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Kinetics of Amyloid β monomer to oligomer exchange by NMR Relaxation

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Abstract

Recent studies implicating non-fibrillar oligomers of the amyloid β (A β) peptide as the primary toxic species in Alzheimer's disease have made A β oligomers the subject of intense study. Detailed structural and kinetic characterization of these states, however, has been difficult. Here we use NMR relaxation measurements to address the kinetics of exchange between monomeric and large, polymorphic oligomeric species of A β (1–40). ^{15}N -R $_2$ and ^1H -R $_2$ data at multiple magnetic fields were recorded for several peptide concentrations subsequent to the establishment of a stable pseudo-equilibrium between monomeric and NMR invisible soluble oligomeric species. The increase in ^{15}N - and ^1H -R $_2$ rates as a function of protein concentration is independent of nucleus and magnetic field and shows only a small degree of variation along the peptide chain. This phenomenon is due to a lifetime broadening effect arising from the unidirectional conversion of monomer to the NMR invisible oligomeric species ('dark' state). At a total A β (1–40) concentration of 300 μM , the apparent first order rate constant for this process is $\sim 3\text{ s}^{-1}$. Fitting the McConnell equations for two dipolar-coupled spins in two-site exchange to transfer-of-saturation profiles at two radiofrequency field strengths gives an estimate for k_{off} of 73 s^{-1} and transiently-bound-monomer ^1H -R $_2$ rates of up to 42,000 s^{-1} in the tightly bound central hydrophobic region and $\sim 300\text{ s}^{-1}$ in the disordered regions such as the first nine residues. The fraction of peptide within the 'dark' oligomeric state undergoing exchange with free monomer is calculated to be $\sim 3\%$. The relatively rapid exchange between the monomer and the polymorphic oligomeric form suggests that therapeutic efforts aimed at altering the equilibrium distribution between these species may be more successful than for the extremely stable fibril form.

A hallmark of Alzheimer's disease (AD) is the formation of plaques composed of amyloid- β (A β) fibrils characterized by an elongated highly ordered β -sheet structure.¹ The link, however, between amyloid fibrils and the etiology of the disease is not well understood.² Recent evidence suggests that smaller, less ordered oligomers of A β may be primarily responsible for neurotoxicity,³ and the presence of non-fibrillar A β oligomers (ranging from 40 to 200 kDa) in the cerebrospinal fluid correlates with AD.⁴ Elucidating the mechanism of conversion of non-toxic monomers to toxic oligomers or fibrils may be critical to the design of therapeutic interventions that steer the equilibrium away from the buildup of toxic species.⁵ While A β amyloid fibrils have been studied at the atomic level by fiber diffraction,⁶ solid state NMR,⁷ electron microscopy,⁸ and H/D exchange combined with mutagenesis,⁹ characterization of the non-fibrillar oligomeric states has proven difficult due to their heterogeneous nature.⁵ Recent work has provided some structural information on A β oligomers,¹⁰ but the chemical cross-linking reagents, ionization conditions, and organic

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Supporting Information Available: ^{15}N and ^1H R $_2$ values and supplementary methods. This material is available free of charge on the internet at <http://pubs.acs.org>.

solvents and detergents used in these studies make comparison with oligomers formed in their absence difficult.⁵⁻¹¹ Several fundamental questions concerning the nature of A β oligomers remain to be answered, including whether the oligomers are permanently stable, or if they are constantly forming from and dissociating back into monomers in a dynamic equilibrium preceding the formation of the extremely stable amyloid fibrils.¹² In this study, we make use of solution NMR relaxation measurements to directly observe rapid exchange under pseudo-equilibrium conditions between monomeric A β peptide and non-fibrillar oligomers formed spontaneously in a standard buffer without organic solvents or detergents. These data provide significant details concerning the pre-fibrillar equilibrium that are difficult to probe by other biophysical techniques.

