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Hydrogen/Deuterium Exchange Mass Spectrometry—A Window into Amyloid Structure

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ABSTRACT
The β-sheet network of the amyloid fibril is a dominant structural feature of this class of protein structures. An attractive way to view the protein misfolding events that lead to the formation of fibrils and other aggregates is to consider how native protein secondary structure rearranges to yield the H-bonding relationships within the aggregate structure. We describe here the application of hydrogen−deuterium exchange mass spectrometry (HX-MS) methods to probe the secondary structure of protein aggregates. This includes exploration of the structures of monomers, protofibrils, and fibrils, the structural relationships among these states, the energetic contribution of H-bonding to fibril stability, and the plasticity of the H-bond network.

Introduction

Amyloid fibrils have been recognized for over a half century as the fibrillar substructure of pathogenic protein-rich deposits associated with disease.[1] Mature fibrils possess an unbranched fibrillar structure and a cross-β substructure, exhibit a unique tinctoral signature, and are highly insoluble.[1] In addition to over 25 different proteins associated with different amyloid pathologies of the brain[2] and periphery,[3] many other nonpathogenic proteins can be induced to form the polymeric amyloid structure in vitro.[4] These attributes make the amyloid structure broadly interesting, not only from the point of view of human disease but also in terms of fundamental aspects of protein folding.

Centrally important questions in the amyloid field are the structures of amyloid and other off-pathway protein aggregates, the energetic basis of amyloidogenesis, the nature of the amyloid assembly mechanism, and the structural basis of protein aggregate toxicity. Structural biology studies on amyloid fibrils have always been challenging due to their high molecular weight, insolubility, and noncrystalline nature. The problem has become even more complex with the recent realization that amyloid fibrils of a single polypeptide can exist in multiple conformations.[5,6] These conformational variants may be responsible for biological effects such as strain effects in prion phenomena.[5] An additional, immense complexity is the recognition that in almost all cases, spontaneous assembly of amyloid involves a series of non-amyloid intermediates, including spherical oligomers and various kinds of protofibrils. The structures of these assemblies, how they differ from each other, and whether and how they may interconvert, are critical questions for understanding the mechanism of fibrillogenesis. The structures of the intermediates are also important because of growing evidence that in some cases these non-native aggregates are more likely to be responsible for pathogenesis than the mature amyloid fibril.[7,8] The amyloid question has thus grown to include a wide range of structures and possible structural interconversions.

The analytical toolbox for addressing such questions has grown considerably in the past decade.[9] Low-resolution views of amyloid morphology are provided by electron microscopy,[10] cryo-EM,[11] and AFM.[12] Insight into gross amyloid secondary structural content comes from FTIR,[13] X-ray fiber diffraction,[14] and, in favorable circumstances, circular dichroism.[15] Information on the arrangement and register of β-strands within the fiber is provided by electron spin resonance[16] and solid-state NMR,[17] with the latter also providing backbone angles and distance restraints that allow construction of realistic models of the protofibril substructures of amyloid. X-ray analysis of amyloidogenic peptides that form crystals possessing expected aspects of amyloid structure are providing models for what the interior structure of an amyloid protofilament might look like.[18,19] The ability to characterize the thermodynamic[20] or kinetic[21] consequences of mutations provides insight into the roles of different sequence positions in the amyloid structure. Limited proteolysis[22] and chemical modification[23] approaches are also providing important information on fibril structure.

An additional approach added to the amyloid toolbox in 2000[24] is hydrogen−deuterium exchange (HX). HX methods offer insights into the extent of stable secondary structure and are, therefore, especially important for studying structures like amyloid and certain amyloid assembly intermediates that possess significant, highly stable β-sheet. In this Account, we review the structural insights gained through the application of on-line mass spectrometry for evaluation of HX.

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Ronald Wetzel obtained a B.S. in Chemistry from Drexel University in 1969 and a Ph.D. in Physical Organic Chemistry from the University of California, Berkeley, in 1973. He then did postdoctoral training at the Max Planck Institute for Experimental Medicine and at Yale University. He has served as a senior scientist at Genentech and as a senior research fellow at SmithKline Beecham and is now Professor in the Graduate School of Medicine at the University of Tennessee. His main scientific interest is protein misfolding and aggregation, especially as it relates to human disease.

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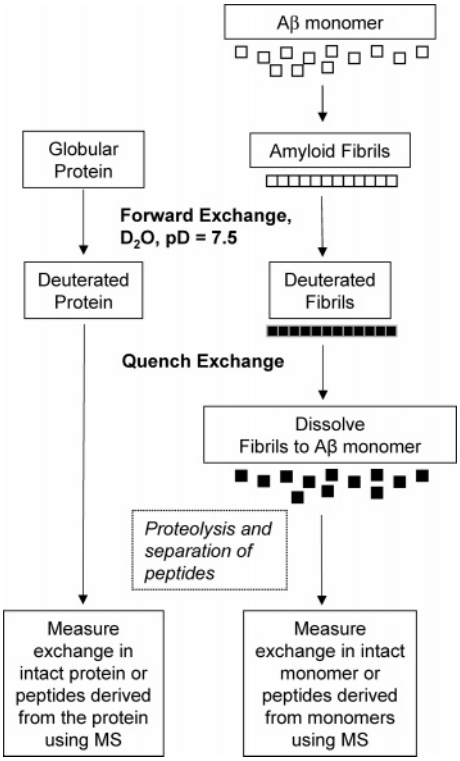

FIGURE 1. Comparison of HX-MS methodology for globular proteins and amyloid fibrils.

