Scanning Cysteine Mutagenesis Analysis of Aβ-(1–40) Amyloid Fibrils*

Shankaramma Shivaprasad and Ronald Wetzel†

From the Graduate School of Medicine, University of Tennessee, Knoxville, Tennessee 37920

We describe here the use of cysteine substitution mutants in the Alzheimer disease amyloid plaque peptide Aβ-(1–40) to probe amyloid fibril structure and stabilization. In one approach, amyloid fibrils were grown from Cys mutant peptides under reducing conditions and then challenged with an alkylation agent to probe solvent accessibility of different residues in the fibril. In another approach, monomeric Cys mutants, either in the thiol form or modified with iodoacetic acid or methyl iodide, were grown into amyloid fibrils, and the equilibrium position at the end of the amyloid formation reaction was quantified by determining the concentration of fibrils, and the equilibrium position at the end of the amyloid formation reaction was quantified by determining the concentration of fibril elongation obtained. This method was compared in order to provide information on the environment of each residue side chain in the fibril. In general, Cys residues in the N and C termini of Aβ-(1–40) were not only accessible to alkylation in the fibril state but also, when modified in the monomeric state, did not greatly impact fibril stability; these observations were consistent with previous indications that these portions of the peptide are not part of the amyloid core. In contrast, residues 16–19 and 31–34 were not only uniformly inaccessible to alkylation in the fibril state, but their modification with the negatively charged carboxymethyl group in monomeric Aβ also destabilized fibril elongation, confirming other data showing that these segments are likely packed into a hydrophobic amyloid core. Residues 20, 30, and 35, flanking these implicated β-sandwich regions, are accessible to alkylation in the fibril indicating a location in solvent exposed structure.

Amyloid fibrils and other non-native protein aggregates are now regarded as an important alternate universe of protein structures formed by normal proteins exposed to conditions of environmental or mutational stress. Fibrils, protofibrils, and other aggregates are associated with a variety of serious human diseases of the brain (1) and periphery (2). Aggregate formation has also long been recognized as a major technical problem in the industrial production and use of proteins (3–6). The intrinsic tendency of polypeptide polymers to generate off-pathway aggregates can be viewed as a design flaw in the structural biology of the cell. Since up to 50% of newly synthesized protein chains are aggregated and/or misfolded (7), it is not surprising that major biochemical pathways have evolved in cells and organisms to manage the misfolding and aggregation processes, as exemplified by the chaperone (8), ubiquitin-proteasome (9, 10), aggresome (11), and autophagy (12) systems. In at least a few cases, nature has exploited the ability of polypeptide chains to form amyloid by evolving specific polypeptide sequences and the required cellular machinery for developing functional amyloids, for example as a means of cell attachment (13) or gene regulation (14).

Given the growing importance of non-native protein aggregates, it is desirable to gain an increased knowledge of their structures and how these structures might differ from those of typical globular proteins. Protein aggregates are not amenable to standard methods of structure determination, however, being too large and heterodisperse for solution phase NMR or x-ray crystallography. The inability of conventional methods to extract many details of aggregate structure has led to a search for alternative approaches. Some of these methods, such as those described here, prove to be derivatives of methods previously used to dissect the structures of globular proteins before structure determination by x-ray crystallography and solution phase NMR became routine.

The non-native aggregate most amenable to structural analysis is the amyloid fibril, and the most widely studied amyloid fibrils are those of the Alzheimer plaque peptide Aβ, in particular the 1–40 form of the peptide. Like other amyloid fibrils, the Aβ-(1–40) fibril consists of a bundle of protofibrils (15). Like other amyloid fibrils, Aβ-(1–40) fibrils exhibit a cross-β pattern in x-ray fiber diffraction (15), indicating a β-sheet-ricb structure in which the β-extended chains are displayed perpendicular to the fibril/protofibril axis with the β-sheet H-bonds between these extended chains parallel to the fibril axis (16). Although some amyloid fibrils, including fibrils of some Aβ fragments, appear to consist of an anti-parallel β-sheet arrangement (17), fibrils of the 1–40 peptide are in parallel sheet, with the chains in-register (18–20), as indicated in the structural models shown in Fig. 1. Studies by a variety of techniques have provided additional detail about Aβ-(1–40) amyloid fibril/protofibril structure and energetics, focusing on the questions of how the Aβ peptide folds to engage that structure and which residues are involved in the H-bonded core. These include EPR of derivatized Cys mutants (20), solid state NMR (18, 19), limited proteolysis (21), chemical accessibility of wild type residues (22), hydrogen-deuterium exchange (23–28), and proline (26), alanine, and disulfide (30) mutagenesis.