Samples of uniformly ^{15}N -labeled A β (1–40) peptide, were prepared from NaOH treated stocks to remove fibril seeds.¹³ NMR samples comprised 60, 150 and 300 μM A β (1–40) in 50 mM HEPES, pH 6.8 and 90% $\text{H}_2\text{O}/10\%$ D_2O . All solutions were pre-filtered in the presence of the chelating agent Chelex 100 (Sigma-Aldrich) to remove any potential trace metal contamination.¹⁴ Samples were prepared and maintained between 4 and 10°C , and all NMR experiments were conducted at 10°C . Under these conditions, A β (1–40) at a concentration of 60 μM remains stable for many weeks as monitored by following the $^1\text{H}_\text{N}/^{15}\text{N}$ cross-peak intensities in ^1H - ^{15}N HSQC correlation spectra (Figures 1A,B) over time. At peptide concentrations of 150 and 300 μM , however, the signal intensities decay uniformly across the peptide over a period of about one week after which a pseudo-equilibrium is established with integrated intensities for the backbone amide ($^1\text{H}_\text{N}$) envelope (measured from the first t_1 increment of an HSQC spectrum) of 70 and 40% of their original values, corresponding to monomer concentrations of 105 and 130 μM , respectively (Figure 1B). Since the $^1\text{H}_\text{N}/^{15}\text{N}$ observed cross-peaks arise solely from monomeric peptide¹³⁻¹⁵⁻¹⁶ and no new cross-peaks appear, the decrease in signal intensity must arise from the conversion of monomer to a species whose NMR signals are broadened beyond detection due to large oligomer size and correspondingly long rotational correlation times. These large species remain in solution as the samples are clear. Once equilibrated, the presence of large polydisperse aggregates was confirmed by dynamic light scattering, and transmission EM revealed the presence of elongated morphologically disordered non-fibrillar aggregates with possibly some small needle-like fibrils present (Figure 2) similar to those observed for other proteins.¹⁷

^{15}N and $^1\text{H}_\text{N}$ transverse relaxation (R_2) rates (Figure 1C) were measured for the equilibrated A β (1–40) samples using 2D ^1H - ^{15}N HSQC-based experiments.¹⁴⁻¹⁸ The R_2 rates increase as a function of total peptide concentration (Figure 3). The difference in R_2 rates, ΔR_2 , between high (150 or 300 μM) and low (60 μM) concentration samples is independent of nucleus or magnetic field (Figure 3B and 3C), as evidenced by the linear ΔR_2 correlation plots with a slope of ~ 1 (Figure 4). In addition, the variation in ΔR_2 across the peptide chain is small with average $\Delta R_2(150\ \mu\text{M} - 60\ \mu\text{M})$ and $\Delta R_2(300\ \mu\text{M} - 60\ \mu\text{M})$ values of 0.7 ± 0.3 and $2.4 \pm 0.5\ \text{s}^{-1}$, respectively. The ΔR_2 values are slightly higher for the hydrophobic (green lettering) segments spanning Leu17 to Ala21 and Ala30 to Val40 with the former being systematically elevated relative to the latter. These two regions correspond to the exterior and interior in-register parallel β -strands, respectively, of the cross- β core of A β (1–40) amyloid fibrils.^{7a,b}

The observation that the R_2 rate enhancements as a function of total A β (1–40) concentration are independent of nucleus and magnetic field and, in addition, vary only slightly along the peptide, indicates that the R_2 rate increases cannot be due to exchange line broadening on the fast/intermediate chemical shift time scale. The latter is dependent on the difference in the resonance frequencies of the spins involved and therefore strongly affected by the nucleus' chemical environment and the external magnetic field used in the measurements.¹⁹