HX Methods in Protein Structure Analysis

HX methods exploit the fact that protons either involved in H-bonded secondary structures or buried in a protein's core structure, exchange with water more slowly than protons in solvent-exposed and non-H-bonded regions. These methods have been used to elucidate the secondary structure and dynamics of globular proteins for several decades.[25,26] Initially, these methods were based on NMR analysis,[25] which detects the loss of individual N−H peaks in the spectrum as the H is exchanged with D. Although NMR can provide resolution at the single residue level, the limited mass range of NMR, as well as requirements for relatively long analysis times and high sample concentrations, led to the development of HX mass spectrometry (MS)-based methods.[27] Since deuterium is 1 Da heavier than hydrogen, an increase in mass of the polypeptide measured by MS as the H is exchanged with D represents the number of protons exchanging with deuterium. Mass spectrometric methods are fast, sensitive, and amenable to analysis of large proteins; however, obtaining single-residue resolution is challenging. Some spatial resolution can be obtained by proteolyzing the protein under exchange quench conditions and measuring the extent of exchange observed in the derived peptide fragments (Figure 1).[26,28] By obtaining overlapping fragments, one can define exchange, in principle, at single amino acid resolution.

HX-MS and HX-NMR techniques have recently been applied to investigate the structure of amyloid fibrils.[24,29−38] Since amyloid fibrils are too large, insoluble, and heterogeneous to be detected directly using these methods, fibrils have to be rapidly dissolved after initial exchange to prepare them for analysis (Figure 1). In the analysis step, the amount of HX occurring at each exchangeable position in the peptide (when the peptide was resident in the fibril) is determined. This is in contrast to measuring HX into globular proteins, where typically the structure of the protein remains largely intact during real-time analysis.

The requirement for dissolution of fibrils prior to analysis leads to the generation of monomers and, hence, the exposure of protons that may have been protected in the amyloid structure, making them transiently accessible to exchange during the analysis time. This requires identifying a solvent that dissolves fibrils rapidly, quenches any further exchange during analysis, and is compatible with the analysis technique (NMR or MS). The time from dissolution to analysis must furthermore be minimized and controlled to obtain accurate and precise results. These challenges have been overcome, and the HX methods in conjunction with MS and NMR have been extended to the study of amyloid fibrils generated from various forms of Aβ, α-synuclein, β2-microglobulin, prion proteins, etc.[24,29−38] These studies have provided data that have facilitated building and testing of models of amyloid fibril structure.

Our work has focused on the development and application of on-line HX-MS methods for structural determination of amyloid fibrils and protofibrils of the Aβ(1−40) peptide associated with Alzheimer's disease.[24,39−44] This intermediate resolution method has provided insight into the structures of monomers, protofibrils, and fibrils and the structural relationships among these different aggregation states. Additionally, these methods have provided insight into the surprising structural dynamics of the amyloid fibril.

The HX-MS Experiment

The HX-MS experiment on protein aggregates is divided into two steps (Figure 2).[24,44] In the first step, protonated fibrils are collected by centrifugation, washed, resuspended in deuterated buffer, and incubated at room temperature to promote exchange of labile protons with deuteriums in analogy to the process used for globular proteins. In the second step (Figure 2), fibrils are disaggregated into monomers and exchange is quenched on-line by a low pH processing solvent prior to MS analysis. The amount of deuterium incorporated is determined on the basis of the time-dependent increase in mass of the polypeptide as monitored by MS. At various times during the deuteration reaction in step 1, an aliquot of the deuterated sample is infused into one arm of the T, while a processing solvent is infused through the second arm of the T. This processing solvent is capable of performing three functions simultaneously: disaggregating fibrils, quenching exchange, and allowing for efficient analysis by MS. Step 2 is performed in the spray capillary (tube A in Figure 2) connected to the third arm of the mixing T. The sample flows for ∼10 s through this capillary before

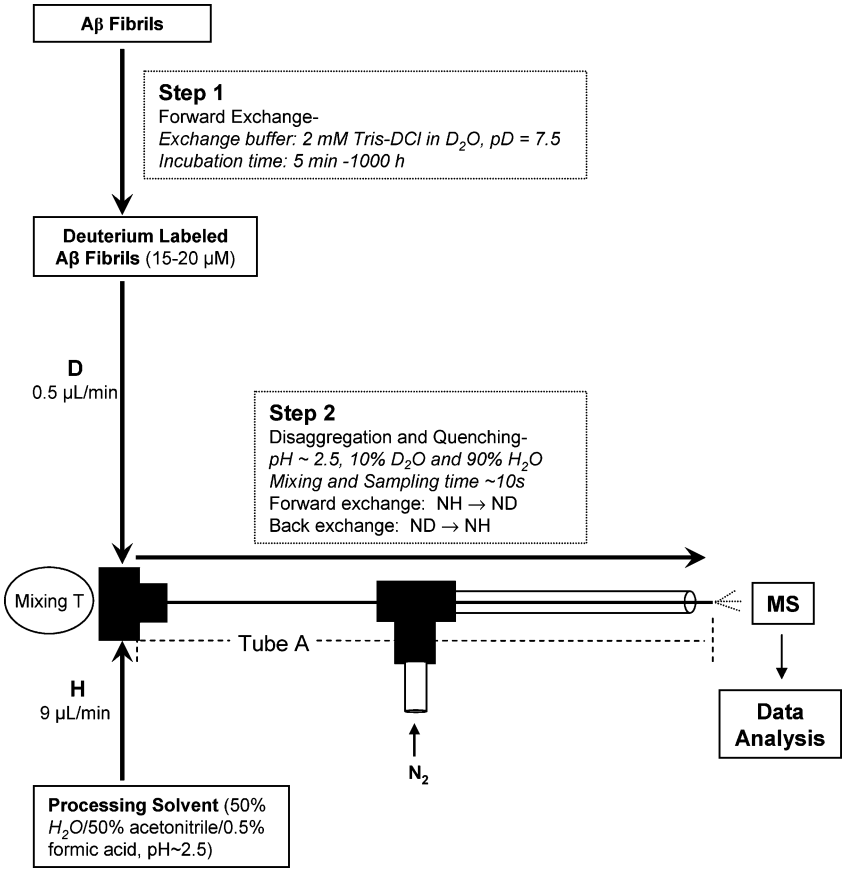

FIGURE 2. Schematic of the HX-MS experiment using a coaxial probe. Adapted from ref 44 with permission. Copyright 2006 Elsevier.

