Supporting Information for: High Pressure NMR Reveals Conformational Perturbations by Disease-Causing Mutations in Amyloid β-Peptide

Methods

Overexpression and Purification of Aβ Peptides

Isotopically labelled samples of Aβ are required for multidimensional solution NMR experiments. ^{15}N labeled recombinant HFIP-treated Aβ40 and Aβ42 peptides were ordered from rPeptide LLC (Bogart, GA, USA), but other variants of Aβ not provided by this company at the time of this work, as well as $^{13}C/^{15}N$ labeled peptides, were produced in lab.

This methodology is adapted from Glockshuber *et al.*;[1] deviations from this protocol were implemented to improve peptide yield. The wild type Aβ40 plasmid used for overexpression, originally provided as a gift and described by Glockshuber *et al.*,[1] encodes an N-terminal 6xHis tag, a $(NANP)_{19}$ repeat sequence included to improve solubility, a site for TEV protease digestion (RSENLYFQ), and finally, a codon-optimized wild type Aβ40 sequence. Vectors encoding D23N and E22G mutants of Aβ40 were generated through site-directed mutagenesis by GenScript USA Inc. (Piscataway, NJ, USA). All plasmids were transformed into BL21(*DE3*) *Escherichia coli* cells. The culture was used to make cell stocks in 15% glycerol, which were then stored at -80°C. Success of the mutagenesis and transformation procedures were confirmed by purifying plasmid from cultured cells derived from the stock through Miniprep and then sequencing the product, a service performed by Eurofins MWG Operon (Huntsville, AL, USA).

The medium used for overexpression of labeled peptides is OD2 growth medium with the desired labeling (either ^{15}N or $^{13}C/^{15}N$), provided by Silantes GmbH (Munich, Germany). This medium was chosen to avoid reductions in yield observed with standard minimal media such as M9. Unlabeled peptides were instead prepared using Luria broth (LB) medium. Overnight cultures were prepared from the cell stocks using 50 mL of the appropriate medium with 50 μg/mL fresh

ampicillin and incubating at 37°C and 225 RPM. Cells were transferred directly into a larger volume of the same medium (also containing 50 μg/mL fresh ampicillin); the volume of this transfer was selected to start this overexpression culture with an OD_{600} of 0.1 measured through absorbance spectrophotometry. This culture was again incubated at 37°C and 225 RPM until an OD_{600} of 0.5-0.6 was measured, then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a total concentration of 1 mM to induce expression of the protein. Following this step, the culture was incubated at 30°C and 225 RPM for 16 hours. Cells were then harvested from the medium by centrifugation at 4000 RPM for 30 minutes, and either frozen at -80°C or used immediately for subsequent purification.

Cells were thawed in a water bath (if needed) and resuspended in a "urea lysis buffer" (8 M urea, 100 mM potassium phosphate buffer (using a dibasic K_2HPO_4 stock), pH 8.0, hereafter called ULB); 5 mL ULB was used for each 1 g of cells. Lysis through sonication was performed by using a sonicator with a microtip at 35% power with 30 seconds on and 1 minute off for a combined total of 4 minutes "on" time, while keeping the sample on ice. This sonication step was repeated twice, and fresh ice was added in between steps. The soluble components of the lysate were then removed from the insoluble pellet through centrifugation at 20,000 RPM for 45 minutes. Nickel sepharose beads obtained from GE Healthcare Biosciences (Pittsburgh, PA, USA) were washed three times in ULB, and then nutated with the supernatant of the lysate obtained above for at least one hour. The bead mixture was then added to a drip column and the flowthrough was discarded. The column was further washed two independent times with 3 column volumes of ULB each time. Finally, the column was eluted with 3 column volumes of "urea elution buffer" (8 M urea, 100 mM potassium phosphate buffer (using a dibasic K_2HPO_4 stock), pH 8.0, 500 mM imidazole), which was carefully collected for the future steps.