It is also important to stress that the observed resonances arise only from the monomeric A β (1–40) peptide and not from any aggregates present. Indeed, intermolecular paramagnetic relaxation enhancement (PRE) measurements, which provide a highly sensitive probe for the presence of low populations of self-associated species in fast (μ s to ms) exchange with monomer,^{20a,b} showed no transverse PRE rate enhancements above a reliable detection limit of $\sim 5 \text{ s}^{-1}$ for mixtures of ^{15}N -labeled A β (1–40) and nitroxide spin-labeled A β (1–40) at natural isotopic abundance (Figure 5). Thus, either the population of lower order, transient, self-associated states, if present, is less than 1–2%,^{20c} or such exchange is slow on the PRE time scale ($< \text{ca. } 1 \text{ ms}$). We conclude that the observed R_2 rate enhancements must be due to a lifetime broadening effect arising from direct incorporation of the NMR visible monomer into NMR invisible oligomers (i.e. a ‘dark’ state).

The transverse magnetizations of any large oligomeric species will decay very rapidly owing to the very large R_2 rates associated with their high molecular weights. Hence, the observed increase in R_2 rates at high concentration of A β (1–40) can be interpreted as the unidirectional monomer to oligomer conversion rate under pseudo-equilibrium conditions. The maximum observed ^{15}N ΔR_2 rates of 1.1 and 3.1 s^{-1} at total A β (1–40) concentrations of 150 and 300 μM , respectively, provide estimates of the apparent first order association rate constants, k_{on}^{app} , for this process. A similar phenomenon has been observed for the equilibrium between monomeric and lipid bound α -synuclein.²¹

To probe the invisible oligomeric state, we carried out a series of ^1H saturation transfer experiments. Using 1 s off-resonance continuous wave (CW) pulses with radiofrequency (RF) field strengths of 180 Hz or 350 Hz at a series of offsets (ranging from +35 kHz to –35 kHz) from the water resonance, the underlying broad resonances of the large oligomers were partially saturated and transfer of saturation from the ‘dark’ oligomeric states to the monomer was measured from the overall decrease in intensity of the $^1\text{H}_\text{N}$ backbone amide envelope (7.8 to 9 ppm) of the monomer in a one-dimensional ^1H -NMR spectrum relative to that in a reference spectrum obtained without saturation.

For the 60 μM A β (1–40) sample, the off-resonance saturation pulse has no effect on the signal intensity of the amide resonances until the saturation pulse approaches resonances of the protein (Figure 6, black), as expected for a monomeric peptide or protein. For the 300 μM A β (1–40) sample, however, the amide resonances are uniformly attenuated by the CW pulse at RF offsets far off-resonance (Figure 6, orange and blue), due to transfer of saturation from the invisible ‘dark’ state with large R_2 .

Exchange between free monomer (M_{free}) and transiently bound monomer (M_{bound}) in the NMR invisible ‘dark’ state, can be represented phenomenologically by a pseudo-first order process (see Supplementary):

(Scheme 1)

The values of the dissociation rate constant, k_{off} , and the R_2^{dark} rate for the ‘dark’ state can be obtained by simultaneously fitting the experimental saturation profiles for the 300 μM sample at the two RF field strengths to a solution of the McConnell equations²² for two dipolar-coupled spins in two-site exchange in the presence of a CW saturation field (see

Supplementary). Inclusion of two dipolar-coupled spins with different resonance frequencies was necessary to account for the ~5 kHz width of the saturation profile observed for the 60 μM sample (black circles in Figure 6) where the fraction of oligomers is negligible (Figure 1B); the width of the saturation profile spans the ^1H chemical shift range of the monomer and is ascribed to saturation transfer arising from cross-relaxation among protons in the monomer.