ionization and introduction into the MS. During step 2, the sample is in a mixture of both H_2O and D_2O based on the relative flow rates used for infusion of solvent and sample, respectively. This results in a continuous forward and back exchange of protons/deuterons between the dissolved fibrils and the bulk solvent. Forward exchange is the replacement of protons in the dissolved fibrils with deuterium in the bulk solvent, while back exchange is the replacement of a proton from the bulk solvent with a deuterium in the dissolved fibrils. This continuous forward and back exchange during the transit through the spray capillary is collectively termed "artifactual exchange".

The goal of these studies is to determine the number of deuteriums present in the fibrils after the initial exposure to D_2O (step 1) without losing any information during sample workup (step 2), so that the extent of H-bonding network in the fibril structure can be determined. Unfortunately, even under the optimized experimental conditions, some artifactual exchange takes place, which compromises the desired exchange information of the fibril state. This artifactual exchange is observed in all HX-MS methods for both globular and fibrillar samples.[28,32,33,36,37,40] We have developed methods to correct for this artifactual exchange and applied them to HX data on amyloid fibrils and protofibrils.[40,43,44] These methods involve measuring back and forward exchange independently using the same sample processing conditions as used in analyzing the exchange reaction.

Amyloid Fibril Structure Using HX into Aβ Fibrils

As mentioned above, multiple conformations of amyloid fibrils can be produced from the same polypeptide, for example, by varying growth conditions.[5,6] In the experiments described here, amyloid fibrils were grown from Aβ(1−40) in PBS (10 mM phosphate, 138 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4) at 37 °C without agitation. Figure 3a presents a representative mass spectrum obtained after incubation of Aβ(1−40) fibrils in deuterated buffer for 24 h. The spectrum obtained from these partially deuterated fibrils shows two Aβ populations represented by peaks A and B. The centroid position (mass/charge) of peak A increases with time, whereas the position of peak B remains constant and is superimposable with that of fully deuterated monomer.

Peak A clearly represents the Aβ(1−40) population protected from exchange because it is incorporated within the fibril architecture during the exchange reaction; however, peak B must also be accounted for to completely understand the data. A fully deuterated component in the HX analysis of fibrils has been observed in other preparations of Aβ aggregates, as well as for other amyloid systems.[24,32,37] We have demonstrated previously that after 3.5 min of incubation in deuterated solvent, a single peak A is observed;[24] peak B, representing the fully deuterated species, emerges and grows as HX time increases. One hypothesis to explain the fully exchanged peak B would

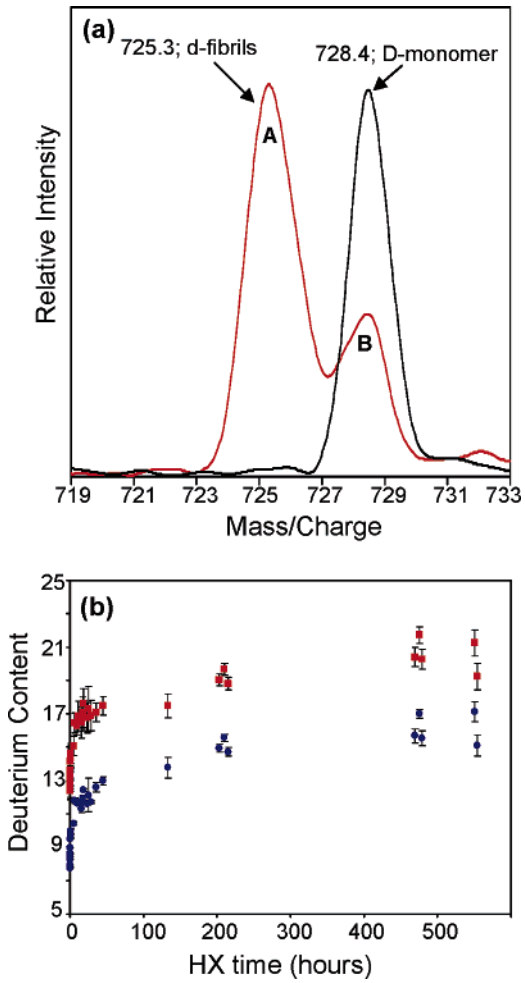

FIGURE 3. (a) ESI-MS of partially deuterated fibrils (red) after 24 h of HX time and fully deuterated monomer (black). All MS data were obtained using a Micromass Quattro II triple quadrupole mass spectrometer. The data shown here have been smoothed using Savitzky Golay method implemented in the MassLynx program (Waters) and normalized in a spreadsheet program. Several charge states were obtained, but only the +6 charge state is shown here for clarity. Adapted from ref 44 with permission. Copyright 2006 Elsevier. (b) Number of backbone amide protons (corrected (red squares) and uncorrected (blue circles)) exchanged with deuterium vs HX time in Aβ(1−40) fibrils.

