Characterizing Methyl-Bearing Side Chain Contacts and Dynamics Mediating Amyloid β Prototibril Interactions Using $^{13}$C$_{methyl}$-DEST and Lifetime Line Broadening**

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Abstract: Many details pertaining to the formation and interactions of protein aggregates associated with neurodegenerative diseases are invisible to conventional biophysical techniques. We recently introduced $^{15}$N dark-state exchange saturation transfer (DEST) and $^{2}$H lifetime line-broadening to study solution backbone dynamics and position-specific binding probabilities for amyloid β (Aβ) monomers in exchange with vast transverse relaxation rates ($^{15}$N-DEST) and kinetic partitioning within the prototibril-bound state of Aβ.[4] $^{13}$C-methyl-DEST, 15N-DEST, and $^{15}$N lifetime line-broadening exploit the binding/unbinding of monomeric Aβ peptides to/from large prototibrillar aggregates and the vastly different transverse relaxation rates ($^{15}$N-DEST) between monomers and prototibril-bound species to imprint atomic resolution information for the NMR-invisible (“dark”) prototibril-bound state onto the easily observed resonances of the free monomer. Unlike Aβ fibrils that are stabilized by β-sheets spanning ten residues in length in both the central and C-terminal hydrophobic regions, transient contacts with Aβ prototibrils are concentrated only in stretches of four to five hydrophobic residues. Since $^{13}$N-DEST reports only on the backbone, $^{13}$C-based experiments are required to probe side-chain contacts within the prototibril-bound states and to ascertain whether the transient interactions on the surface of the prototibrils are directly mediated by side chains or involve edge-on β-sheet contacts with effectively disordered side chains. Here we use $^{13}$C$_{methyl}$-DEST and lifetime broadening to refine the atomic-level picture of the interactions between monomeric and prototibril-bound Aβ40. We show that methyl groups in hydrophobic side chains directly mediate interactions on the prototibril surface and are sensitive reporters of dynamics in the bound-state ensemble.

The formation of protein aggregates lies at the heart of numerous degenerative diseases, including Alzheimer’s disease in which the peptide amyloid β (Aβ) forms fibril plaques.[3] Because smaller aggregates of Aβ, including oligomers and prototibrils, have been implicated as the primary toxic species, they are critical targets for structural characterization.[2] Their large size, transient nature, and high level of structural disorder and heterogeneity, however, preclude characterization by standard structural techniques. Several new methods have been developed in an attempt to reveal structural details of these complexes.[3] We recently reported a new solution NMR technique, dark-state exchange saturation transfer at backbone nitrogen positions ($^{15}$N-DEST), to probe, with residue-level resolution, dynamic information ($^{15}$N-R) and kinetic partitioning within the prototibril-bound state of Aβ.[4] $^{15}$N-DEST and $^{15}$N lifetime line-broadening exploit the binding/unbinding of monomeric Aβ peptides to/from large prototibrillar aggregates and the vastly different transverse relaxation rates ($^{15}$N-DEST) between monomers and prototibril-bound species to imprint atomic resolution information for the NMR-invisible (“dark”) prototibril-bound state onto the easily observed resonances of the free monomer. Unlike Aβ fibrils that are stabilized by β-sheets spanning ten residues in length in both the central and C-terminal hydrophobic regions, transient contacts with Aβ prototibrils are concentrated only in stretches of four to five hydrophobic residues. Since $^{13}$N-DEST reports only on the backbone, $^{13}$C-based experiments are required to probe side-chain contacts within the prototibril-bound states and to ascertain whether the transient interactions on the surface of the prototibrils are directly mediated by side chains or involve edge-on β-sheet contacts with effectively disordered side chains. Here we use $^{13}$C$_{methyl}$-DEST and lifetime broadening to refine the atomic-level picture of the interactions between monomeric and prototibril-bound Aβ40. We show that methyl groups in hydrophobic side chains directly mediate interactions on the prototibril surface and are sensitive reporters of dynamics in the bound-state ensemble.


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assembly, the monomeric population remains in pseudo-equilibrium with the protofibril form for about one month, during which time the monomeric peptide reversibly binds to and unbinds from the protofibrils, resulting in near-zero net further assembly.[4]

The protofibril-bound species exchanging with the remaining monomeric pool is invisible to standard solution NMR methods, because it is sparsely populated (≈5% of the remaining monomer) and characterized by very rapid transverse relaxation rates (15N-τ1 up to ca. 19 000 s−1) due to its very large size.[6] Interactions, however, between monomeric and protofibrillar Aβ are evident by measuring backbone-lifetime-broadening effects (ΔR2), independent of field and nucleus, from the difference in 13Cmethyl-DEST measurements confirm the presence (high concentration) and absence (low-concentration control) of protofibrils.[6b] The apparent first-order association rate constant (Kapp) for monomer-binding to the protofibril, given by the maximum value of 15N-ΔR2,[8] is 2.5 ± 0.3 s−1 for U-[13C/15N] Aβ40 at 240 μM (SI, Figure S2).

