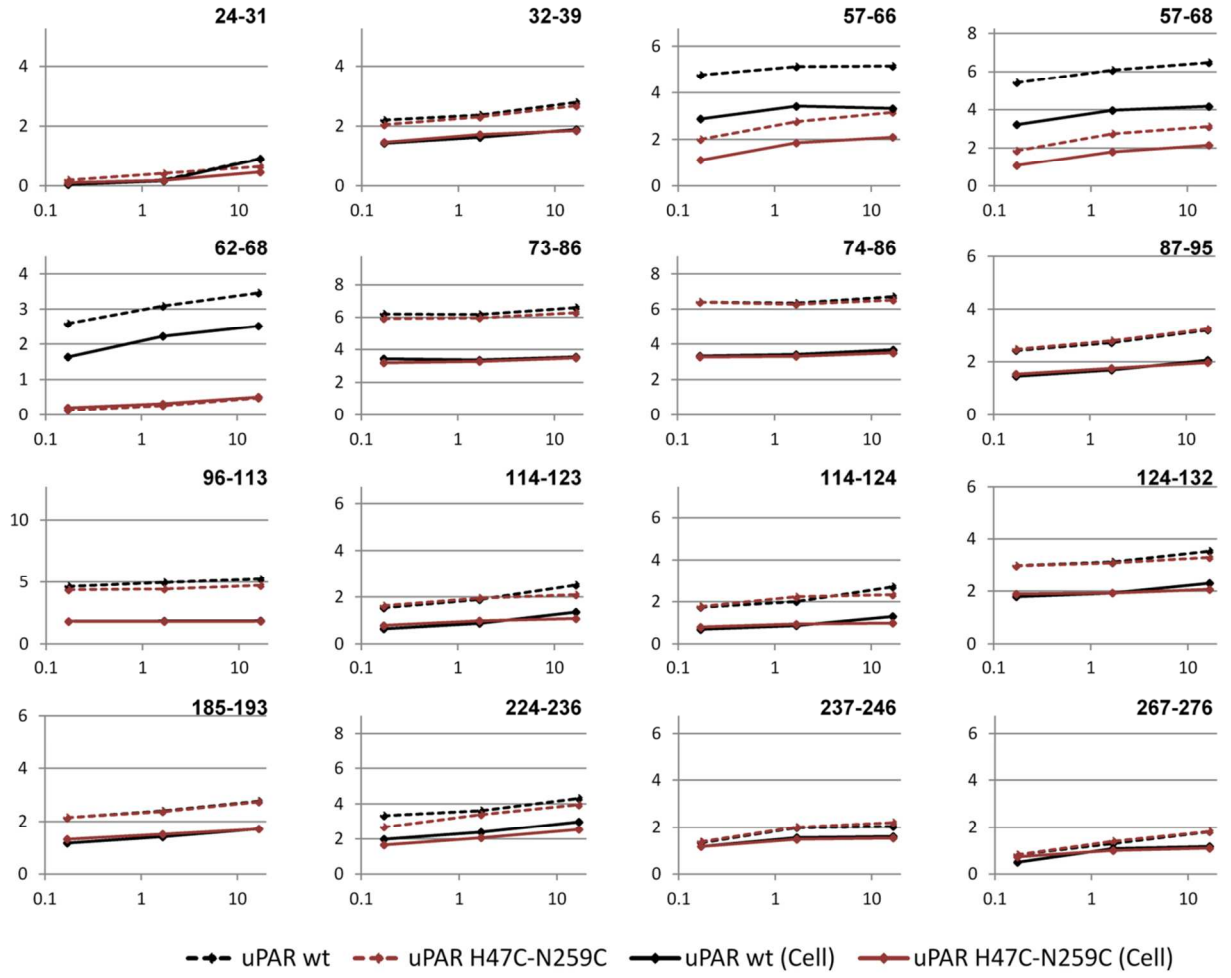

Figure S5. Deuterium uptake in peptides derived from uPAR wt and H47C-N259C, following chemical and on-line electrochemical reduction. Both methods yield the same qualitative deuteration patterns. It is important to note that these data should not be taken as a reporter for back-exchange induced by electrochemical reduction, compared to TCEP-reduction, as samples were prepared and reduced differently. Firstly, the electrochemically reduced uPAR samples were deuterated in 80% D_2O. In contrast, the TCEP-reduced samples were deuterated in 95% D_2O, resulting in greater initial deuterium incorporation. Secondly, TCEP-reduced samples were subject to forward-exchange during the reduction step. More specifically, the chemical reduction was initiated by diluting the quenched samples (still in a 95% D_2O solution) 1:1 