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Supporting Material

Stoichiometry and Affinity of Human Serum Albumin – Alzheimer's Aβ Peptide Interactions

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For

Stoichiometry and Affinity of Human Serum Albumin – Alzheimer's Aβ Peptide Interactions

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Confirmation of the structural integrity of the HSA constructs. The refolding and secondary structure elements of the HSA mutants prepared through bacterial expression were validated by acquiring far-UV CD spectra (Figure S1). Based on the measured molar ellipticities at 222 nm (20), the α-helical content of full length wt HSA as well as of domains 1 and 3 are calculated to be 68 %, 45 % and 56 %, respectively (Table S1). These values are in good agreement with those previously published for corresponding HSA mutants purified from yeast *P. Pastoris* cells (Table S1) (S1). However, no reference data is available for the two domains constructs and therefore for these deletion mutants a lower limit to the α-helical content was calculated as a weighted average of the α-helical percentages reported for the individual domains (Table S1). The experimental α-helical percentages measured for the two-domain constructs are slightly higher relative to the values calculated using the single domain constructs (Table S1). These minor differences of about 7-8 % units are expected based on the structure of HSA (Figure 1a) and are rationalized in terms of partial local unfolding of inter-domain helices due to the elimination of domain 2 from the two-domain constructs. Overall, the CD data (Figure S1) support a successful refolding of all the prepared HSA constructs (Figure 1b).

Equation S1: The concentration of free HSA is derived from $[HSA]_{Tot.}$, $[A\beta_n]_{Tot}$ and $K_{D,HSA,app.}$ according to equation S1:

$$[HSA] = [HSA]_{Tot} - 0.5\{ b - \sqrt{(b^2 - 4[A\beta_n]_{Tot}[HSA]_{Tot.})}\} \quad (S1)$$

where $b = [HSA]_{Tot.} + [A\beta_n]_{Tot} + K_{D,HSA,app.}$.

Material and Methods

Aβ (12-28) Peptide Sample Preparation. Samples of 0.65 and 1 mM Aβ (12-28) were prepared as previously described (4,5) by dissolving a lyophilized powder in 50 mM acetate buffer–d_4 at pH 4.7, with 10% D_2O. At this pH, Aβ (12-28) undergoes chemical exchange between the monomeric and oligomeric states. This exchange is detectable by NMR through line-broadening, STD and ORR, while at higher pH this exchange is significantly reduced. This pH is also relevant for HSA, since at this pH albumin is still present in the physiologically relevant N form as previously reported (4). The Aβ (12-28) peptide used in this investigation was purchased from Genscript Corp., Piscataway, NJ with a purity of 98 %. After the peptide was dissolved, the solution was filtered through a Ultrafree-MC Millipore with a 30 kDa cutoff filter in 5 min intervals at 3,000 rpm to remove aggregates possibly serendipitously formed during the peptide lyophilization and dissolution processes. To induce aggregation in a controlled manner, a 1 M NaCl solution prepared in 50 mM acetate buffer at pH 4.7 was then added to the filtered peptide sample up to a final concentration of 25 or 40 mM NaCl. The resulting aggregated Aβ (12-28) solutions were allowed to equilibrate for seven days. This equilibration time was determined to be sufficient for sample stability, as assessed through line width analysis and through 1D-STD NMR experiments. Care was taken to ensure that all the Aβ (12-28) samples used in the titrations came from the same peptide stock solution, so that any

difference observed in the protein titrations cannot be attributed to differences in the peptide preparations. For this reason we prepared an aggregated peptide stock solution, from which 500 µL aliquots were obtained and used for each titration.

Aβ (1-42) Peptide Sample Preparation. The Alzheimer's peptide Aβ(1-42) was purchased from EZBiolab Inc., with a purity greater than 95 %. Soluble 90 µM Aβ(1-42) samples were prepared by dissolving 1 mg of the Aβ (1-42) peptide in 500 µL of 10 mM NaOH. After sonication for 1 min, the peptide sample was then placed on ice for a two minute interval, after which it was sonicated again for another minute. This solution was then diluted with 2 mL of a 15 mM potassium phosphate buffer at pH 7.4, with 10 % D_2O and 0.02 % NaN_3. The final peptide concentration was 90 µM at a pH of 7.4. Immediately after preparation, the 90 µM Aβ(1-42) solution was divided into several fractions to which wild-type HSA or HSA deletion mutants, dialyzed in the same buffer, were added prior to the acquisition of NMR data at 37 ^oC. During the time between NMR data acquisition sessions, the NMR samples were stored in water bath at 37 ^oC without stirring or mixing.