After confirming the NANP-Aβ fusion protein was present by SDS-PAGE, the elution fraction was dialyzed overnight with "TEV digest buffer" (50 mM Tris-HCl, 0.5 mM EDTA, 30 mM NaCl, 1 mM DTT, pH 8.0). After assessing the appropriate activity concentration ratio through test cleavage of a NANP-MBP fusion protein, TEV protease purified in lab was added to the buffer exchanged sample. The sample was nutated at 4°C, allowing the cleavage reaction to

occur for ~30 hours. Any precipitate formed by the digest was then dissolved through addition of 6 M urea.

Confirming success of the digest reaction through SDS-PAGE, reversed-phase chromatography was used to separate cleaved Aβ from uncleaved fusion protein, NANP tag, TEV protease, and other contaminants. The Shimadzu HPLC (Kyoto, Japan) was equipped with a Zorbax SB-C18 semi-prepative RP-HPLC column, obtained from Agilent Technologies (Santa Clara, CA, USA). The column was heated to 80 °C, washed with a buffer containing 10% acetonitrile and 0.1% triflouroacetic acid (TFA), and loaded with the sample containing Aβ. The second line was connected with a buffer of 90% acetonitrile and 0.1% TFA, and the two buffers were combined with a stepwise gradient to encompass the range 20-30% acetonitrile that was used to elute the Aβ peptide. A flow rate of 3 mL/min was used throughout the procedure. Fractions with pure Aβ were determined and confirmed through MALDI-TOF mass spectrometry, then lyophilized and frozen at -80°C. Lyophilization removes water, acetonitrile, and other volatile organic solvents.

NMR Sample Preparation of Aβ Peptides

Powdered Aβ samples (including both in lab and rPeptide samples, see previous section) were disaggregated using a HFIP-NaOH treatment, demonstrated to produce Aβ in primarily monomeric form.[2-4] This entails fully dissolving the powder in hexafluoro-2-propanol (HFIP), then allowing the sample to fully evaporate under a stream of N_2, leaving a film of disaggregated peptide. By fully evaporating the HFIP, we ensure that there is no contamination by organic solvents. The film was re-dissolved in 10 mM NaOH with enough volume to produce a storage concentration of 1 mg/mL, then either used directly for NMR or stored at -80 °C to be used in future experiments. NMR samples were produced by dissolving this solution in pressure compatible deuterated Tris-HCl buffer, which we use in place of the more pressure sensitive potassium phosphate buffer that are typically used for standard NMR experiments.[5-7] This produced the following final conditions: 100 μM Aβ, 10 mM deuterated Tris-HCl buffer, 2 mM NaOH, 10% D_2O, pH 7.3, with a total volume of 500 μL.

NMR assignment of Aβ mutants.

NMR backbone assignment were carried out with standard triple resonance experiments using ^{13}C, ^{15}N labeled samples.

High Pressure NMR Data Acquisition

Coupled with the monomer-driven sample preparation above, NMR signals of Aβ have been demonstrated to be primarily (>90%) from the monomeric state, as demonstrated by analysis of NMR relaxation and pulse field experiments.[3, 4, 8] The NMR experiments discussed here were run on a 600 MHz Bruker NMR spectrometer, equipped with a TXI probe, located at the NMR core facility in the Center for Biotechnology and Interdisciplinary Studies at Rensselaer. All experiments were run at 277 K to minimize signal loss due to peptide aggregation and the effects of solvent exchange.