The problem of structural analysis of amyloid fibrils has been made even more complicated by the revelations of recent years that single amino acid sequences can form multiple conformational forms of amyloid fibrils (31–33). The structural bases of these conformational differences remain to be elaborated in full. Interestingly, perhaps the best characterized example of conformational variation is in amyloid fibrils of Aβ-(1–40). Fibrils grown under conditions of stirring (‘agitated’) differ from fibrils grown without stirring (‘quiescent’), both in their intramolecular architecture and in their EM morphology (33). It is

* This work was supported by Grant AG 18416 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Graduate School of Medicine, University of Tennessee, 1924 Alcoa Highway, Knoxville TN 37920. Tel.: 865-544-9168; Fax, 865-544-9235; E-mail: rwetzel@mc.utmck.edu.

2 The abbreviations used are: Aβ, amyloid-β; ThT, thioflavin T; TCEP, Tris(2-carboxyethyl)phosphine; PBS, phosphate-buffered saline; PBSA, PBS containing 0.05% sodium azide; HPLC, high pressure liquid chromatography; RP-HPLC, reverse-phase-HPLC; WT, wild type; MS, mass spectrometry; EM, electron microscopy.

3 A. D. Williams, S. Shivaprasad, and R. Wetzel, manuscript submitted.
Cysteine Mutagenesis of Amyloid Fibrils

therefore likely that some differences in published structural data on Aβ-(1–40) fibrils, such as among the studies cited above, can be attributed to real structural differences in the fibrils due to variations in fibril growth conditions.

We describe here several uses of cysteine mutants of the Aβ-(1–40) sequence, on the one hand exploiting the unique chemical reactivity of the Cys sulfhydryl group to assess side chain accessibility in the fibril and on the other hand taking advantage of Cys reactivity to create multiple chemical mutants of Aβ-(1–40) that can be assessed for their relative abilities to make fibrils. The data provided new, unique information about the solvent accessibility of various residue positions in Aβ-(1–40) amyloid fibril grown under the physiological conditions of PBS buffer, 37 °C, and the absence of stirring or shaking. The data also provided additional information on the chemical environments within the fibril for the amino acid side chains at most residue positions. The data allowed us to further refine our understanding of amyloid structure in general and of one important conformer type of the Aβ-(1–40) fibril in particular.

MATERIALS AND METHODS

All the peptides used in this study were purchased from Keck Biotechnology Center at Yale University. WT Aβ-(1–40) (for amino acid sequence, see Fig. 1) was obtained as a pure peptide. Single cysteine replacement mutants of Aβ-(1–40) (for representation of which mutant peptides were obtained, see Fig. 1) were obtained unpurified. The mutant peptides were purified on Bio-Rad RP-HPLC using a semi-preparative C3 column and a water/acetonitrile gradient with 0.05% trifluoroacetic acid. The identity and purity of the final peptides were confirmed by mass spectrometry and analytical RP-HPLC on an Agilent 1100 series mass spectrometry detector. Bond breaker TCEP solution (Tris(2-carboxyethyl)phosphine) was purchased from Pierce. Iodoacetate, iodoacetamide, and iodomethane were purchased from Sigma.

**Covalent Modification of Cys Sulphydryl Group at the Monomer Level—**

The side chain sulphydryl group of Cys in each of the mutant peptides was modified by treating the monomeric peptide with two different alkylation agents. For modification of the side chain, each purified mutant peptide at a concentration of 1–2 mg/ml was incubated in argon-purged 8 M urea buffer at 37 °C with either iodoacetic acid (10 mM; 20 min) or iodomethane (10 mM; 60 min) at pH 8.0. Reaction mixtures were immediately injected onto RP-HPLC and purified. Eluate fractions were pooled based on mass spectrometric analysis. The final purity of each pooled modified peptide was confirmed by analytical HPLC and covalent modification confirmed by electrospray ionization mass spectrometry.