be that it represents a monomeric component of the fibril preparation that independently undergoes exchange; however, in the experiments described here, fibrils are isolated from monomer by centrifugation prior to subjecting them to deuterium exchange. Moreover, analysis of the supernatant of partially deuterated fibril suspension shows that the proportion of material observed in peak B cannot be accounted for by the dissociation of Aβ from the aggregate during the exchange period (Portelius E., Kheterpal I., Cook K. D., Wetzel R., unpublished). Another possible mechanistic source of the peak B material could be Aβ molecules associated with the fibrils but not involved in the H-bonded core structure. A recycling model encompassing dissociation of monomer from fibril, full exchange of monomer in the deuterated solvent, and then its reassociation into the fibril structure has also been invoked to account for a fully exchanged species in HX-MS analysis of another amyloid system.[37]

In contrast to peak B, exchange information in partially protected peak A contains structural information about the aggregate. The extent of deuterium incorporation into Aβ(1−40) amyloid fibrils is determined from the centroid of peak A in Figure 3a. The details on how to calculate and correct deuterium incorporation for artifactual exchange have been described previously.[40,44] The corrected data show that 17 deuteriums are incorporated into backbone amide protons of fibril-incorporated Aβ(1−40) after 24 h of incubation in deuterated buffer. Kinetics studies of HX from ∼5 min to ∼600 h show that the number of deuteriums incorporated into Aβ(1−40) fibrils reaches a plateau of ∼20 (Figure 3b). This is in contrast to the kinetics of exchange observed for Aβ(1−40) monomer under similar conditions, where all 39 backbone amide protons were found to completely exchange with deuterium in <5 min.[24] The comparison of corrected and uncorrected data presented in Figure 3b shows that corrections affect only the amplitude of the measurement of deuterium incorporation and are relatively small. While uncorrected data provide a fingerprint of exchange into the amyloid fibrils and can be used to qualitatively compare fibril preparations, corrected data provide quantitative information on the extent of deuterium exchange and can be directly applied toward understanding details of amyloid structure.

Thus, HX-MS results show that PBS-quiescent Aβ(1−40) amyloid fibrils consist of a very rigid core structure and most likely an H-bonded β-sheet structure, which involves ∼50% of the 39 Aβ backbone amides. The HX kinetics of the fibrils suggest a number of kinetic phases: (1) Thirteen amide protons of Aβ(1−40) fibrils exchange with deuteriums at the same rate ($K_{ex} \geq 9\ h^{-1}$) as that observed in the monomeric state. We believe these protons are exposed and not involved in β-sheet formation. (2) A set of ∼5 protons exchange with rates comparable to those observed for protons involved in secondary structure in globular proteins. (3) Another set of ∼20 protons are very resistant to exchange indicating a very rigid core structure in the amyloid fibrils. These data were further confirmed by HX-NMR studies on Aβ(1−40) amyloid fibrils.[35] These data demonstrate that the involvement of the entire Aβ sequence in H-bonded secondary structure within these amyloid fibrils is highly unlikely. In fact, most current structural models of various Aβ(1−40) amyloid fibril conformations feature a peptide possessing a balance of H-bonded β-sheet and more flexible regions.

Although determining the number of backbone amide groups involved in stable H-bonded structure is important, it would also be useful to determine the H-bonding states of the backbone amides of each individual amino acid in the sequence. In principle, spatial resolution in HX-MS methods can be increased by either proteolyzing proteins prior to exchange determination or applying tandem MS. The use of tandem MS to measure HX at single-residue level is clouded, however, by the reports of scrambling of backbone amide hydrogens and

deuteriums in the gas phase,[45,46] and in fact, we have encountered problems using this approach to analyze positional exchange in Aβ amyloid fibrils (Kheterpal I., DaGue B., Cook K. D. and Wetzel R., unpublished). In contrast, proteolytic fragmentation followed by LC separation and MS analysis of the peptide fragments has been very informative in localizing HX to specific regions and residues of proteins.[26,28] These methods have been used widely with globular proteins and have also recently been applied toward structural studies of aggregates.[32,36]

Amyloid Fibril Structure Using HX-MS Coupled to On-Line Proteolysis

We have developed and applied an alternative method to incorporate proteolysis into our on-line approach to obtain segmental exchange information in Aβ(1−40) incorporated into amyloid fibrils.[43,44,47] Details of this methodology utilizing a triaxial probe that sequentially mixes three independent streams of solutions (sample, enzyme, and organic modifier) prior to MS analysis have been described.[43,44,47] Acetonitrile has been shown to reduce digestion efficiency, and therefore, its addition was delayed using the triaxial probe.[47] This on-line coupling of proteolysis with MS analysis minimizes and controls the time from fibril dissolution to HX measurement and allows spectra for all fragments to be acquired simultaneously. For the HX analysis of large proteins, it may be necessary to employ an on-line chromatographic step to simplify complex peptide mixtures prior to analysis. This separation step adds 10−30 min to the total analysis time.[26,28,32,36] As with methods without proteolysis, unavoidable artifactual exchange in HX experiments (with and without the chromatography step) must be corrected for. We have confirmed that the methods developed to correct for this exchange in Aβ(1−40) peptide using the coaxial probe[40] are applicable to the data obtained using a triaxial probe.