Samples were loaded in a 2500 bar standard ceramic pressure cell connected to an Xtreme-60 syringe pump system provided by Daedalus Innovations (Aston, PA, USA). This is an in-line system, allowing us to change the pressure without removing the sample from the NMR probe. Temperature calibration in the high pressure cell at ambient pressure was specifically performed by measuring the ^{1}H chemical shift difference between the methyl and hydroxyl groups of 100% methanol[9] to gauge the actual temperature in the sample. This was done 7 times, yielding sample temperatures that encompassed the range 273-307 K in a roughly uniform manner; these data collectively yielded a linear fit between sample and sensor temperatures with an R^2 of 0.999 (Supplemental Figure 1). This fit allowed the interpolation of appropriate sensor temperature to reach a 277 K sample temperature. ^{15}N-^{1}H HSQC and ^{1}H NMR spectra were measured for multiple samples (Aβ40, Aβ42, Aβ40-D23N, Aβ40-E22G) at 250 bar increments, from ambient pressure (1 bar) to 2500 bar. ^{1}H chemical shifts were referenced to the remnant methylene signal from the 10 mM deuterated Tris-HCl buffer across the different pressures. This Tris signal itself was, in turn, referenced to DSS through a second set of ^{1}H spectra collected across the different pressures, with a sample of 0.1 mM DSS added to the same buffer at the same conditions (10 mM deuterated Tris-HCl buffer, pH 7.3) but without Aβ peptide. This 2-step referencing was performed because

DSS has been observed to interact with Aβ and affect its aggregation;[10] this method avoids using DSS as a buffer component. (Tris chemical shifts, meanwhile, are not observed to not change upon addition of Aβ.) All peaks involved in the 2-step referencing were observed to be non-overlapped. ^{15}N chemical shifts were then also referenced based on the extrapolated DSS shift using the indirect method proposed by Wishart and colleagues.[11] HSQCs were collected at 1 bar, and then every 250 bar increment up to 2500 bar. Each HSQC was collected successively, using the same sample in an in-line fashion. 16 scans were employed for each HSQC, requiring a full acquisition time of about 1 hour, except for Aβ40-E22G, which used 32 scans, employed to mitigate losses due to low signal to noise ratio, and thus required ~2 hours for NMR data acquisition per pressure point.

Pressure coefficients from the positions of ^{15}N-^{1}H HSQC peaks were calculated through a similar method as the one documented by Munte *et al.*[12] This entails subtracting random coil chemical shifts at each given pressure by extrapolating the random coil amide nitrogen and hydrogen chemical shifts at ambient pressure with the known pressure dependence of these nuclei for each amino acid X in the model peptide Ac-Gly-Gly-X-Ala-NH2, as determined by Koehler *et al.*[13]. The set of these corrected shifts were used as the objective variable δ^* to fit the pressure coefficients B_1^* and B_2^* in the following second order Taylor expansion:

$$\delta^*(p,T_o) = \delta_o^*(p_o,T_o) + B_1^*(p-p_o) + \frac{1}{2}B_2^*(p-p_o)^2 \qquad (1)$$

where p_o is the ambient pressure and δ_o^* is the random chemical shift at this pressure. This second order model was previously observed to best represent the movement of ^{1}H chemical shifts with pressure[14]. Least squares fitting over all pressures was used to obtain pressure coefficients for both nitrogen and hydrogen nuclei separately.

Further, we define chemical shift perturbations (CSP) between two ^{1}H-^{15}N HSQCs to be:

$$CSP = \sqrt{(10\Delta\delta_H)^2 + (\Delta\delta_N)^2} \qquad (2)$$

where $\Delta\delta_H$ and $\Delta\delta_N$ are the change in chemical shift of a given resonance in the hydrogen and nitrogen dimensions, respectively.

All spectra were processed using nmrPipe[15] and analyzed with Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco, CA).

MD simulation

Details of the all-atom, explicit solvent replica exchange molecular dynamics[16, 17] (REMD) simulations compared in this paper are described in detail in our previous publications.[18, 19] The simulation parameters are briefly summarized below for the reader's convenience.