Protein Sample Preparations – Sub-Cloning. The HSA gene in the pUC19 vector was purchased from ATCC (Cat. number 61356). Sequencing results indicated that the HSA gene has an internal NdeI restriction enzyme site (5'- ACA TAT G-3') located in domain 2 of HSA. This site was mutated using PCR to 5'- ACT TAT G-3', which does not change protein amino acid sequence but prevents NdeI digestion at domain 2. The NdeI restriction enzyme site was instead introduced in the pUC19 vector at the beginning of domain 1 of full length HSA. This vector was then digested with the NdeI and BamH1 (pUC19 cloning site) enzymes to release the HSA gene. The isolated HSA gene was purified from a 1% agarose gel and was ligated into the pET 15B vector, which encodes for a His-tag used in the protein purification. While the his-tag is always removed by thrombin cleavage, it leaves behind four residues (Gly-Ser-His-Met), which are present at the beginning of the HSA constructs. Sequencing confirmed the correct insertion of the HSA gene into the pet15B vector. Individual domains and HSA gene constructs were then obtained as follows. The domain 1 gene (Figure 1b) was obtained by introducing two stop codons at the end of the domain 1 through PCR mutagenesis. The construct for domains 12 (Figure 1b) was obtained by introducing a stop codon at the end of domain 2, while the domain 23 fragment (Figure 1b) was obtained by introducing an NdeI restriction enzyme site at the end of domain 1. This mutated DNA was then digested with the NdeI enzyme, which resulted in the removal of domain 1. Ligation of the purified vector resulted in the domain 23 construct (Figure 1b). Similarly, the DNA sequence of domain 3 (Figure 1b) was generated by introducing an Nde1 restriction enzyme site at the end of domain 2. Digestion with the Nde1 enzyme resulted in the removal of the sequences of domains 1 and 2 and subsequent ligation generated the domain 3 construct. The sequence of each constructs was confirmed through PCR based sequencing. The exact positions of the domain boundaries are indicated in Figure 1b.

Protein Sample Preparations – Expression and Purification. The main protocol for the expression and purification of our albumin constructs was adapted from reference (20). In brief, each construct was expressed in BL21-CodonPlus (DE3) cells. Protein expression was induced with 1 mM IPTG at 37 ^oC for 3 hours after the optical density reading at 600 nm reached a 0.9 value. After induction cells where collected and re-suspended in 20 mM Tris HCl buffer at pH 7.9 with 150 mM NaCl. Cells where then lysed using a French press/emulsifier (EmulsiFlex-C5).

