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Supporting information

A systematic study on the effect of protonation and deuteration on T_m/T_2 in a cellular context

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1. Materials and Methods

1.1. Protein expression and purification

Protonated Ubiquitin - A pET-21a vector encoding for human Ubiquitin (Ub) (bearing the double mutation S20G35C) was used to transform BL21 (DE3) cell strain. E. coli cells which were grown to mid-log phase (OD_{600}: 0.6) at 37° C in LB medium, and then induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG). After induction the cells were grown for other 5 hours at 37° C. The cells were then harvested, resuspended in lysis buffer (50 mM sodium acetate, pH 4.5) and lysed by sonication (10 seconds ON, 30 seconds OFF, at 60% of amplitude for 40 minutes). The suspension was ultracentrifuged at 40000 rpm for 40 minutes and was loaded onto a HisTrap™ SP HP column (Cytiva). Further purification was performed using size exclusion chromatography with a HiLoad 16/600 Superdex 75 pg column (GE Healthcare) exchanging the buffer with 100 mM sodium phosphate, 150 mM NaCl, 1 mM TCEP, pH 6.5.

Deuterated Ubiquitin- A pET-21a vector encoding for human Ubiquitin (Ub) (containing the double mutation S20G35C) was used to transform BL21 (DE3). E. coli cells which were grown to mid-log phase at 37° C in minimal cell growth medium (6.5 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L ammonium chloride, 2 g/L D-Glucose-d12, 120 mg/L $MgSO_4$, 11 mg/L $CaCl_2$, 10 ml MEM (100x)) in deuterated water. The expression conditions and the purification steps are the same as for the protonated protein.

1.2. Cell preparation for heat shock delivery

Deuterated *E.Coli* -

1 stock of XL1 blue competent cells was inoculated in 1 ml of LB in H_2O at 37°C over-day. The suspension was then transferred in 20 ml of minimal cell growth medium (6.5 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L ammonium chloride, 2 g/L D-Glucose-d12, 120 mg/L $MgSO_4$, 11 mg/L $CaCl_2$, 10 ml MEM (100x)) in deuterated water and incubated overnight at 37°C in a shaking incubator. The next day, the incubated cells were transferred in 200 ml of the same minimal culture medium. The growth was carried on at 37°C reaching a final OD_{600} of 0.35-0.4. Afterwards, the culture was chilled in ice for 30 minutes and the cells were harvested by centrifugation at 3000 x *g*. The pellet was resuspended with 100mL of a deuterated ice-cold solution of 100mM $MgCl_2$, and the bacteria cells were collected again by centrifugation at 3000 x *g*. The process was repeated with 100 mL of a pre-chilled deuterated solution of 100 mM $CaCl_2$, the *E.coli* cells were once again harvested by centrifugation at 3000 x *g*. Consecutively, bacterial cells were washed with 100 mL of a cold deuterated solution containing 85 mM of $CaCl_2$ and 15% v/v of glycerol. Finally, the cells were harvested by centrifugation at 1000 x *g*, resuspended in the aforementioned buffer, stocked in aliquots and stored at -80°C after snap freezing with liquid nitrogen.

***E.Coli* protonated**

From a stock of 100 µL of XL1 blue competent cells a 5 mL overnight culture was grown in sterile LB medium at 37°C in a shaking incubator. The next day, the overnight culture was transferred in 500 mL of sterile LB medium. The cells were grown at 37°C until the OD_{600} reached 0.35-0.4. The harvesting, washing, and storing procedures are the same than the one applied for the deuterated cells, using protonated water and not the deuterated one.

***P.Pastoris* protonated**

From a stock of 100 µL of X-33 *Pichia Pastoris* a 5 mL overnight culture was grown in 1 mL of YPD (10 g yeast extract, 20 g peptone, and 20 g glucose in 1L of water) at 30°C in a shaking incubator. The next day, the overnight culture was diluted to approximatively an OD_{600} of 0.15–0.20 in 200 mL of YPD. The growth was carried on at 30°C to an OD_{600} of 0.8-1.0, the cells were harvested by centrifugation at 500 x *g* at RT and the supernatant was poured off. The cell pellet was resuspended in PBS and stored in small aliquots of the same buffer at -80°C, after snap freezing in liquid nitrogen.