The "OPLS" simulations use a combination of the OPLS-AA/L force field[20] and the TIP3P water model,[21] while the "ILDN" simulations were run using the AMBER99sb-ILDN force field[22] and the TIP4P-Ew water model.[23] The Aβ systems were each individually built as fully extended structures with zwitterionic termini and protonation states that correspond to pH 7. All simulations were solvated in a 5.4 nm cubic box with 4947 water molecules, then equilibrated according to the protocol described in Rosenman *et al.* 2013.[18] Production REMD runs were then run using 52 replicas, encompassing the temperatures 270.0 K to 601.2 K. More details about the parameters of the REMD runs can also be found in Rosenman *et al.* 2013.[18] The three simulations referenced in this paper ("OPLS" Aβ40, "ILDN" Aβ40, and "ILDN" Aβ40-E22G) were each run to 1000 ns/replica, representing a cumulative simulation time of 52 μs per system. All simulations described above were run either using GROMACS 4.5.3 or 4.5.5.[24] The REMD simulations were each performed using either 208 or 416 CPUs of a Linux cluster based at Rensselaer Polytechnic Institute or the Stampede cluster at the Texas Advanced Computing Center at the University of Texas as part of the Extreme Science and Engineering Discovery Environment (XSEDE).

Figures

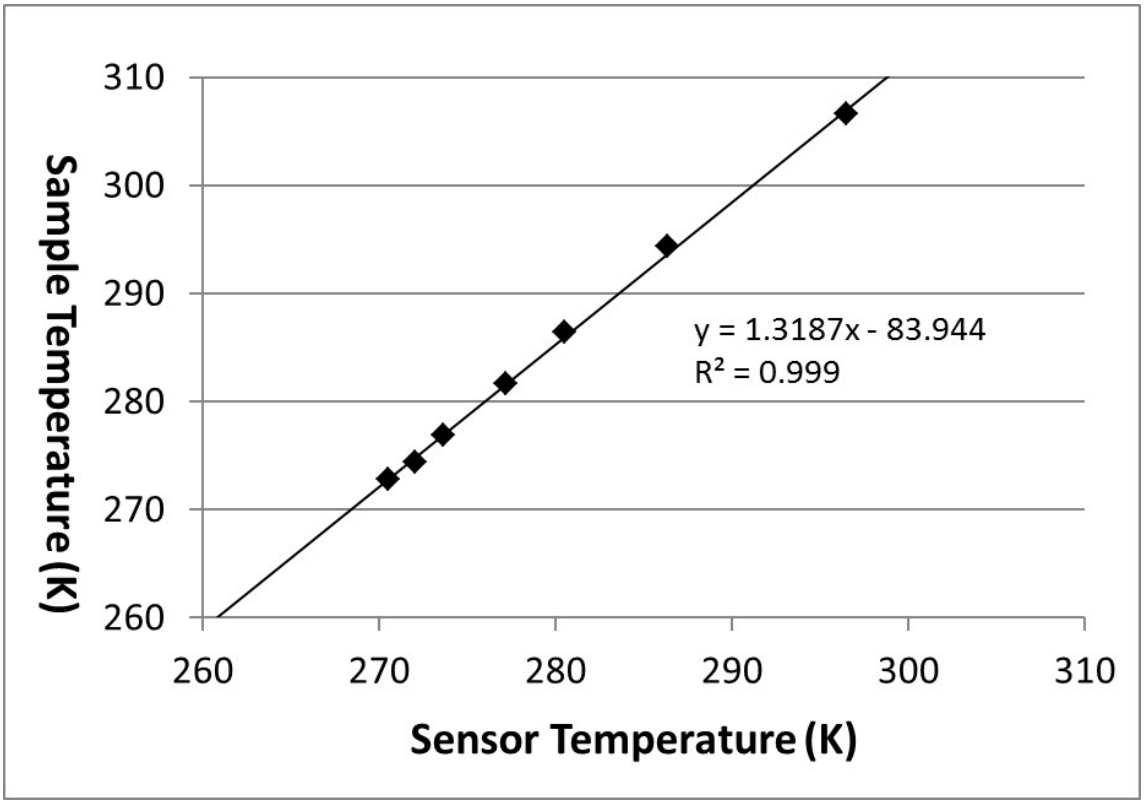


Figure S1. Temperature calibration for a sample in the 600 MHz Bruker spectrometer inside the high pressure cell used for the high pressure NMR experiments in Chapter 4. Sample temperature was determined by converting the ^{1}H chemical shift difference between the methyl and hydroxyl groups of 100% methanol[9].