HX-MS analysis of the intact fibrils using a triaxial probe (by omitting enzyme) showed that 17 amide protons exchange with deuterium into the Aβ(1−40) monomer when it is incorporated into amyloid fibrils (Figure 4a, Figure 5), a value consistent with that obtained for 24 h of exchange using a coaxial probe (Figure 3b). Representative mass spectra of three non-overlapping fragments after 24 h of exchange are presented in Figure 4b−d. Since the exchange into intact fibrils shows the presence of a fully deuterated component peak B (Figure 4a), all proteolytic fragments of these fibrils are also expected to contain a fully exchanged component. Recognition of this feature of the data is important for correct analysis and interpretation. In accordance with this observation, a cleanly distinguished peak B is observed in the 20−34 peptide fragment that is analogous to the fully deuterated species observed for the intact peptide (Figure 4b). The fact that fragments 1−19 and 35−40 do not show two distinct peaks in the B region (Figure 4c,d) suggests that the amount of protection in these peptide components within the fibril is quite low, such that the observed peak

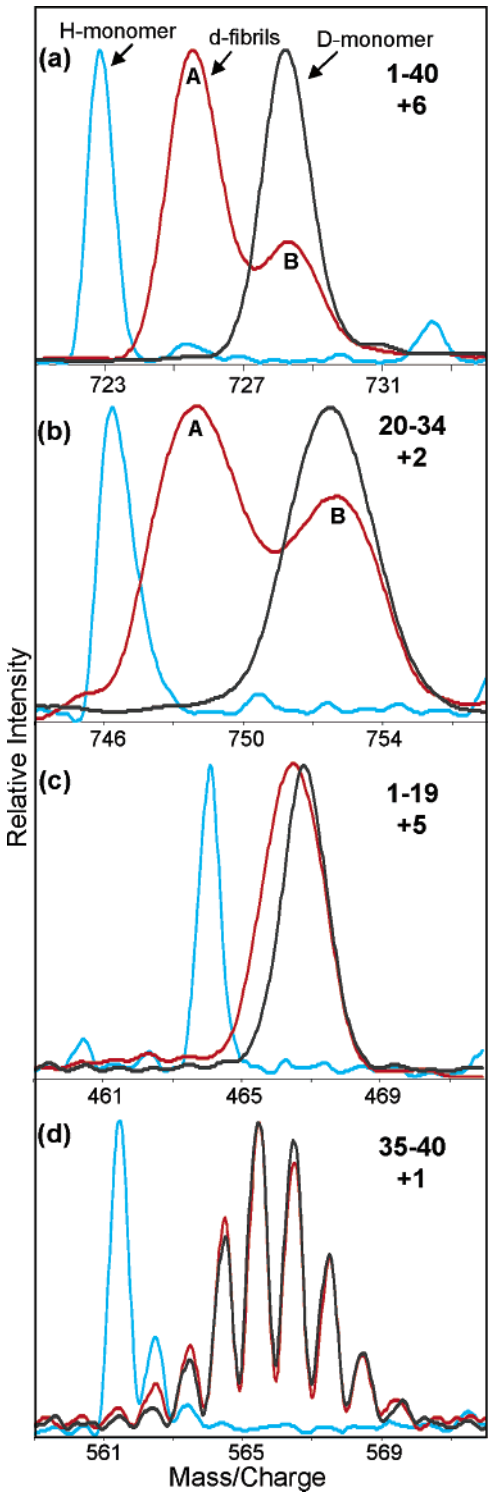

FIGURE 4. ESI-MS of (a) intact Aβ(1−40) fibrils and proteolytic fragments (b) 20−34, (c) 1−19, and (d) 35−40 after 24 h of exchange. The corresponding spectra for protonated monomer (blue) and fully deuterated monomer (black) are overlaid for each fragment. Adapted from ref 43 with permission. Copyright 2006 Elsevier.

is a weighted average of partially protected (A) and fully exchanged (B) peaks. Recognition of this phenomenon allowed us to implement a deconvolution scheme that resolves the mass spectra of the 1−19 and 35−40 fragments into A and B components and determines the centroid position of A[43,44] (Table 1).

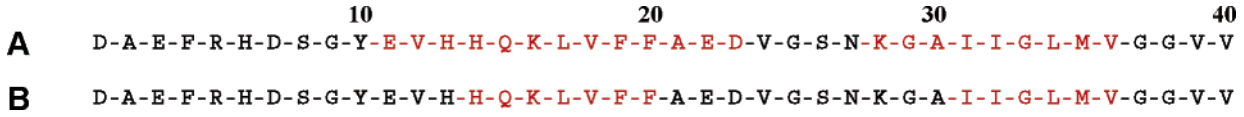

FIGURE 5. Amino acid sequence of Aβ(1−40) showing the backbone amide protons protected from HX (red) in Aβ(1−40) fibrils (A) and CLC-stabilized protofibrils (B). These models for β-extended chain segments in mature fibrils (A) and protofibrils (B) are derived from data shown in Figures 3 and 4 and Figure 8, respectively.

Table 1. Summary of Segmental Protection Data for Aβ(1−40) Aggregates after 24 h of HX Time

	amyloid fibrils			CLC protofibrils		
fragment	amide protons exchanged	protected NH/total	percentage of protected NH	amide protons exchanged	protected NH/total	percentage of protected NH
1−40	17.2 ± 0.7	21.8/39	56	26.2 ± 1	12.8/39	33
1−19	10.4 ± 0.6	7.6/18	43	12.4 ± 0.6	5.6/18	31
4−19	6.6 ± 0.5	8.4/15	57	9.9 ± 0.6	5.1/15	34
20−34	4.3 ± 0.3	9.7/14	69	9.5 ± 0.4	4.5/14	32
35−40	3.9 ± 0.2	1.1/5	22	4.1 ± 0.1	0.9/5	18

These data show that both the N- and C-terminal segments (1−19 and 35−40) are readily exchangeable (and therefore not involved in the core structure), whereas the fragment containing residues 20−34 is highly protected from exchange. This result is in agreement with HX-NMR[35] and scanning proline[41] and scanning alanine[20] analysis of PBS-quiescent amyloid fibrils of Aβ(1−40). These data for a free C-terminus are in contrast with ssNMR results showing that the entire C-terminal segment of Aβ(1−40) is involved in H-bonded β-sheet structure in amyloid fibrils.[6,48] One possible explanation for this discrepancy is that the fibrils analyzed using ssNMR[6,48] were prepared with agitation in low salt buffer, potentially leading to formation of an alternative amyloid conformation.[6] Detailed analysis of these differences in fibril conformations requires further investigation. Clearly, models for fibril structure may only apply to fibrils grown under particular growth conditions.