Given measured values of $^1\text{H}_\text{N}\text{-R}_1 = 1 \text{ s}^{-1}$ and $^1\text{H}_\text{N}\text{-R}_2 = 10 \text{ s}^{-1}$ for the A β (1–40) monomer, $k_{\text{on}}^{\text{app}} = 3.1 \text{ s}^{-1}$, and assuming the cross-relaxation rates are small in the monomer (-0.5 to -3 s^{-1}) but large in the oligomer ($\sim -500 \text{ s}^{-1}$), the saturation profiles for the 300 μM sample can be fit with $k_{\text{off}} = 73 \pm 6 \text{ s}^{-1}$ and two distinct values of R_2^{dark} : $42,000 \pm 3000 \text{ s}^{-1}$ and $\sim 300 \text{ s}^{-1}$ with weights of 0.40 ± 0.03 and 0.60 ± 0.03 , respectively (Figure 6, thick orange and blue lines). The saturation profiles for the 300 μM sample cannot be adequately fit by a model with only a single R_2^{dark} rate (Figure 6, thin orange and blue lines), but are not inconsistent with a distribution of larger and smaller R_2^{dark} rates.

If R_2^{dark} varies along the peptide chain, ranging from ~ 300 to $\sim 40,000 \text{ s}^{-1}$, the residues with the largest R_2^{dark} rates would have broad saturation profiles while those with smaller R_2^{dark} rates would experience saturation profiles close to that of the free monomer. Because only a small fraction of monomer is involved in transient interactions with the oligomer ($k_{\text{off}} \gg k_{\text{on}}^{\text{app}}$), $^1\text{H}_\text{N}$ resonances with smaller R_2^{dark} rates will make a negligible contribution to a standard 1D ^1H -NMR spectrum such that only signals of the free monomer are observed. Further, because k_{off} is large, the lifetime broadening effect (ΔR_2) will be smaller in the case of residues having smaller R_2^{dark} rates (Figure 7), thereby explaining the small variation in ΔR_2 as a function of residue (Figure 3). Thus, the lower observed ΔR_2 rates for the first nine N-terminal residues (which are disordered in A β fibrils_{6a}:23), as well as for residues 24–29 (which form a turn between the two hydrophobic segments in A β fibrils_{7a}:24), can be attributed to their higher mobility in the oligomer-bound state. Regions in intimate contact with the oligomer exhibit maximal ΔR_2 rates equal to the unidirectional on-rate $k_{\text{on}}^{\text{app}}$, as exemplified by the central hydrophobic region. The optimized weights of 40 and 60% for the large and small R_2^{dark} rates obtained from the fits to the saturation profiles (Figure 6) are fully consistent with the number of residues exhibiting larger and smaller ΔR_2 values, respectively (Figures 3 and 4).

The fraction, f_{ex} , of peptide within the oligomeric ‘dark’ state that exchanges with free monomer, is given by the ratio of $[\text{M}_{\text{bound}}]$ to total peptide sample concentration. f_{ex} is readily calculated from the values of k_{off} , $k_{\text{on}}^{\text{app}}$ and $[\text{M}_{\text{free}}]$, and found to be 3.5 and 3.3% for the 150 and 300 μM A β (1–40) samples, respectively, indicating that the data at the two sample concentrations are self-consistent. Thus, only 1 in about 30 peptides within the oligomer undergoes exchange with free monomer suggesting that exchange occurs predominantly from the ends of the oligomers.

In conclusion, we have demonstrated that large oligomers of the A β (1–40) peptide are in dynamic equilibrium with the monomeric state on a timescale of 10 to 15 ms, and that monomers are constantly binding and being released from NMR invisible oligomers. This process is slower than the fast transient (μs to ms time scale) self-association of monomers that gives rise to sizeable intermolecular PREs observed for α -synuclein.²⁴ The small regional specificity for ΔR_2 indicates that the N-terminal 9 residues remain highly mobile while the central and C-terminal hydrophobic regions are largely immobilized upon association of monomer onto the surface of the oligomeric species. Rapid exchange between