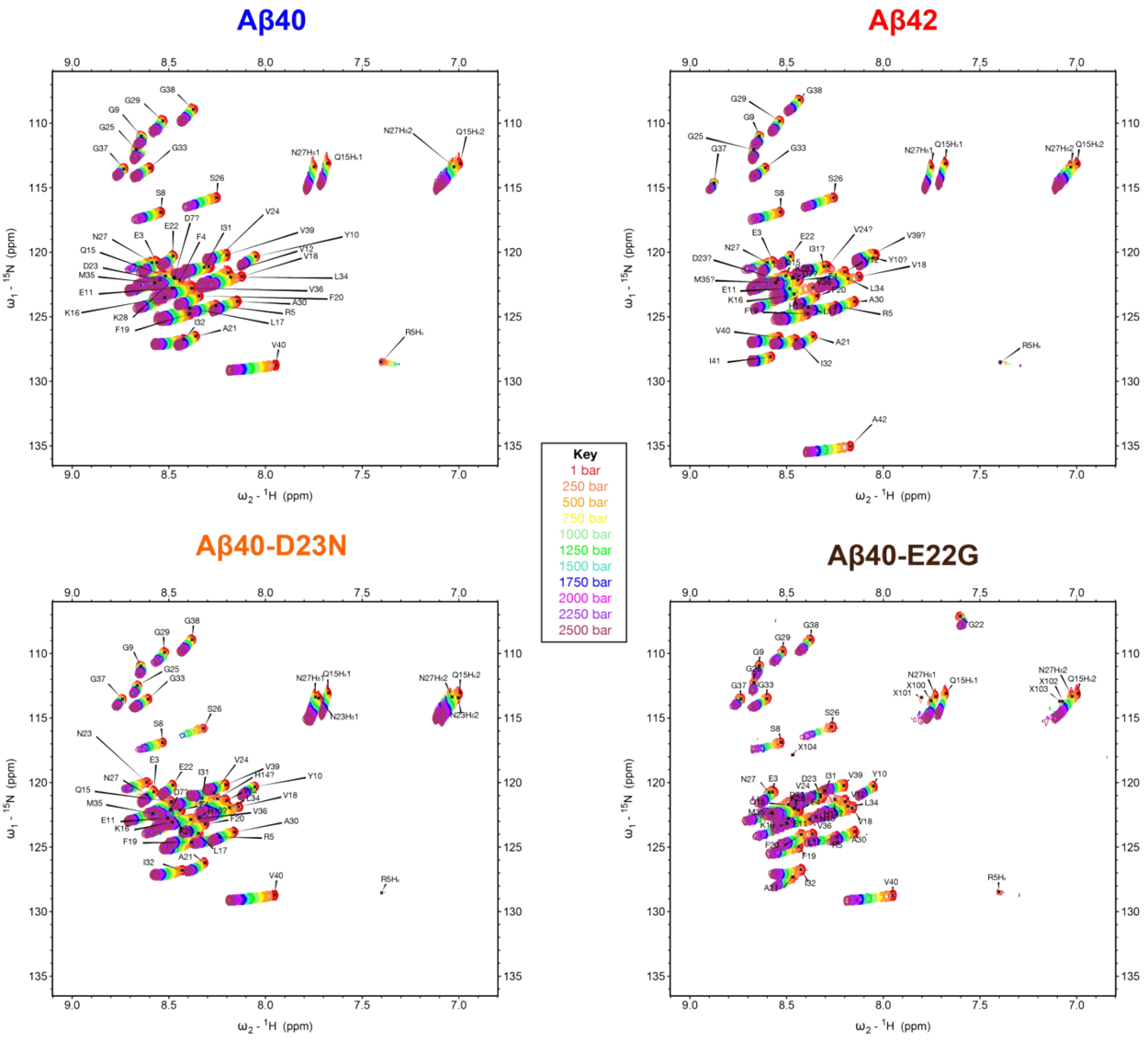
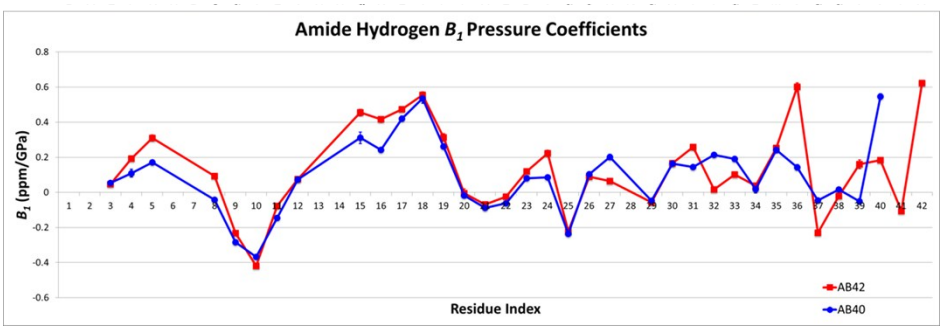