Protein inclusion bodies were collected by centrifugation at the 13,000 rpm. Protein pellets were solubilized using a 6M guanidine hydrochloride solution (20 mM TrisHCl, 2 mM beta mercaptoethanol (BME), 6M guanidine HCl, pH 7.9) and were loaded onto Nickel beads for 16 hours at 4 °C. Non-specifically bound proteins were washed from the beads using a 20 mM imidazole buffer (20 mM imidazole, 8M urea, 20 mM Tris, 2 mM BME, pH 7.9). The target protein was then eluted using a 1 M imidazole solution (8M urea and 20 mM Tris, 2 mM BME, pH 7.9). Nickel ions where removed by dialyzing the protein in water and then 0.5 % acetic acid. The precipitated protein was solubilized in 8M urea and reduced with 50 mM DTT at 37 °C for 30 minutes. The protein was then diluted to a 0.5 mg/mL concentration with a solution of 8M urea, 50 mM sodium bicarbonate at pH 10. Removal of urea and subsequent protein refolding was performed by dialyzing the solution in 50 mM sodium bicarbonate, 1 mM EDTA buffer at pH 10. The pH of the final solution was adjusted to the 7.0 and the properly folded protein was collected using a HiTrap Blue Sepharose column (GE Healthcare). The protein was then digested with thrombin and further purified using a Superdex 75 preparation grade XK26/60 column (GE Healthcare) pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.5) containing 150 mM NaCl, 0.1 mM EDTA, and 0.02 % NaN_3. The thrombin digested monomeric protein construct was collected and delipidated using a hydroxyalkoxypropyl dextran type VI resin (Sigma Chemical) at pH 3 for 90 min at room temperature. The collected protein was then dialyzed in 50 mM sodium phosphate buffer, pH 6.5, 150 mM NaCl, 0.02 % NaN_3. Fatty acid free and essentially globulin free full length HSA was purchased from Sigma with a 99% purity. All final protein samples were dialyzed either in a 20 mM acetic acid, 25 mM NaCl solution to be used for the Aβ (12-28) titration experiments or in a 15 mM potassium phosphate buffer (pH 7.4) to match the conditions of the Aβ (1-42) samples. 20 mM acetic acid, 25 mM NaCl buffer was used as opposed to 50 mM acetic-d_4 buffer, 25 mM NaCl in peptide solution to avoid excessive acetic acid signal in the NMR spectrum during peptide titration at high protein concentrations. The final protein concentrations were measured using the absorbance at 280 nm on a Hewlett Packard 8453 UV–vis spectrophotometer and in a 6 M guanidine hydrochloride solution. The extinction coefficient used to calculate protein concentrations was obtained using the biology workbench software (http://workbench.sdsc.edu/). All HSA constructs were run through SDS-PAGE and in addition their secondary structure was probed by far-UV circular dichroism (CD) measurements performed between 195 nm and 260 nm on an AVIV Circular dichroism (model 215) spectrometer at 25°C after dilution in 50 mM sodium phosphate buffer (pH 6.5) containing 150 mM NaCl, and 0.02% NaN_3.

NMR Spectroscopy – Saturation Transfer Difference (STD) Experiments. 1D-STD experiments were used to monitor the effect of HSA and its constructs on the Aβ(12-28) samples. All STD experiments were acquired using previously described pulse sequences (13,14) and a Bruker Avance 700 MHz spectrometer equipped with 5 mm TCI Cyroprobe at 20 ^oC. Selective saturation was achieved using a train of 40 Gaussian-shaped pulses of 50 ms each and separated by a 1 ms inter pulse delay, resulting in total saturation time of ~2 seconds which was preceded by a 100 ms inter-scan delay. The strength of each saturating Gaussian pulse was 110.23 Hz with a 1 % truncation and 1000 digitization points. On-resonance saturation was achieved by setting the carrier frequency of the Gaussian pulse train at 0.75 ppm, while off-resonance saturation was obtained by saturating at 30 ppm. The saturation transfer difference spectra were obtained by subtracting on-resonance and off-resonance spectra through phase cycling. In all experiments the water magnetization was suppressed using the 3-9-19 Watergate

gradient spin-echo (S2). A 30 ms spin lock pulse with strength of 2.6 kHz was applied to suppress the residual protein signal in all STD and saturation transfer reference (STR) experiments. For the STD experiments 128 scans and 8 dummy scans were acquired, which were reduced to 32 scans and 32 dummy scans for the more sensitive STR spectra. For each titration point two STR and four STD replica spectra were collected. All STD and STR replicas were then added to increase the S/N ratios. The 6.88-7.25 ppm spectral region was used to determine the I_{STD}/I_{STR} ratios and the related errors were evaluated as standard deviations of the signal/noise ratios of the individual replicas. Before each titration, STD experiments were preformed on a control aggregated Aβ(12-28) sample without protein to confirm sample stability, and to ensure differences observed in the titration profiles for different proteins are not due to the different oligomer populations in different Aβ samples. The titration curves were fitted using a Scatchard-like model as outlined in the Results section. Due to the transient nature of the oligomers formed by the longer Aβ (1-42) peptide, the STD experiments were not performed for this longer peptide. However, the Aβ (1-42) self-association was monitored through the signal loss occurring over time after sample preparation in a 1D Watergate experiment incorporating a 30 ms long spin lock pulse with a 2.6 kHz strength prior to acquisition to suppress the residual protein signal. These 1D time-profiles for Aβ (1-42) were acquired at 600 MHz using 128 scans and 64 dummy scans. All 1D spectra were processed using an exponential multiplication window function prior to zero filling.