To compare the values measured at the backbone to those measured at the side-chain methyl positions, effective 13Cmethyl-ΔR2 values were determined from 13Cmethyl (166 Hz spin-lock field-centered at 20.7 ppm) and 13C-τ1 values using standard experiments and procedures (SI, Figures S3 and S4). [9,10] 13Cmethyl lifetime-broadening, (13Cmethyl-ΔR2), given by the difference in 13Cmethyl-ΔR2 values between Aβ40 samples in the presence and absence of protofibril aggregates (Figure 1A), shows a position-specific pattern of interactions with the protofibrils. 13Cmethyl-DEST measurements confirm the residue-specific nature of the interactions (Figure 1B). Briefly, the 13Cmethyl-DEST pulse sequence (SI, Figure S5) resembles a 2D 1H-13C HSQC-based 13Cmethyl experiment, in which 13C polarization is prepared; however, the variable delay used to measure R1 is replaced by a fixed 480 ms 13C saturation pulse (radiofrequency field strengths of 763 and 1523 Hz) applied at a series of offsets from the 13C carrier frequency at 19 ppm (−23.7, −20, −16, −10 −8, −6, −4, −2, 0, 2, 4, 6, 8, 10, 16, 20, 23.7 26 kHz). Whereas the 13Cmethyl-DEST pulse sequence is similar to the recently described 13Cmethyl-CEST experiment,[10] the DEST and CEST experiments are not the same and should not be confused with one another: CEST relies on differences in chemical shifts between slowly exchanging species with the same or approximately the same R1 rates, whereas DEST is entirely reliant on the exchange between species with very large differences in R1 rates and requires no differences in chemical shifts. Further, distinct chemical shifts for the minor species will not be observed in a CEST experiment when the R1 values for the minor species are very large. Thus, the experimental considerations for and quantitative analysis of the DEST and CEST experiments are quite different.

The data from 13Cmethyl-ΔR2 and DEST experiments were fit simultaneously to the same kinetic model previously used for the corresponding 15N data[10] to provide atomic-resolution details on the role of methyl-side-chain interactions in protofibril-bound states. Our previous 15N data, which probed the backbone of Aβ, showed that the ensemble of binding modes in the protofibril-bound state could not be described by a simple global two-state exchange model.[4] The same is true for the current 13Cmethyl data, for which the simple two-state exchange model predicts 13Cmethyl ΔR2 values and DEST profiles that are larger and narrower, respectively, than measured. However, a simple extension, incorporating slowly interconverting exchange between distinct bound states for each residue, described by a residue-specific partition coefficient Kij for the population ratio of direct-contact to tethered states (Figure 2), is sufficient to reproduce the 15N-DEST and 13Cmethyl-ΔR2 data.[4] This phenomenological extension of a two-state exchange model concisely captures the average behavior of the potentially very large ensemble of bound conformations using only two distinct residue-specific parameters, the partition coefficients Kij and the R2tethered values for the tethered states. Here, we show that without expanding the model, we capture the 13Cmethyl experimental data by constraining the global values of Kapp to the value of 15N-ΔR2max measured on the 240 μM 13C/15N-labeled Aβ40 sample (SI,
that $k_{\text{off}}$ is consistent with a range of values from about 20 to 200 s$^{-1}$ with statistically equivalent fits vis-à-vis residuals; hence we chose to fix $k_{\text{off}}$ to the same value as determined previously,\textsuperscript{[4]} which should be independent of the Aβ40 concentration and facilitate the comparison between the current results and our previous work.

A comparison of the experimental and computed $^{13}$C-methyl $R_2$ (Figure 1A) and $^{13}$Cmethyl-DEST profiles (Figure 1B) for the best-fit solution demonstrates that the model in Figure 2B describes the ensemble of interactions well. The ability to quantitatively match the observed $^{13}$Cmethyl-DEST profiles and $^{13}$Cmethyl-$R_2$ values (Figure 1) with $K_3$ partition coefficients similar to those determined for the backbone (Figure 3A) demonstrates the ability of the model to capture the essential interactions present in transient monomer–protofibril binding. The high correlation between the population of direct contact states (i.e., $K_3$) obtained from the $^{13}$Cmethyl and $^{15}$N data for all residues (Fig-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Interaction of monomeric Aβ on the surface of Aβ protofibrils. A) Schematic of overall exchange process. B) The direct-contact/tethered bound-state model, which incorporates tethered and direct-contact states for each residue when bound to the protofibril surface, superimposed on a global two-state exchange model.}
\end{figure}