Figure S2. ^{1}H-^{15}N HSQC spectra for different monomeric Aβ species collected with high pressure NMR, ranging from ambient pressure to 2500 bar at 250 bar increments. Assignments are shown on the spectra marked at their locations at 1 bar. Unknown peaks are labelled X100-X104. All spectra are shown at the same contour levels and with the same axes to facilitate comparison, both between pressures and between species. Data collected on a 600 MHz spectrometer at 277 K, using the high pressure pump and buffer conditions as described in the Methods.

A



B

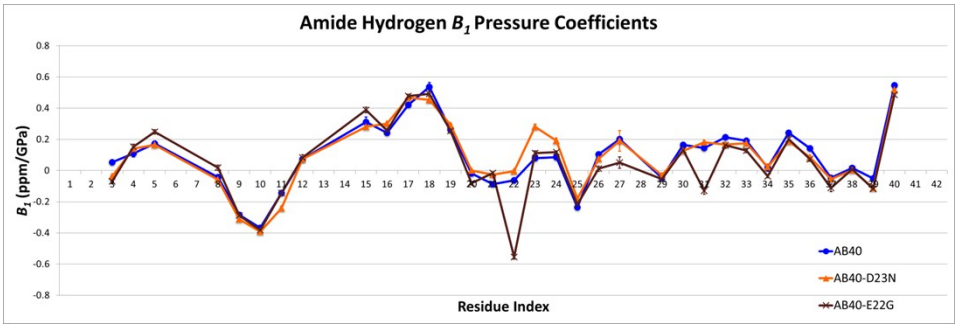
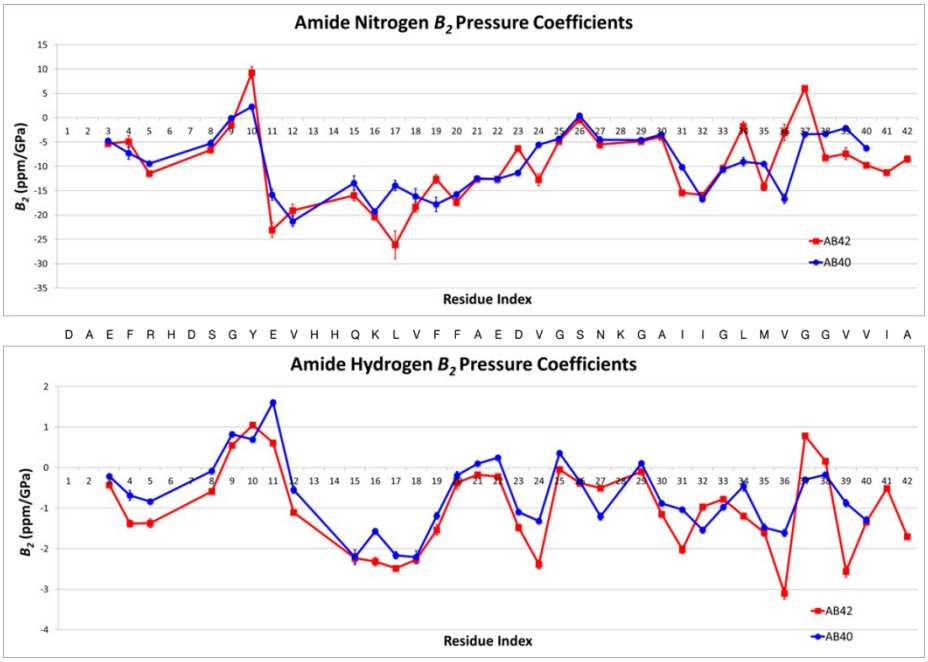