NMR Spectroscopy – Off-Resonance Relaxation (ORR) Experiments. Nonselective off-resonance relaxation 2D-TOCSY experiments (15,16) were acquired at 700 MHz with an off-resonance trapezoidal spin-lock including two adiabatic pulses of 4 ms duration and applied at the angle of 35.5° for 13 ms and 88 ms. The strength of the off-resonance and TOCSY spin locks were 8.25 and 10 kHz, respectively. The spectral widths for both dimensions were 8389.26 Hz with 256 and 1024 t_1 and t_2 complex points, respectively. The interscan delay was 2 seconds long. The water magnetization was suppressed using the binomial 3-9-19 Watergate gradient spin-echo (WG) (S2). Each experiment was collected with 8 scans and 128 dummy scans. For each spin-lock duration two data sets were acquired. 2D replica sets were then added to increase the S/N ratios and processed with Xwinnmr (Bruker Inc.) using a 90^o phase shifted squared sine bell window function for both dimensions prior to zero filling. The 2D cross-peak intensities were measured with Sparky 3.11126 (S3) by Gaussian line fitting using the fit peak heights. The standard deviation of the differences in fit heights between two copies was used to estimate the error of the individual spectra. The error of the sum was scaled up proportionally to the square root of the total number of scans. For all residues, the $H_{\alpha,i}$-$H_{N,i}$ cross-peaks were used for data analysis, with the exception of G25 and of the N-terminal V12 (15,16). G25 was omitted from the analysis due to the overlap of its degenerate H_α protons, while for V12, the $H_{\alpha,12}$-$H_{Me,12}$ cross-peak was used to probe Hα relaxation rates. The non-selective off-resonance relaxation rates were related to the experimental fit heights as previously explained (15,16). The measured rates and the related errors were normalized with respect to the maximum observed rate.

Dynamic Light Scattering (DLS) Measurements. DLS measurements were preformed on a Zetasizer Nano S (Malvern Instruments, Malvern UK) using a detection angle of 173° at a temperature of 25 °C and with a 4 mW He-Ne laser operating at a wavelength of 633 nm. All measurements were performed using a 12 μL (ZEN2112) quartz cell. The particle diameter detection limit is 0.6 – 6 μm. The viscosity value for water was used in the analysis of all

measurements. The intensity size distributions were obtained from the analysis of the correlation functions using the cumulant algorithm in the Zetasizer Nano S software. A stock 1 mM albumin sample was filtered using a 0.1 μm filter, while a 0.1 mM Aβ (1-42) stock solution was centrifuged for 10 minutes at 5,000 rpm prior to protein addition to remove possible dust particles. Measurements were preformed on a 0.1 mM Aβ (1-42) samples in the absence of and in the presence of 200 μM HSA, immediately after sample preparation and 48 hour after incubation at 37 ^{o}C. For each sample condition three measurements were collected to ensure reproducibility of the intensity distribution.

Table S1. Comparison of α-Helical Content in Different HSA Constructs

Constructs	Residue Ranges	Reference Values[a]	Calculated Values[b]	Measured Values[c]
Wt full length HSA[d]	1-585	66 +/- 0.6%	N/A	68 %
Domain 1	1-197	46 +/- 0.5%	N/A	45 %
Domain 3	381-585	53 +/- 1.5%	N/A	56 %
Domain 2	189-385	37 +/- 0.5%	N/A	N/A
Domain 23	189-585	N/A	45 %	52 %
Domain 12	1-385	N/A	41 %	49 %

[a]From (S1) based on constructs expressed in yeast.

[b]Weighted average of the reference values reported for the isolated domains.

[c]All data was recorded at 25°C on an AVIV Circular Dichroism spectrometer. The percentages of the α-helical structures were calculated from the molar ellipticities at 222 nm.