***P.Pastoris* deuterated**

From a stock of 100 µL of X-33 *Pichia Pastoris* a 1 mL over-day culture was grown in 1 ml of YPD (10 g yeast extract, 20 g peptone, and 20 g glucose in 1L of water) at 30°C in a shaking incubator. Afterwards, the harvested cell pellet was transferred in 20 ml of the final M9 minimal medium prepared with both deuterated water and fully deuterated glucose and were incubated overnight at 30°C in a shaking incubator. In addition to the glucose and deuterated water we prepared the M9 as described above (See preparation of *E.coli* deuterated) using thiamine and biotin as vitamins source instead of 10 ml of MEM (100x) in water. The following day, the incubated cells were transferred in 200 ml of the same medium. The growth was carried on at 30°C to an OD_{600} of 0.8-1.0. Afterwards, the cells were harvested by centrifugation at 500 x *g* at RT and the supernatant was poured off. The cell pellet was resuspended in PBS deuterated and stored in small aliquots of the buffer at -80°C, after snap freezing in liquid nitrogen.

1.3. Site Direct Spin Labelling reactions and CW-EPR spectroscopy

Site directed spin labelling reactions were carried out using 3-maleimido-proxyl label. Both protonated and deuterated ubiquitin S20G35C protein solution, were individually incubated with an excess of DTT (1:10 protein: DTT molar ratio) at RT in order to reduce the cysteine thiol group. After 1 hour, the reducing agent was removed through a PD-10 desalting column. A 10-fold molar excess of 3-maleimido-proxyl (Sigma Aldrich), dissolved in dimethyl-sulfoxide (DMSO), was then added to the protein solution. The labeling reaction was kept at 4° C overnight, under stirring. The unreacted spin label was removed using a PD-10 column and the purified fractions were checked acquiring X-band cw-EPR spectrum. The final protein concentration was estimated from the UV/vis spectrum at 280 nm by a Varian Cary 50 spectrophotometer (ε_{Ub} = 1490 M-1 cm-1). In vitro samples were

inserted into EPR quartz tubes (1.6 mm O.D., 1 mm I.D.) and used to record cw-EPR spectra by a Bruker ELEXYS E580 spectrometer equipped with a super HIGH Q ER4122SHQE operating at X-band. cw-EPR spectra were recorder with the following instrument settings: v= 9.874 GHz; center field= 3500 G; sweep width= 150 G; microwave power= 20 mW; modulation frequency= 100 kHz; modulation amplitude= 1 G; conversion time= 25 ms; sweep time= 25.6 s; scans= 25). For the in-cell samples, cells were transferred into EPR quartz tubes (1.6 mm O.D., 1 mm I.D.) and after a preparation time of ~10 minutes, RT cw-EPR spectra were recorded with the same spectrometer setup as above.

1.4. In cell sample preparation via heat shock delivery

E.coli

For E.coli cells we employed 50 μL of 500 μM doubly labelled human ubiquitin (protonated or fully deuterated) solution in PBS buffer at pH 7.4 (Gibco®) to resuspend a pellet containing approximately 10^9 cells/mL E. coli XL1 blue competent cells. The cells were incubated with the protein solution at 42 °C for 1 min, using a thermic bath, to induce the internalization of the external protein. Afterwards the sample was incubated in ice for 2 minutes and then centrifugated for 30 seconds. The supernatant was removed and the cells were then washed 4 times with an isotonic phosphate buffer solution to ensure complete removal of the protein which was not internalized into bacteria. Every wash step involved the addition of 200 μL of buffer, to completely resuspend the cell pellet, and a centrifugation step. The supernatant solution of each wash step was checked by EPR to ensure the complete removal of the not-internalized spin labelled protein. After the last wash step the sample was resuspended in 50 μL of phosphate buffer and was directly inserted in Q-band tubes and snap-freeze by complete immersion in liquid nitrogen. Every step was repeated for both protonated and fully deuterated bacterial cells.

It is worth mentioning that the whole thermal treatment needs to be carried out as fast as possible to avoid as much as possible the disappearance of the EPR nitroxide signal due to reduction phenomena that are present inside the intracellular environment. Previous works showed that the complete disappearance of the signal occurs after 40 minutes[1]. The procedure was repeated using both protonated and fully deuterated bacterial cells.