Figure S3. Hydrogen B1 pressure coefficients for (A) Aβ42 vs Aβ40 (B) Aβ40-E22G and Aβ40-D23N vs. Aβ40.

A



B

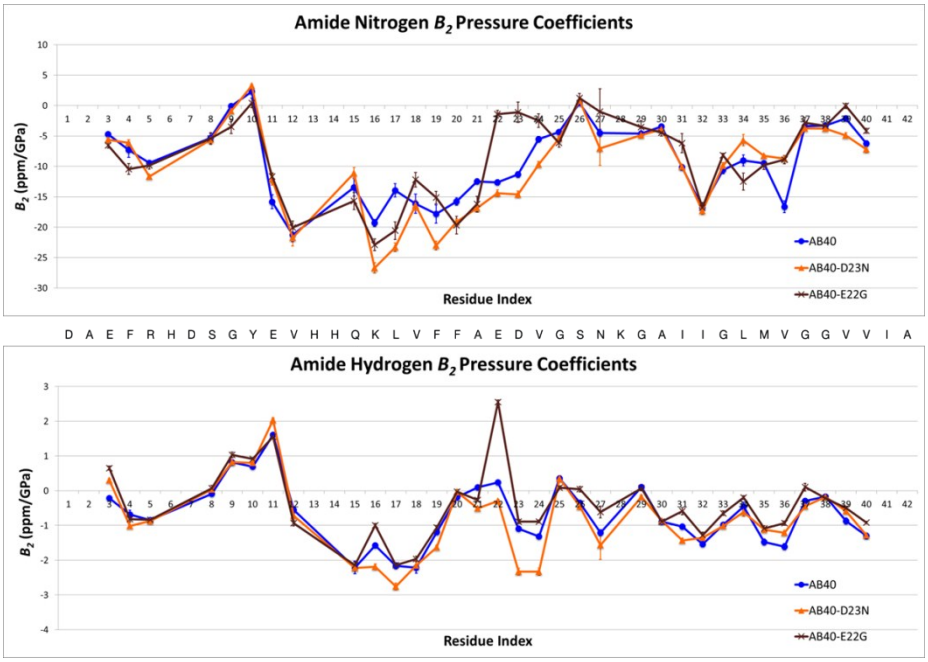


Figure S4. Plot of the second order (B_2) pressure coefficients for the nitrogen and hydrogen nuclei for (A) Aβ42 vs Aβ40 (B) Aβ40-E22G and Aβ40-D23N vs. Aβ40. The sequence of wild type Aβ42 is presented in between each nitrogen and hydrogen graph.

References

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