monomeric and polymorphous oligomeric forms suggests that therapeutic efforts aimed at altering the equilibrium between these species may be more successful than for highly stable amyloid fibrils.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) 900 MHz ^1H - ^{15}N HSQC correlation spectrum of 60 μM A β (1–40) at 10°C. (B) Time dependence of the integrated intensity of the $^1\text{H}_\text{N}$ envelope (measured from the first t_1 increment of a ^1H - ^{15}N HSQC spectrum) for 60 (black), 150 (red) and 300 (blue) μM A β (1–40) samples. The solid lines represent single or double exponential fits to the 150 or 300 μM A β (1–40) data, respectively. The double exponential fitting function is given by $I(t)/I_0 = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + (1 - A_1 - A_2)$, where τ_1 and τ_2 are characteristic time constants, and A_1 and A_2 are the associated amplitudes. The parameter A_2 is set to 0 for the single exponential fit. For the 150 μM sample, $\tau_1 = 51$ h and $A_1 = 0.31$; for the 300 μM sample, $\tau_1 = 6$ h, $\tau_2 = 93$ h, $A_1 = 0.36$ and $A_2 = 0.21$. At both 150 and 300 μM total concentrations, the samples

reach pseudo-equilibrium after about 100 h, after which the signal intensity does not change more than a few percent on the timescale of a complete NMR experiment (4 to 12 h). (C) ^{15}N - R_2 (top) and ^1H - R_2 (bottom) relaxation data for the 60 μM A β (1–40) sample recorded at 900 MHz. The solid lines are single exponential best-fits.

Figure 2. Transmission EM images of negatively stained (A) 150 and (b) 300 μM A β (1–40) NMR samples after >3 weeks of equilibration.

Figure 3.

Transverse relaxation rates (R_2) measured on equilibrated samples of $A\beta(1-40)$. (A) ^{15}N - R_2 at 900 MHz for 60 μM $A\beta(1-40)$. Cross-peaks for Ala2, His6 and His14 are not sufficiently resolved to permit accurate determination of R_2 rates. The difference in R_2 relaxation rates, ΔR_2 , between 300 (blue) or 150 (red) μM samples and the 60 μM sample for (B) ^{15}N (top, 900 MHz; bottom, 600 MHz) and (C) ^1H (top, 900 MHz; bottom, 600 MHz). Large R_2 rates and uncertainties in their values preclude reliable determination of ΔR_2 for His13, Gln15 and Asn27 (A, open circles), and hence are excluded in (B) and (C). The peptide sequence, with hydrophobic residues colored in green, is presented at the top of the figure. (Error bars, 1 s.d.)

Figure 4.

ΔR_2 is independent of nucleus and magnetic field. Correlation plots of $^{15}\text{N}-\Delta R_2$ at 900 MHz vs. $^1\text{H}_\text{N}-\Delta R_2$ at 900 MHz (left panel) and $^{15}\text{N}-\Delta R_2$ at 600 MHz (right panel). The ΔR_2 (300 μM - 60 μM) and ΔR_2 (150 μM - 60 μM) rates are colored in blue and red, respectively. A line with slope of unity is displayed for comparison. A histogram of the distribution of $^{15}\text{N}-\Delta R_2$ rates is shown as an inset.

Figure 5.

Intermolecular PRE profiles for Aβ(1–40). Transverse $^1\text{H}_\text{N}$ -PRE rates, $^1\text{H}_\text{N}-\Gamma_2$, were determined from the difference in transverse relaxation rates, $^1\text{H}_\text{N}-R_2$, between paramagnetic and diamagnetic samples. The samples comprised 100 μM U- ^{15}N -labeled Aβ(1–40) and 170 μM single-cysteine variant Aβ(1–40) peptides at natural isotopic abundance conjugated to either a nitroxide spin-label or a diamagnetic analogue. A Cys residue was introduced preceding the N-terminal Asp residue (Cys-Aβ, black) or as a F20C mutation in the center of the peptide (red). For the Cys-Aβ variant only small intermolecular PREs ($^1\text{H}_\text{N}-\Gamma_2 < 5 \text{ s}^{-1}$) were observed, while essentially no intermolecular PREs were observed for the F20C sample.

Figure 6.