P.Pastoris

P. pastoris X-33 cells, previously stored in PBS solution, were incubated at 30 °C for 10 min, using a thermic bath. The cells were then centrifuged at 2800 g for 3 min and then resuspended in 50 μL of TE buffer (10mM Tris-HCl ,1mM EDTA pH: 8) supplemented with 0.1% v/v lithium acetate. Then, 50 μL of 500 μM doubly labelled human Ubiquitin (protonated or fully deuterated) solution was added to 2 x 10^8 cells/mL of P. pastoris X-33 cells. The yeast cells were finally incubated at 42 °C for 10 min to promote protein internalization. The sample was then stored on ice for 3 minutes. The cells were

then centrifuged, the protein solution was removed and the pellet was washed 4/5 times with isotonic PBS buffer. Every wash step involved the addition of 200 µL of buffer, to completely resuspend the cell pellet, and a centrifugation step. The supernatant solution of each wash step was checked by EPR to ensure the complete removal of the not-internalized spin labelled protein. After the last wash step the sample was resuspended in 50 µL of phosphate buffer, directly inserted in Q-band tubes and snap-freeze by complete immersion in liquid nitrogen. All steps were repeated for both protonated and fully deuterated bacterial cells

1.5. X.-band Room Temperature measurements

In vitro and in cell samples were inserted into EPR quartz tubes (1.6 mm O.D., 1 mm I.D.) and used to record cw-EPR spectra by a Bruker ELEXYS E580 spectrometer equipped with a super HIGH Q ER4122SHQE operating at X-band. The in-cell samples were inserted in the resonator after an average handling time of around 12-14 minutes. cw-EPR spectra were recorder with the following instrument settings: v= 9.874 GHz; center field= 3500 G; sweep width= 150 G; microwave power= 20 mW; modulation frequency= 100 kHz; modulation amplitude= 1 G; conversion time= 25 ms; sweep time= 25.6 s; scans= 25). In-cell samples were transferred into EPR quartz tubes (1.6 mm O.D., 1 mm I.D.), In the case of in cell samples, in order to follow the bioreduction of the nitroxide spin label inside cells, a set of cw-EPR experiments covering a time period of 120 minutes were recorded with the above spectroscopic parameters.

1.6. Q-band T_2 measurements

All experiments were acquired using a Bruker ELEXYS E580 X/Q-band spectrometer equipped with a pulse 10 W Amp Q amplifier, an EN 5107D2 Q-band EPR/ENDOR probe-head and a continuous He-flow cryostat (Oxford Instruments) coupled with a temperature controller (Oxford Instruments). The temperature was kept at 50 K. All the T_2 experiments were acquired according to this pulse sequence: [$\pi/2 - \tau - \pi - \tau -$ echo] under overcoupling condition. The experiments were acquired off resonance, 70 MHz from the resonator dip, to be consistent with the detection frequency in the DEER experiment. All pulses were optimized for each experiment using the regular mw-nutation sequence optimizing the pulse at the maximum of the nitroxide field. They were found in the range of π: 60 - 70 ns. A regular two step phase cycling was applied during the echo acquisitions.

1.7. *T_2* echo decays fitting procedure

The fitting of the T2 echo decays were performed using a MATLAB home written script. Equation 1 (of the main manuscript) was used to fit all the reported data by fitting the T_m and c exponent parameters to the experimental data (See Table S1-S2-S3).

In the specific case of the in vitro protonated and deuterated ubiquitin samples resuspended in deuterated buffer the best data fitting was found by eliminating the first part of the echo decay curve from t=0 to variable times (from 2 to 2.5 μs as shown in **Figure S2**) for each decay trace, to eliminate the contribution of the deuterium modulation from the extracted values of T_2. This procedure was introduced by M.Huber and coworkes[2]. We exploited the same approach to fit the T_2 decay curve for protonated ubiquitin delivered inside protonated *P. pastoris*. Errors were determined from the confidence intervals for each fitted value as half of the width of its 95 % confidence interval.