Figure S2), and $k_{\text{off}}$ to that determined previously,\textsuperscript{[4]} and varying the residue-specific $^{13}$Cmethyl-$R_2$ relaxation rates and partition coefficients $K_3$, as well as a single global value of the transverse relaxation rate ($^{13}$Cmethyl-$R_2$) when a methyl group is in direct contact with the protofibril surface. (Note

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Best-fit values of A) the partition coefficients $K_3$ (blue) and B) $^{13}$Cmethyl-$R_2$ (blue). Values for the corresponding $K_3$ values obtained previously from $^{15}$N data\textsuperscript{[4]} (black) and experimental values of $^{13}$Cmethyl-$R_2$ for the monomeric state (black) are also shown in panels (A) and (B), respectively. Values for residues (Val and Leu) with two methyl groups are averaged. For reference, $^{13}$Cmethyl-$R_2$ when a methyl group is in direct contact with the protofibril surface, is $1330 \pm 80$ s$^{-1}$. Error bars are 1 s.d. The amino acid sequence is displayed on top with hydrophobic residues colored in green.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Correlation between A) experimental side-chain $^{13}$Cmethyl-$R_2$, and backbone $^{15}$N-$R_2$ values, B) best-fit direct contact/tethered partition coefficients $K_3$ obtained from $^{13}$Cmethyl and backbone $^{15}$N data; and C) best-fit $^{13}$Cmethyl-$R_2$ and $^{15}$N-$R_2$ values for Aβ40. Values for residues (Val and Leu) with two methyl groups are averaged. Color coding: black, Ala; red, Ile (C$_\text{g}$methyl); green, Leu; blue, Met; and grey, Val. $^{15}$N values could not be determined for Ala2 owing to water exchange and are represented here by values for Glu3. Error bars are 1 s.d.}
\end{figure}
ure 4B), including the long, highly flexible methionine side chain, indicates that the entire side chain plays a role in mediating direct contacts with the protofibrils. The values of $K_i$ determined from the $^{13}$Cmethyl data are systematically larger than those from the $^{15}$N data. Whether this truly reflects a different partitioning of bound and tethered states as viewed by the methyl groups, or whether these differences, which are less than a factor of 2 in an equilibrium constant, simply reflect sample variation, cannot be ascertained, because almost equally good fits to the data can be obtained using the $K_i$ values determined previously from the $^{15}$N data[6] (SI, Figure S6), further emphasizing the role of the side chains in mediating direct contacts at the protofibril surface.

The global best-fit value of $^{13}$Cmethyl-$R_{\text{contact}}$ (at a 1H frequency of 600 MHz) is $1330 \pm 50$ s$^{-1}$. This value, which corresponds to a homogenous line width of 3 ppm, is consistent with direct methyl group interactions with the surface of large (2-80MDa) protofibril aggregates. Importantly, these direct interactions, though less frequent, are present even for residues such as Ala2 (Figure 1B), in the hydrophilic N-terminal region of Aβ.

The most important additional observations enabled by the $^{13}$Cmethyl-$\Delta R$ and DEST experiments relate to the behavior of the side chains in the tethered states. Our previous $^{15}$N results suggested that the backbone $R_2$ in the tethered state varies as the average number of residues between the direct contact residue(s) and the residue of interest varies, with the highest $^{15}$N-$R_{\text{tethered}}$ values (shortest tether) for residues 17-20. However, the role of side-chain atoms in tethered states could not be ascertained, and the relative contribution of a single side chain is obscured, because backbone motions are highly correlated along the peptide chain.[4]

The good correlation between the experimental backbone $^{15}$N-$\Delta R_2$ and side chain $^{13}$Cmethyl-$R_2$ values (correlation coefficient of 0.88) demonstrates the consistency between lifetime line-broadening at backbone and side-chain positions (Figure 4A). However, the $^{13}$Cmethyl-$\Delta R_2$ values are about half of those of the $^{15}$N-$\Delta R_2$ values, as expected since $^{13}$Cmethyl-$R_{\text{contact}}$ is only about 20-fold higher than $k_{\text{off}}$, such that $^{13}$Cmethyl-$R_2$ max does not reach the limiting value of $k_{\text{on}}$. The validity of the model used to analyze the $^{13}$C-methyl data is further evidenced by the high degree of correlation between $R_{\text{tethered}}$ values for side-chain methyl groups and the $^{15}$N backbone of alanine residues (correlation coefficient = 0.99). The effective values of $^{13}$Cmethyl-$R_{\text{tethered}}$ (Figure 3B, blue circles) for the three alanine residues, whose methyl groups at Cβ reflect the backbone motion, are much higher for Ala21 compared to Ala30 and Ala2 (Figure 3B), just as those at the corresponding $^{15}$N positions (Figure 4C, black circles, and solid black line).