Protofibril Structure Using HX-MS

The structural characterization of protofibrillar intermediates from Aβ fibril assembly is necessary to understand their structural relationship to fibrils and their roles in cytotoxicity and fibril assembly. However, the transient nature of protofibrils places additional constraints on structural determination, over and above the difficulties associated with amyloid structure determination. Two ways to improve stability are to use mixtures of peptides or to use other stabilizers. The arctic mutant of the Aβ peptide (E22G, Aβ_{ARC}) has an increased propensity for forming protofibrils compared with the wild-type Aβ (Aβ_{WT}).[49,50] Protofibrils formed from an equimolar mixture of Aβ_{WT} and Aβ_{ARC} have been shown to be more stable than those obtained by Aβ_{WT} or Aβ_{ARC} alone.[49] Some small molecules such as calmidazolium chloride (CLC) increase protofibril formation and decrease fibril formation with Aβ_{WT}(1−40) so that the protofibrils can be conveniently analyzed.[42]

We used the HX-MS methods described above to characterize the structure of these stabilized Aβ(1−40) protofibrils.[39,42] HX kinetics of protofibrils from Aβ_{ARC} and a Aβ_{WT}/Aβ_{ARC} (1−40) mixture and CLC-stabilized protofibrils are presented in Figure 6, along with fitted curves for repeated runs on Aβ_{WT} monomer and fibrils in the absence of CLC. These data show that exchange into protofibrils prepared from fibril formation reactions without CLC is identical to that from CLC−Aβ aggregates (Figure 6a). These experiments demonstrate that HX-MS methods can easily distinguish among unstructured monomer, protofibrils, and fibrils by their different protection patterns. Based on their poor ThT staining, lack of ability to serve as efficient templates for elongation, and poor general stability, we expected protofibrils to not exhibit stable H-bonded structure. Surprisingly, these data show that there is an element of core structure within protofibrils that is just as stable as fibrils. Given this surprising result,

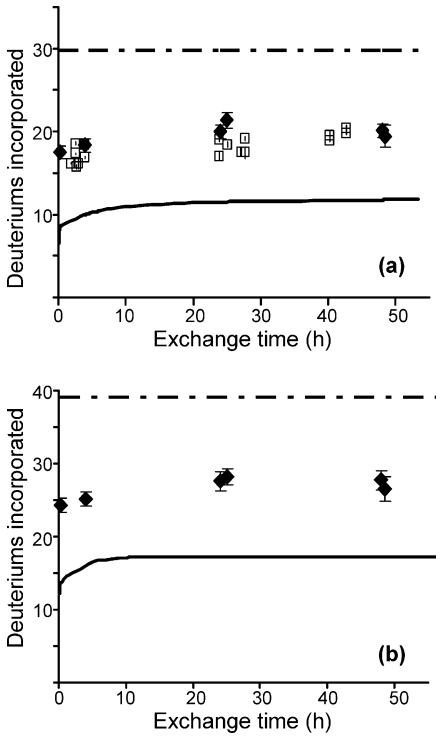

FIGURE 6. HX kinetics of CLC-Aβ(1−40) (diamonds) and Aβ_{ARC} and Aβ_{ARC}/Aβ_{WT} (squares) protofibrils corrected for artifactual exchange into side chains only (a) or into side chains and main chain (b). Fitted curves for repeated runs on Aβ_{WT} monomer (dash) and Aβ_{WT} fibrils (solid) with no CLC are included for comparison. Adapted from ref 42. Copyright 2005 The National Academy of Sciences of the United States of America.