[d]The protein concentrations were 15, 30, 7, 5.5, and 28.7 μM for HSA (commercial), domain 3, domain 23, domain 12 and domain 1, respectively. All proteins samples were in 50 mM sodium phosphate buffer (pH 6.5) containing 150 mM NaCl, and 0.02% NaN_3.

References

S1. Dockal, M., D.C. Carter and F. Ruker 1999. The Three Recombinant Domains of Human Serum Albumin. J. Biol. Chem. 274, 29303-29310.

S2. Piotto, M., V. Saudek and V. Sklenar 1992. Gradient-tailored excitation for single-quantum nmr-spectroscopy of aqueous-solutions. J. Biomol. NMR. 2, 661-665.

S3. Sparky 3.111 Goddard, T. D. and Kneller, D. G., SPARKY 3, University of California, San Francisco

Figure S1

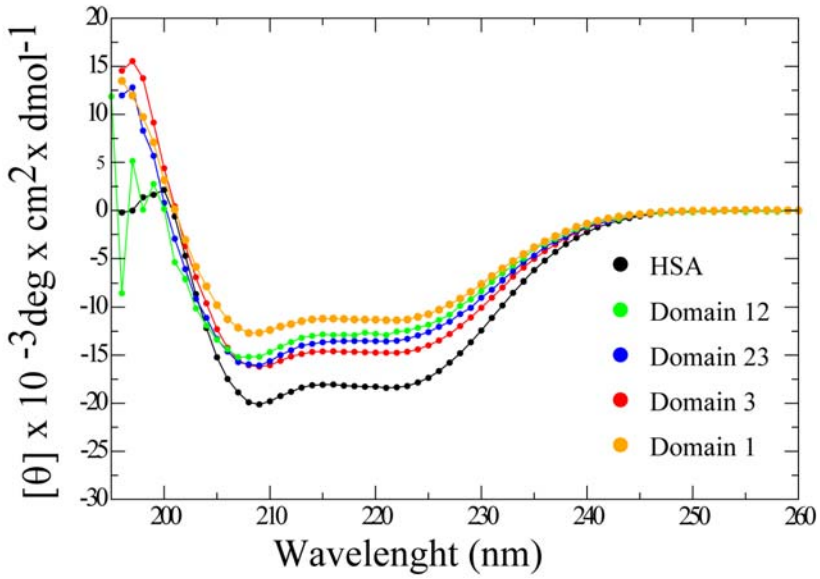

Figure S1: CD spectra of all protein constructs used in this study. Spectra were collected on an AVIV Circular Dichroism spectrometer at 25°C and were used to validate the protein fold, as indicated in Table 1. Measurements were preformed in 50 mM sodium phosphate buffer (pH 6.5) containing 150 mM NaCl, and 0.02% NaN_3.