The deviation of other methyl-bearing residues from the linear correlation between alanine $^{13}$Cmethyl and $^{15}$N $R_2$ gathered values (Figure 4C, solid line) shows the ability of the $^{13}$C-methyl experiments to provide additional information regarding the motion of particular side chains in the tethered state. Some residues, such as Leu34 and Val39, fall along the same correlation line as the alanine residues, indicating that their side chains behave much like the backbone. The remaining residues, however, fall below the alanine correlation with correspondingly smaller, but still well correlated, $^{13}$Cmethyl-$R_{\text{tethered}}$ values relative to the $^{15}$N-$R_{\text{tethered}}$ values (Figure 4C, dashed line with correlation coefficient of 0.85 and a slope value half of that for the alanine correlation) suggesting that the average distance of these methyl groups to the nearest direct contact point is further away than for the backbone.

Within the central hydrophilic region, the values of $^{13}$Cmethyl-$R_{\text{tethered}}$ for Leu17 and Ala21 are high, consistent with our previous observations for the corresponding backbone $^{15}$N-$R_{\text{tethered}}$ values.[4] A lower than expected $^{13}$Cmethyl-$R_{\text{tethered}}$ value, however, is observed for Val18 compared to the adjacent Leu17 despite one fewer dihedral angle degree of freedom separating the methyl groups from the backbone, suggesting exclusion of Val18 from transient-tethered-state interactions. Conversely, although the $^{15}$N-$R_{\text{tethered}}$ values for Val18 are 50% higher than those for Val12, the $^{13}$Cmethyl-$R_{\text{tethered}}$ value for Val18 is about 30% lower, suggesting a greater degree of involvement of the Val12 side chain in transient intermolecular contacts in the tethered states than discernable from $^{15}$N-DEST alone. Finally, the value of $^{13}$Cmethyl-$R_{\text{tethered}}$ for Met35 is significantly lower than that for neighboring residues, indicating that its long side chain retains considerable mobility when not directly in contact with the protofibril surface. (It should be noted that one would not expect to directly observe methyl cross-peaks for the tethered states, even in cases, in which the $^{13}$Cmethyl-$R_{\text{tethered}}$ values are low, as at the C-terminus, because the tethered state may be identical in chemical shift to the monomer state, and more importantly any potential cross-peak for a tethered state would be highly broadened in both $^1$H and $^1$C dimensions by the off-rate of 51 s$^{-1}$ due to the highly skewed populations of free and bound Aβ40 exchanging on the surface of the protofibrils.)

The fits reported for the $^{13}$Cmethyl DEST and $\Delta R_2$ data in Figures 1, 3, and 4 were obtained with optimized values of $K_i$. However, as noted above, almost equally good fits can be obtained by constraining the values of $K_i$ to those obtained previously from analysis of the $^{15}$N data.[4] The $^{13}$Cmethyl-$R_{\text{tethered}}$ values obtained in this manner are highly correlated to those obtained with optimized $K_i$ values (SI, Figure S6) and hence do not impact the conclusions drawn above. The absolute values of $^{13}$Cmethyl-$R_{\text{tethered}}$ as well as the value of $^{13}$Cmethyl-$R_{\text{contact}}$, are about 60-70% higher when $K_i$ is fixed. This is simply due to the fact that the values of $K_i$ and $^{13}$Cmethyl-$R_2$ in the bound state are partially anticorrelated such that higher values of $K_i$ (when optimized) lead to lower values of $^{13}$Cmethyl-$R_2$ in the bound state.

Our current $^{13}$Cmethyl data support a model in which protofibril-bound structures are stabilized by a network of residues (based on the consistency of $^{15}$N and $^{13}$Cmethyl DEST profiles), whereas methyl-bearing residues contribute to substructures in the tethered states. Further, the current work confirms the validity of the “model-free” representation of the very large ensemble of states that can be sampled on the surface of the protofibrils (Figure 2).

In summary, our analysis combining $^{13}$Cmethyl DEST and $\Delta R_2$ measurements brings a new, detailed view of the structural and dynamic properties of side-chain methyl groups in exchanging systems involving very high molecular
weight (> 1 MDa), transient complexes, and provides direct insight into interactions in the vicinity of a given methyl side chain that are inaccessible by other methods. Moreover, the rapid rotation of methyl groups resulting in a large reduction of \( R_{\text{contact}} \) relative to the backbone, should permit \(^{13}\text{C}\) methyl DEST and lifetime line-broadening to be applied to a much larger variety of systems involving both larger NMR-observed species (e.g. greater than 100 kDa) for which the observation of backbone amides would not be feasible, larger dark states (in excess of 80 MDa), and faster exchange kinetics down to the millisecond time scale. Further extension of DEST to all nonmethyl \(^{13}\text{C}\) atoms is also possible, but lower signal-to-noise and significant overlap precluded the complete characterization of other side-chain positions in A\(\beta\)40.

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