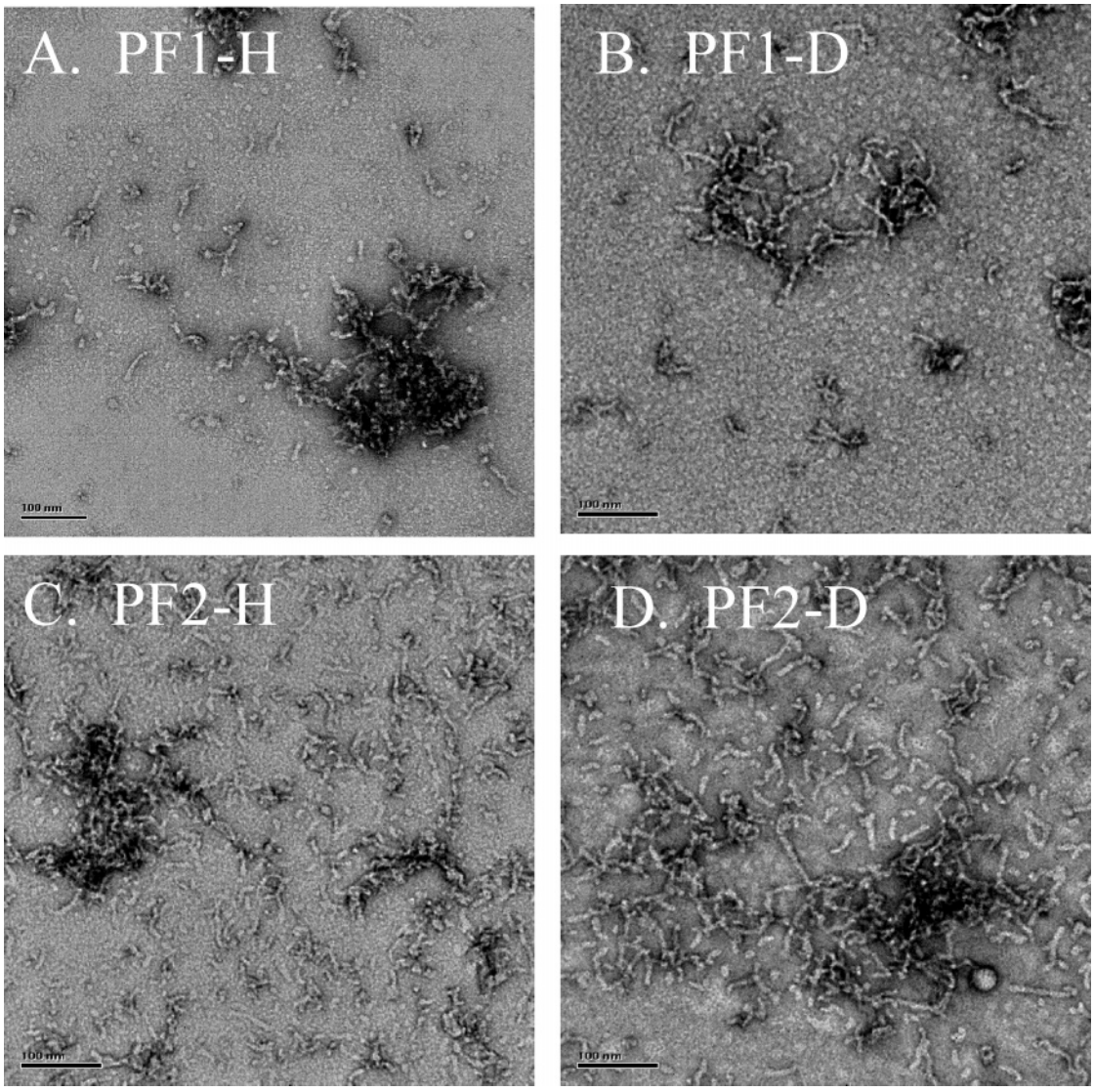

FIGURE 7. EM of negatively stained images of protofibril fractions obtained from a 1:1 equimolar mixture of $A\beta_{ARC}$ and $A\beta_{WT}$ before (A and C) and after HX (B and D) experiments.

we were concerned that, despite our best efforts, the protofibrils may have partially converted to fibrils during analysis time. However, EM analysis well after the end of the HX experiments showed that protofibril structure had not detectibly changed from before analysis (Figure 7).

To complete these analyses, fully deuterated CLC-Aβ aggregates were prepared from fully deuterated Aβ(1−40) monomer and were used to correct for artifactual back exchange. Figure 6b presents these fully corrected data for protofibrils in the context of the exchange levels of mature fibrils (solid) and monomer (dashed). These data indicate that CLC-stabilized protofibrils contain 12 highly protected backbone amide protons after 2 days of exchange time. This is in contrast to mature Aβ(1−40) amyloid fibrils, whose equally stable structure retains ∼22 protected backbone amide protons over similar HX time.

To determine the relationship between the strongly protected H-bonds in protofibrils and fibrils, the on-line proteolysis method was used to obtain the segmental HX protection pattern in CLC-Aβ aggregates.[43] Figure 8 shows representative spectra obtained from intact protofibrils and three non-overlapping fragments after 24 h of exchange. Interestingly, in contrast to mature fibrils (Figure 4), these protofibrils show only the intermediate exchange peak (A) and no fully exchanged peak (B). Thus, the Aβ molecules resident in these protofibrils represent a very homogeneous population. The corrected number of deuteriums exchanged into the intact peptide (1−40) and in the three fragments 1−19, 20−34, and 35−40 are 26, 12, 10, and 4, respectively (Table 1). There is an exact match between the sum of exchange into the three non-overlapping fragments and in the value for intact protofibrils.

These data show that, as is the case with mature fibrils, the N- and C-termini of Aβ(1−40) are not involved in protected structure in protofibrils. Additionally, the protective secondary structure within the 20−34 fragment in protofibrils is not as extensive as in fibrils[43] suggesting that the central 20−34 segment is more ordered in fibrils compared with protofibrils (Figure 5) . This structural relationship between these two forms of aggregates is consistent with an on-pathway role for protofibrils in amyloid fibril assembly, a mechanism also supported by examination