Figure S2

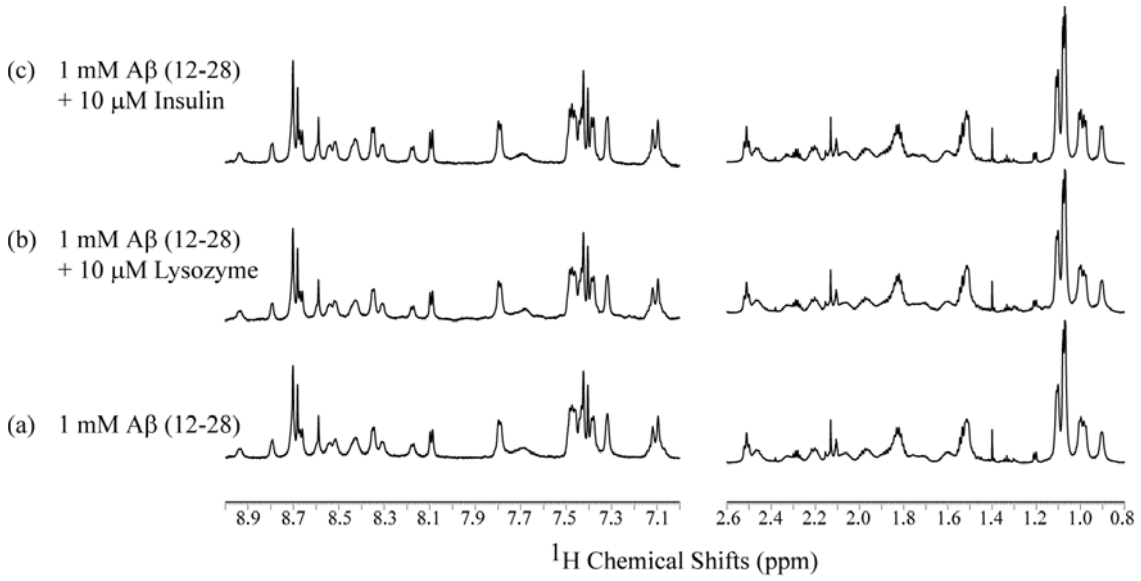

Figure S2: Effect of insulin and lysozyme on the aggregated 1 mM Aβ (12-28) sample. Panel (a) reports aggregated 1 mM Aβ (12-28). Effect of the lysozyme and insulin addition is shown in panel (b) and (c), respectively. Contrary to the HSA these proteins do not interact with Aβ and therefore have no effect on the line widths of the 1D spectrum as it was observed in Figure 2 upon addition of HSA and HSA constructs. All samples were prepared in 50 mM deuterated acetate buffer with 10% D_2O at pH 4.7. Experiments were recorded at 700 MHz using a TCI CryoProbe and at 293K.

Figure S3

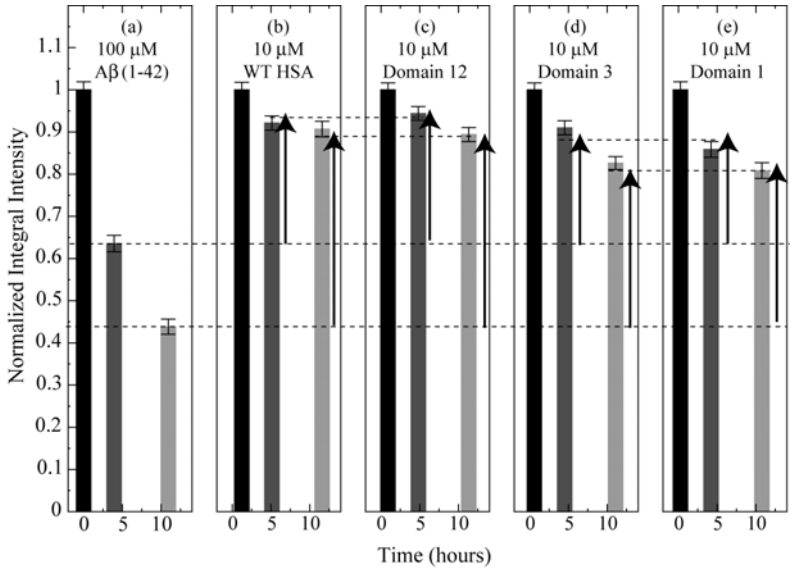

Figure S3: Effect of HSA and of the HSA deletion mutants on the early aggregation of the Aβ (1-42) peptide. Changes in the intensity of the 1D spectra were used to monitor the Aβ(1-42) aggregation state in the absence (a) and presence of 10 μM of HSA (b), the domain 12 construct (c), domain 3 (d) and domain 1 (e), respectively. Experiments were recorded at 600 MHz and 37 ^{o}C using a 30 ms spin lock to suppress protein signals. The integrals reported in this Figure as a function of time are for the methyl spectral region (0.6–1.1 ppm). The error was estimated from the spectral noise. All integrals were normalized to their starting values. In between acquisition sessions, NMR samples were stored in a water bath at 37 ^{o}C.