Attenuation of the integrated intensity of the $^1\text{H}_\text{N}$ envelope of monomeric $\text{A}\beta(1-40)$ by transfer of saturation from the 'dark' state following application of an off-resonance radio-frequency (RF) field as a function of offset from the water resonance. Black circles, 60 μM total concentration and 350 Hz RF field; orange and blue circles, 300 μM total concentration with 180 and 350 Hz RF fields, respectively. The simultaneous best-fits to the experimental saturation profiles for the 300 μM sample at the two RF fields, using the McConnell equations for a dipolar-coupled two spin, two-site exchange model, are significantly better with two distinct R_2^{dark} rates (bold orange and blue lines) than with a single R_2^{dark} rate (thin orange and blue lines). Dotted blue and orange lines indicate the region near-resonance where saturation is not well represented by the model due to the many-spin nature of the experimental system. Plots of the residuals (observed minus calculated) between the experimental saturation profiles and the best-fit curves are shown in the top panel. The model with the single R_2^{dark} underestimates the attenuation between $5 \text{ kHz} < |\text{RF offset}| < 20 \text{ kHz}$ at the lower (180 Hz) RF field, while overestimating the attenuation between $-15 \text{ kHz} < \text{RF offset} < -5 \text{ kHz}$ and between $+5 \text{ kHz} < \text{RF offset} < +10 \text{ kHz}$ at the higher (350 Hz) RF field. The best-fit parameters for the model with a single R_2^{dark} rate are

$R_2^{\text{dark}} = 66,000 \pm 9000 \text{ s}^{-1}$ and $k_{\text{off}} = 320 \pm 20 \text{ s}^{-1}$. The best-fit parameters for the model with two R_2^{dark} rates are $k_{\text{off}} = 73 \pm 6 \text{ s}^{-1}$, $R_2^{\text{dark}}(\text{large}) = 42,000 \pm 3000 \text{ s}^{-1}$ and $R_2^{\text{dark}}(\text{small}) \leq 300 \text{ s}^{-1}$ with population weights of 0.40 ± 0.03 and 0.60 ± 0.03 , respectively.

Figure 7.

Simulation of the dependence of the monomer ΔR_2 rates arising from exchange with an NMR invisible, oligomer bound species as a function of the R_2 rate in the bound state obtained by numerical solution of the McConnell equations. The blue curve is calculated using the experimentally determined values of 3.1 and 73 s^{-1} for k_{on}^{app} and k_{off} , respectively, obtained for the 300 μM A β (1–40) sample. The ΔR_2 predicted for $k_{off} \pm 6 \text{ s}^{-1}$ (the 68% confidence interval, representing ± 1 s.d) is represented by the gray region. Calculated ΔR_2 rates as a function of the R_2 rate in the oligomeric state for k_{off} values of 2 (black), 20 (red) and 200 (green) s^{-1} are also shown for comparison. The effect of ^1H chemical shift differences, between free and oligomer-bound monomers, on the observed ΔR_2 is assumed to be negligible. Residue positions tightly interacting with the oligomer, such as the central hydrophobic region, may have large chemical shift deviations upon binding, but these effects are far exceeded by the large R_2 (ca. 40,000 s^{-1}) at those positions, which causes complete decay of transverse magnetization of these residues in the oligomer-bound monomer. In contrast, residue positions retaining significant mobility in the oligomer-bound form have much lower R_2 values (ca. 300 s^{-1}); hence, their chemical shift changes are likely to be small since the average environment of these highly disordered residues is likely to be very similar to that in the unstructured free state. Further, the ^{15}N CPMG R_2 experiment with a 180° pulse separation time of 900 μs should suppress the effects arising from chemical exchange for moderate shift differences (<200 Hz). The correspondence of the ΔR_2 measured on two different nuclei ($^1\text{H}_\text{N}$ and ^{15}N R_2 relaxation) at two different fields (600 MHz and 900 MHz) (Figure 3) further supports this assumption of negligible effects of shift differences upon chemical exchange.