2. Supplementary information

2.1. X-band Continuous Wave (CW) EPR

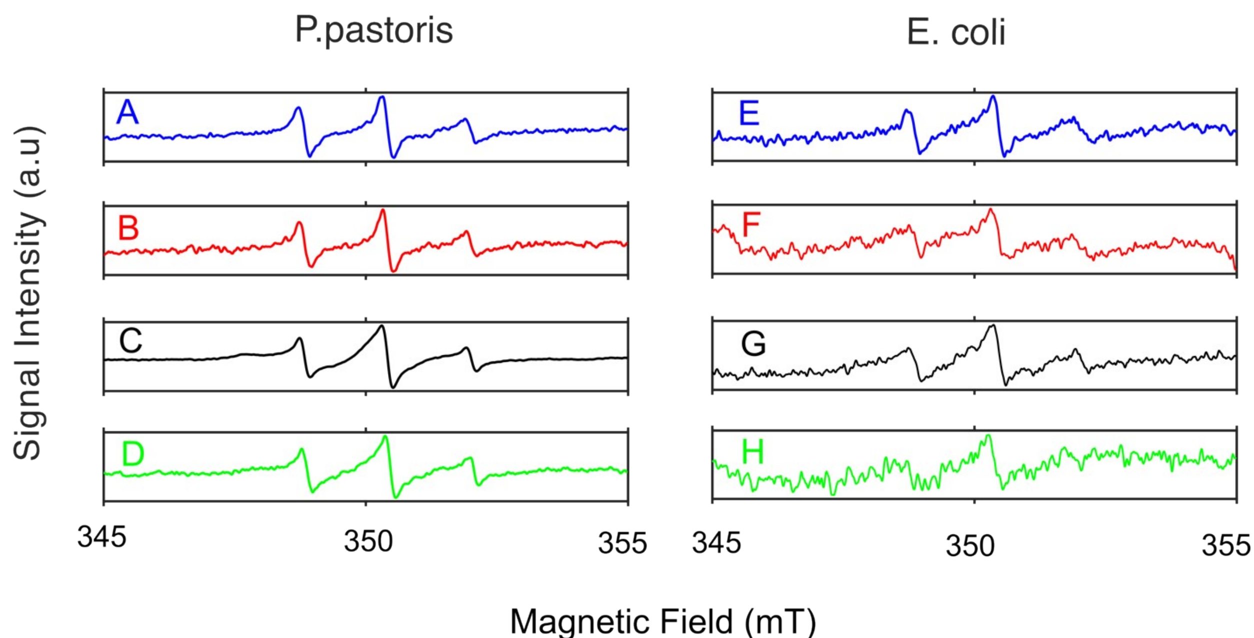

Figure S1: cw-EPR spectra acquired for the in cell sample prepared with both *E. coli* and *P.pastoris,* both in fully protonated and deuterated condition. In particular the blue spectra (A-E) were acquired from the sample with deuterated protein and cells. The red spectra (B-F) were obtained from the in cell sample prepared by delivering the deuterated protein inside the protonated cells. Green spectra (C-G) were measured from the in cell samples prepared delivering protonated protein inside protonated cells. Lastly, the black traces (D-H) were measured from the samples obtained by the delivery of protonated protein inside deuterated cells.