Figure S4

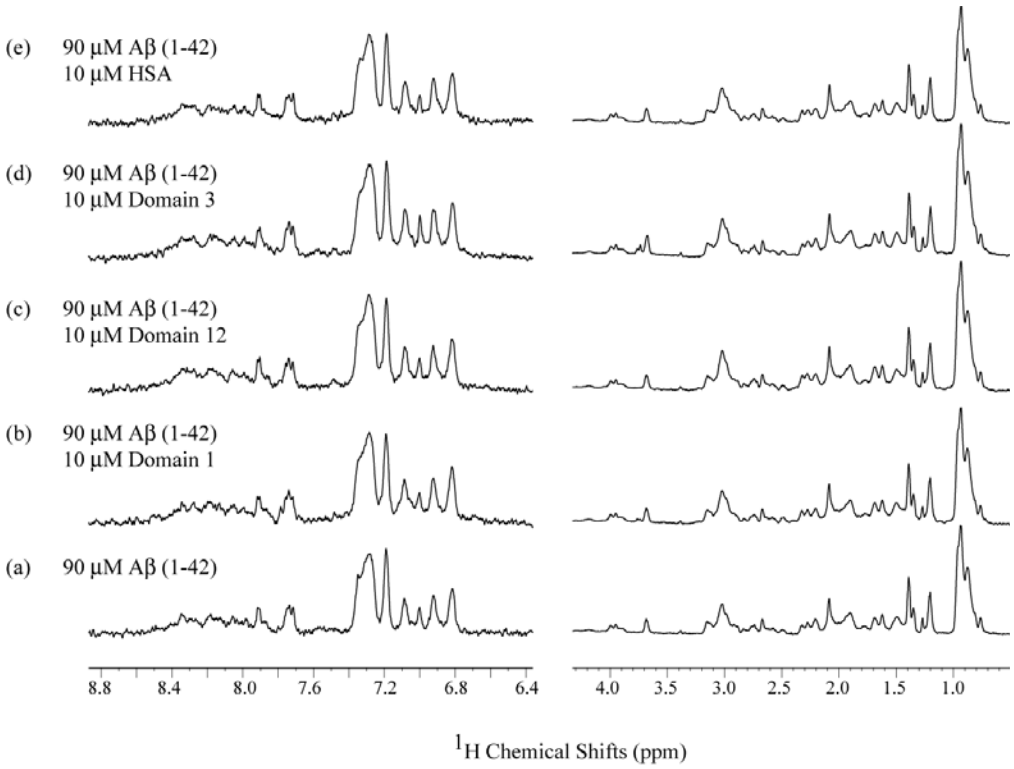

Figure S4: 1D-WG NMR spectra comparisons to show that the HSA deletion mutants do not interact with the monomeric Aβ(1-42) peptide. All samples used for this Figure contain 90 μM Aβ(1-42) either in the absence or in the presence of 10 μM HSA or its deletion constructs indicated in the Figure. These 1D-WG experiments were obtained with a 30 ms spin lock filter to suppress the protein signal and were recorded at 600 MHz at 37 ^{o}C. All spectra were processed using an exponential multiplication window function with a line-broadening coefficient of 3 Hz. Samples were prepared in 15 mM potassium phosphate buffer with 10% D_2O at pH 7.4.