with 800 mM TCEP (pH 2.5). The TCEP-containing samples are incubated on ice for 2 minutes. During these 2 minutes, uPAR is exposed to a 47.5% D_2O solution while the protein is unfolded due to the low pH and the presence of a chemical reductant. This allows for a forward-exchange reaction where backbone amides, which were previously hydrogen bonded or less that 47.5% deuterated, are further deuterated, compared to their state at the time of the quenching.

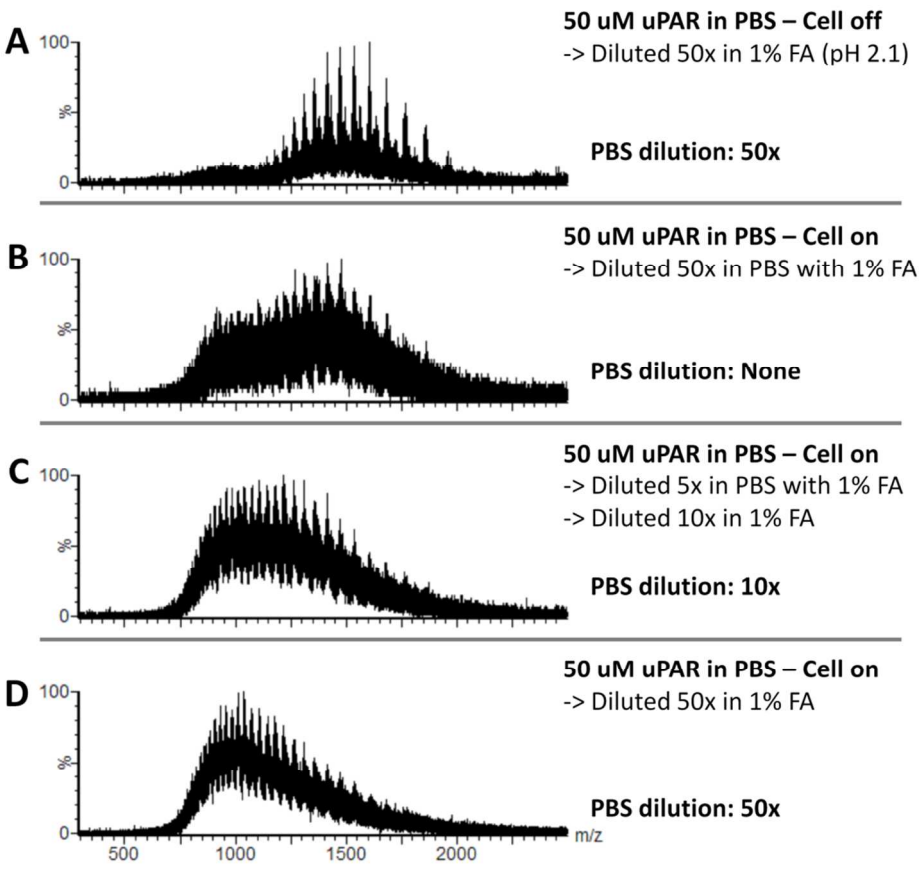

Figure S6. Mass spectra of intact uPAR wt without on-line reduction (A) or when performing electrochemical reduction in increasingly diluted PBS (B-D). Greater reduction efficiencies could be observed as a shift toward higher charge states, indicating an unfolding of the protein. When uPAR was dissolved in undiluted PBS, the reduction efficiency was decreased.