2.2. T_2 decays curves for the in vitro samples

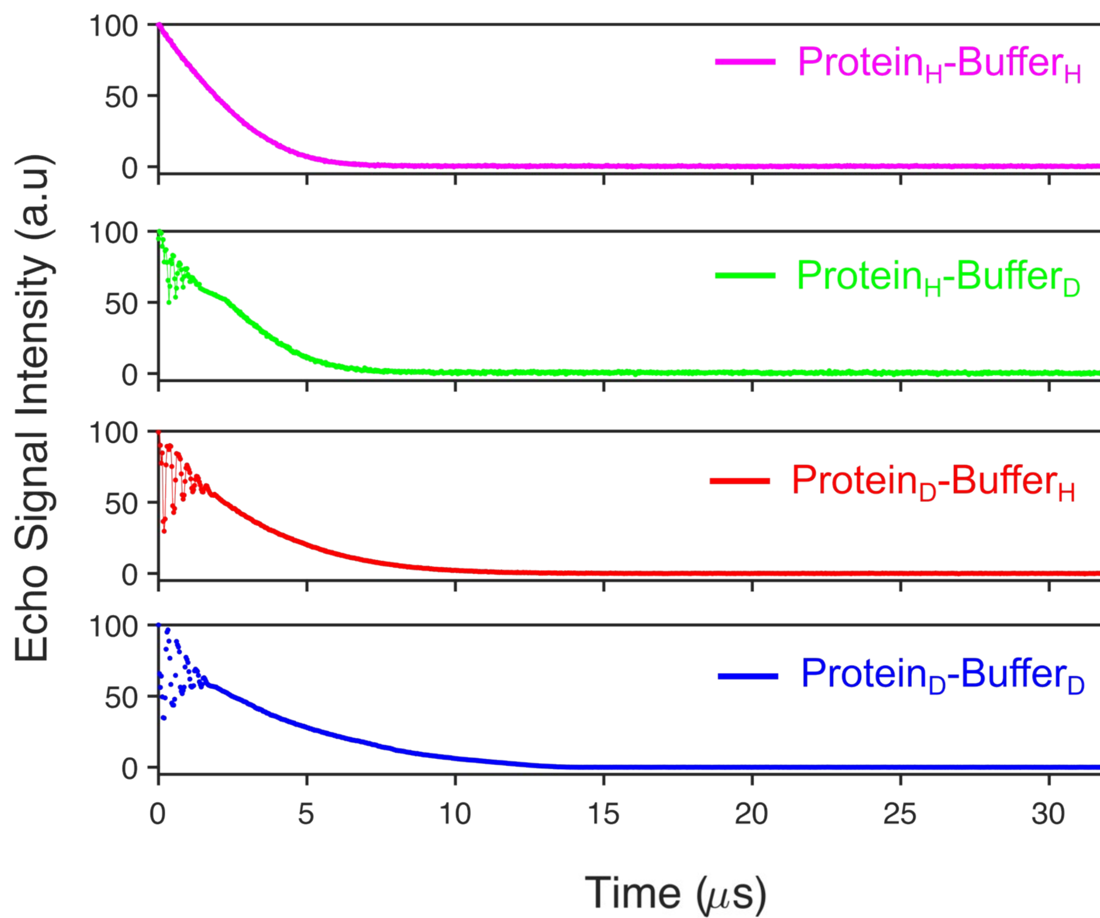

Figure S2: Measured echo decays curves for the Ub protonated/deuterated in the different buffers.