Figure S5

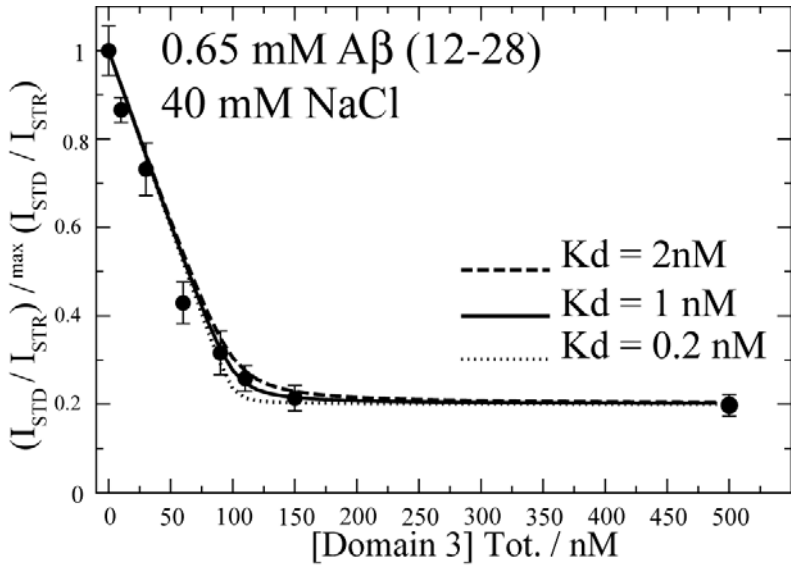

Figure S5: Effect of domain 3 on the relative I_{STD}/I_{STR} ratios measured for the filtered 0.65 mM Aβ (12-28) peptide aggregated through addition of 40 mM NaCl. Experiments were recorded at 700 MHz using a TCI CryoProbe and at 20 ^{o}C. All ratios were normalized to their maximum value measured before domain 3 addition. Dotted lines were used to model dissociation constants in the 2-0.2 nM range.

Figure S6

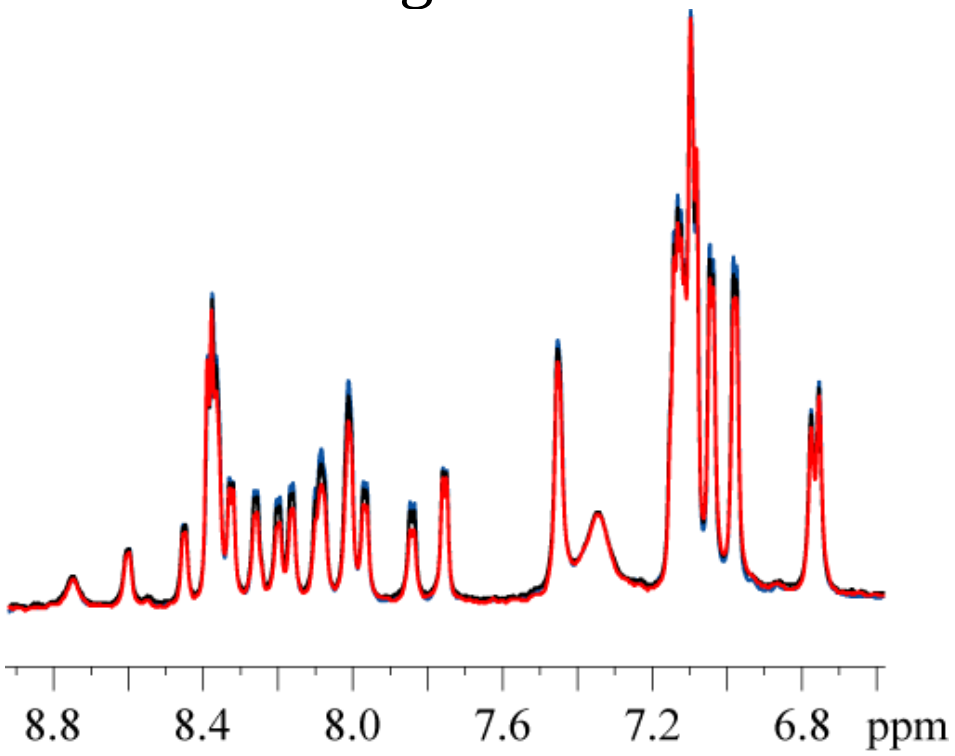

Figure S6: 1D –STR NMR spectra of the Aβ(12-28) peptide in the absence and presence of HSA domain 3 processed using an exponential multiplication window function with a line broadening coefficient of 5 Hz. This line broadening coefficient ensures that the linewidth is approximately independent of the amount of HSA domain 3 added. The spectral intensity can therefore be used to estimate the relative concentrations of monomeric Aβ(12-28). These 1D-WG experiments used a 30 ms spin lock filter to suppress the protein signal and were recorded at 700 MHz at 293K. The spectrum of 0.65 mM Aβ(12-28) in the absence of domain 3 is shown in red, while samples with 60 and 500 nM of domain 3 are shown in black and blue respectively. While addition of the domain 3 affects significantly the STD signal, no major intensity or chemical shift changes were observed in the Aβ STR spectra. All samples were prepared in 50 mM acetic acid-d_4, 40 mM NaCl buffer with 10% D_2O at pH 4.7.