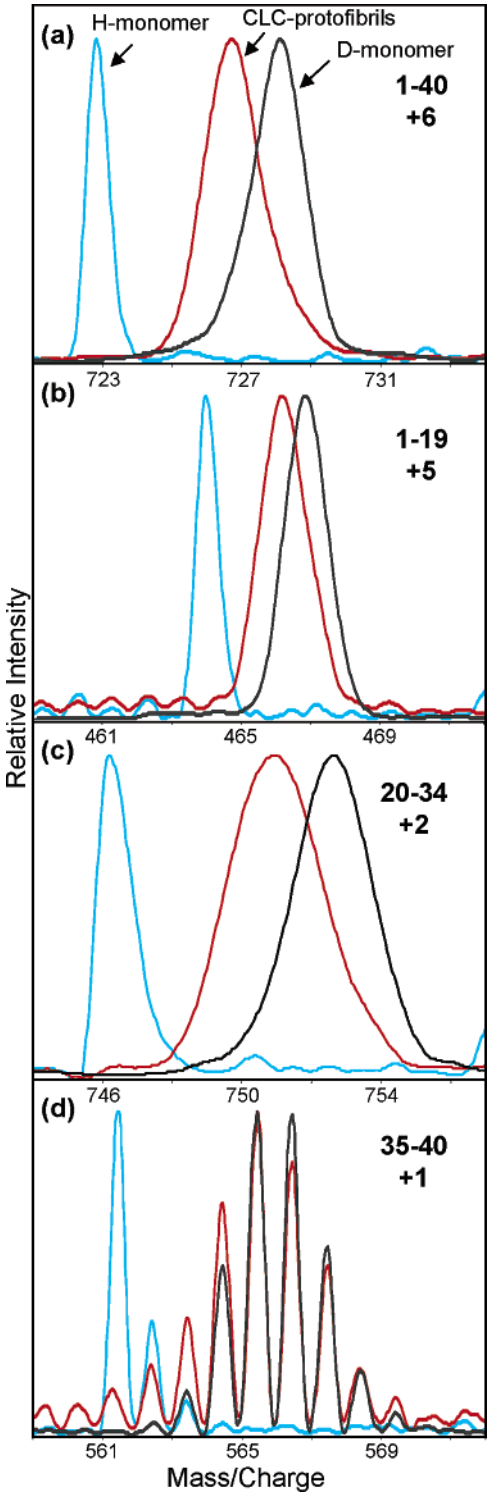

FIGURE 8. ESI-MS of (a) intact Aβ(1−40) CLC-stablilized protofibrils and proteolytic fragments (b) 1−19, (c) 20−34, and (d) 35−40 after 24 h of exchange. The corresponding spectra for protonated monomer (blue) and fully deuterated monomer (black) are overlaid for each fragment. Adapted from ref 43 with permission. Copyright 2006 Elsevier.

of the distribution of Aβ variants in protofibrils and fibrils in experiments containing Aβ_{WT}/Aβ_{ARC} mixture.[39]

Preliminary analysis of populations enriched in individual morphologies of protofibrils (Kheterpal I., Lashuel H. A., Lansbury P. T., and Wetzel R, unpublished) suggest that this HX-MS method may be useful in determining the extent of H-bonding in various assembly intermediates such as spherical oligomers, ADDLs, and linear and annular protofibrils. Such an analysis will facilitate determination of which intermediate species are on- or off-pathway in fibril formation.

Characterization and Comparison of Structures of Mutant Aβ Fibrils

Examination of the response of the amyloid structure to mutations has proved to be a valuable approach in characterizing details of Aβ(1−40) amyloid fibril structure and structural energetics and has led to some surprising observations. Even when a devastating mutation such as a proline replacement is made within a suspected β-sheet segment of amyloid, fibrils invariably grow, although they may be much less stable than Aβ_{WT}.[41] Interestingly, a number of these destabilized fibrils exhibit a greater number of highly protected H-bonds than are found in the more stable Aβ_{WT} fibrils. For example, although the amyloid fibrils grown from the I32P mutant of Aβ(1−40) exhibit a destabilized elongation equilibrium compared with the WT fibrils corresponding to 2 kcal/mol, I32P mutant fibrils contain four or five additional highly protected H-bonds compared with WT fibrils.[41] To account for this paradox, it must be assumed that fibrils rely for structural stability on more than their H-bonded network and, in fact, like globular proteins, achieve stability through a mix of interaction types. Interestingly, however, amyloid appears to be much more plastic than globular proteins, since the latter tend to absorb the effects of mutations locally, through relatively modest structural adjustments, while amyloid seems capable of dissipating the blow of a destabilizing mutation through propagation of major structural changes distally from the site of mutation.

To better understand the nature of the plasticity of the amyloid β-sheet, it is of great interest to apply methods such as on-line proteolysis procedure described here to determine how the H-bonding network within the fibril migrates in response to a disruptive mutation. For example, analysis of amyloid fibrils of the H14P mutant of Aβ(1−40) shows that the increased H-bonding generated by the mutation is localized in the N-terminal 1−19 fragment (Chen M., Kheterpal I., Wetzel R., Cook K. D., unpublished), which is perhaps not surprising because of the site of mutation and the amount of unstructured sequence available in the N-terminus. At the same time, it is fascinating to consider how the structure of the amyloid folding motif can so readily accommodate a segment that, in wild-type fibrils, appears to be uninvolved in β-structure.

Conclusions

H-bonded β-sheet networks are the dominant structural elements of the amyloid fibril, and HX methods promise to give great insights into the location of these secondary structural elements and how they vary between different

aggregated assemblies, including amyloid intermediates, amyloids from mutated peptides, and conformational variants of amyloid from the same peptide. The data obtained using this methodology are complementary to other biophysical and biochemical techniques and aid in building a detailed amyloid fibril structural model, which may be required for full understanding of disease pathogenesis and the design of treatments.

The on-line HX-MS methods described here are rapid, sensitive, and reproducible. These methods provide a quantitative tool for validating reproducibility in fibril preparations and for analyzing a variety of amyloid fibrils such as fibrils grown with and without agitation (Kodali R., and Wetzel R., unpublished), aggregates with different morphologies,[51] and various protofibril and monomeric structures. Future applications will also include detailed analysis of HX kinetics into particular regions of the polypeptides and development of methods to obtain protection data at near single-residue resolution. By giving the experimentalist additional flexibility, the triaxial probe may allow analysis of amyloid fibrils that are resistant to dissolution.

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