2.3 T_2 decays fitting for the in vitro samples and extracted nuclear modulation

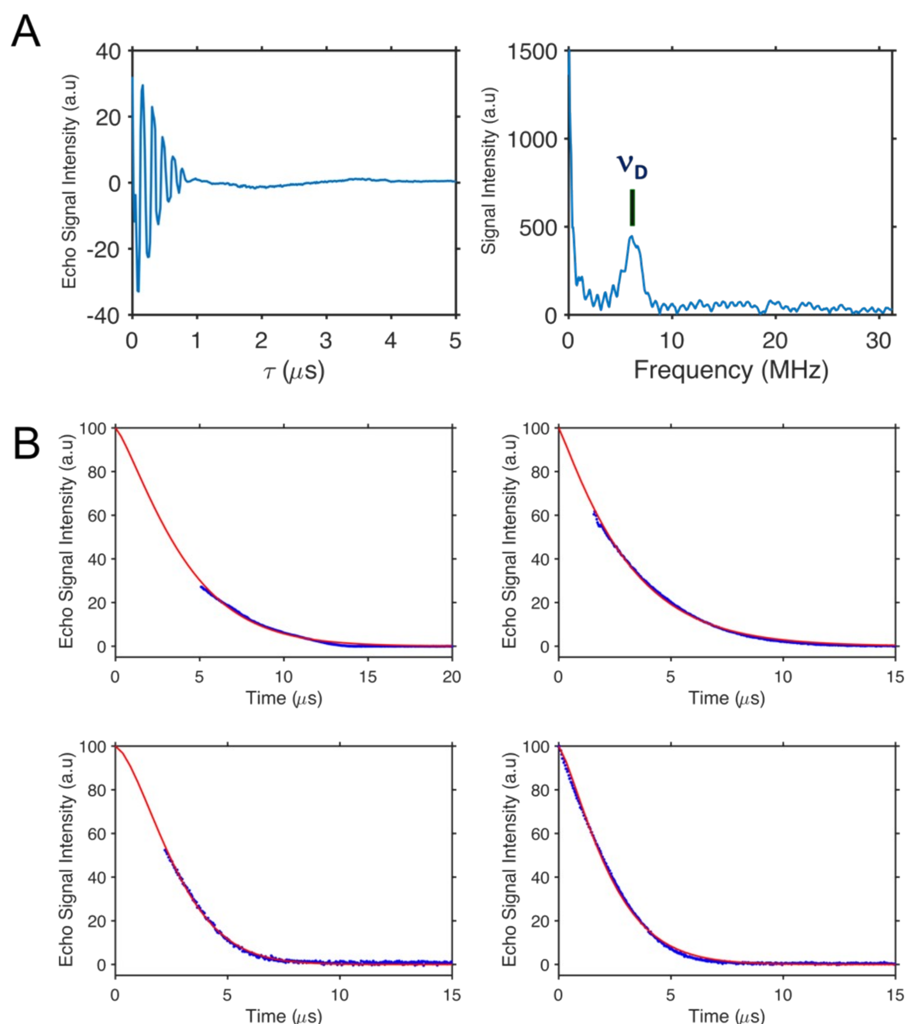

Figure S3: (A) Extracted electron spin echo decay modulation induced by the presence of the deuterium nuclei in close proximity to the nitroxide moiety. The right panel shows the Fourier transform of the ESEEM modulation which indicates the Larmor frequency of deuterium nuclei (ν_D) at the Q-band frequency and field. **(B)** T_2 Fitting for the acquired in vitro samples. The acquired experimental data are represented by blue dots, while the fitted curves are represented by the red lines. On the top left the in vitro sample prepared by placing the spin labelled deuterated Ub in the deuterated solvent, on the top right the sample constituted by the deuterated Ub resuspended in the protonated buffer. On the bottom left, sample prepared by having the protonated Ub resuspended in the deuterated buffer, and, on the bottom right the final sample constituted by the protonated Ub in the protonated buffer. All measurements were conducted as reported above and always in presence of 10 % v/v of glycerol (either deuterated or protonated according to the relative buffer). The first three experiments were trimmed at 4.9 μs, 1.6 μs and 2.3 μs respectively to remove the deuterium nuclear modulation in the context of the fitting.

2.4. Estimated T_2 values and c exponents

Sample	T_2 (μs)	c	Sample	T_2 (μs)	c
Ub_H-$Buffer_H$	2.47 ± 0.01	1.29 ± 0.01	Ub_H-$Buffer_D$	3.03 ± 0.01	1.54 ± 0.01
Ub_D-$Buffer_H$	3.16 ± 0.01	1.10 ± 0.01	Ub_D-$Buffer_D$	4.33 ± 0.02	1.25 ± 0.01

Table S1: Estimated values for the T_2 and the stretched parameter (c) for the four different samples of Ubiquitin protein respectively: protonated ubiquitin (Ub_H) in protonated buffer ($Buffer_H$), protonate ubiquitin in deuterated buffer ($Buffer_D$), deuterated protein (Ub_D) in protonated buffer and deuterated protein in deuterated buffer ($Buffer_D$).

Sample	T_2 (μs)	c	Sample	T_2 (μs)	c
Ub_H-E.$coli_H$	1.97 ± 0.07	0.98 ± 0.05	Ub_H-E.$coli_D$	10.07 ± 0.08	0.58 ± 0.01
Ub_D-E.$coli_H$	1.53 ± 0.02	0.97 ± 0.02	Ub_D-E.$coli_D$	11.15 ± 0.04	0.86 ± 0.01

Table S2: Estimated values for the T_2 and the stretched parameter (c) for the four different samples of Ubiquitin delivered in E.coli cells protein respectively: protonated ubiquitin (Ub_H) in protonated cells (E.$coli_H$), protonate ubiquitin in deuterated buffer (E.$coli_D$), deuterated protein (Ub_D) in protonated cells and deuterated protein in deuterated E.coli cells.

Sample	T_2 (μs)	c	Sample	T_2 (μs)	c
Ub_H-P.$pastoris_H$	3.36 ± 0.01	0.97 ± 0.01	Ub_H-P.$pastoris_D$	4.36 ± 0.01	0.91 ± 0.01
Ub_D-P.$pastoris_H$	3.01 ± 0.03	0.88 ± 0.01	Ub_D-P.$pastoris_D$	7.49 ± 0.07	0.56 ± 0.01

Table S3: Estimated values for the T_2 and the stretched parameter (c) for the four different samples of Ubiquitin delivered in Pichia pastoris cells protein respectively: protonated ubiquitin (Ub_H) in fully protonated cells (P.$pastoris_H$), protonate ubiquitin in deuterated cells (P.$pastoris_D$), deuterated protein (Ub_D) in protonated cells and deuterated protein in deuterated cells.

2.5. In vitro T_2 concentration dependence

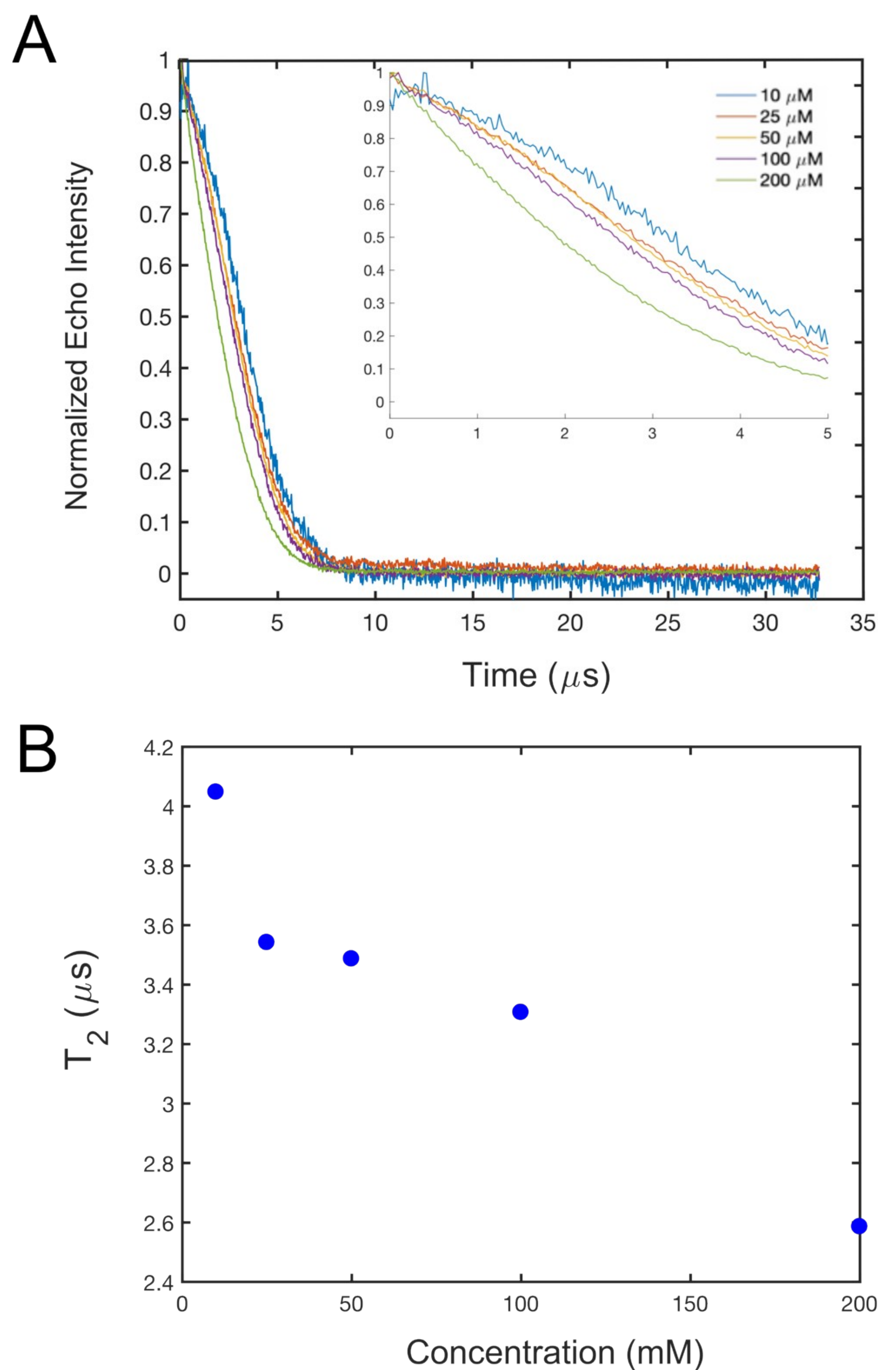

Figure S4: In vitro concentration dependent T_2 decays at Q-band frequency and field acquired at five different protein concentrations. The T_2 decays were acquired following the spectroscopical set-up reported in the Material and Methods section. As shown by the data the electronic T_2 increases its value by the increasing of the dilution of the spin labelled protein.

References

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2. M. Huber, M. Lindgren, P. Hammarström, L. G. Mårtensson, U. Carlsson, G. R. Eaton and S. S. Eaton, *Biophysical Chemistry*, 2001, **94**, 245-256.