**Amyloid Fibril Assembly—**

Fibril formation was carried out on both side chain-modified and -unmodified cysteine mutants. For the side chain-modified mutants, fibril growth was carried out using the standard protocol described (21, 34). Briefly, freshly disaggregated (34, 35) peptide was incubated at 37 °C in PBS (pH 7.5) containing 0.5% sodium azide (PBSA) together with a seed consisting of 0.1 weight percentage of sonicated WT Aβ-(1–40) fibrils. For the unmodified cysteine mutants, the protocol was similar except that the PBSA buffer contained 10 mM TCEP plus 1 mM EDTA and was bubbled with argon gas prior to dissolving the freshly disaggregated peptide, to keep the sulfhydryl group of cysteine in the reduced state during fibril assembly. Fibril formation reactions for all mutants were seeded initially with 0.1 weight percentage of sonicated WT Aβ-(1–40) fibrils. (For some mutants, which exhibited a lag time of more than 15 days, we used 1% seed.) For most of the mutants, assembly reactions were carried out at starting concentrations of monomeric peptide of about 50 μM. For those peptides that did not aggregate at 50 μM concentration, a new fibril assembly reaction was initiated at a higher concentration, in the range of 100–200 μM. Progress of the reaction was monitored by ThT measurement of fibrils (36). When the ThT signal reached a plateau value, reaction progress was further monitored by HPLC, as described previously (21, 34), to determine the concentration of Aβ-(1–40) at equilibrium, the critical concentration (C_c) (34, 37). Equilibrium constants for fibril elongation derived from C_c values were used to calculate free energies using the relationship ΔG = -RTlnK_eq.

**Modification of Sulphydryl Group of Cys at the Fibril Level—**

Unmodified Cys mutant fibrils grown under reducing conditions were centrifuged at 85,000 rpm (31,500 × g) for 30 min to remove any soluble peptide. The pellet was then resuspended and incubated in the dark for 1 h with 10 mM iodoacetamide in 50 mM Tris buffer at pH 8.0 at room temperature. Fibril products were collected by centrifugation (as above) and washed twice with PBS buffer to remove any dissociated monomer and unreacted alkyllation agent. After the last wash, the centrifugation pellet was dissolved in 20% aqueous formic acid, injected on a Zorbax SB-C3 analytical HPLC column linked to an Agilent 1100 electrospray mass spectrometer, and eluted with an acetonitrile-aqueous 0.1% formic acid gradient (formic acid is used instead of the more standard trifluoroacetic acid to improve MS sensitivity). Eluent was passed through a UV detector set at 215 nm and an Agilent 1100 electrospray mass spectrometer. Using A_215 peak area(s) for quantitation, Aβ-(1–40) recovery was typically 80% or better based on the amount of Aβ expected for the mass of fibrils used in the experiment (38).

For most analyses, the amount of Cys modification was quantified using the MS data. Using the Agilent software, the mass distribution data across the HPLC elution peak(s) corresponding to unmodified, singly modified, and multiply modified Aβ molecules were deconvoluted to generate the relative abundance of each species underneath the peak envelope. These relative abundances were used to calculate percentage of modification. In some cases, the unmodified and modified A_215 peaks were sufficiently separated in the HPLC that the relative A_215 peak areas could be used to calculate the percentage of modification. Since, however, for many Aβ mutants, the modified and unmodified peaks were not fully separated, it was necessary in these cases to use the deconvoluted MS data to determine the percentage of modification. Although this requires the assumption of equal MS sensitivity for the modified and unmodified peptides, this appears to be a valid assumption since there is good agreement with the values calculated using peak areas in those cases in which analysis by both means is feasible (38).

**Electron Microscopy—**

Fibrils grown in PBS were adsorbed onto carbon- and Formvar-coated grids and negatively stained with 0.5% (w/v) uranyl acetate solution. The stained grids after washing of the excess uranyl acetate were examined and photographed on a Hitachi-600 EM.

RESULTS

Fig. 1 shows the Aβ-(1–40) sequence (a) and two published models of the Aβ-(1–40) amyloid fibril. The model in b was derived from a combination of scanning probe mutagenesis experiments (26) and a threading approach applied to parallel β-helical domains of globular proteins (39). The model in c was derived from solid state NMR measurements (40). Both models posit a basic unit of fibril structure in which the Aβ molecule folds into a loop composed of 2–3 elements of extended chain oriented in such a way that the amino acid side chains in these segments project either into the interior of the loop or out of the loop. In both models, the inward-projecting side chains form a densely packed interior (although perhaps not evident from the depiction in Fig. 1b, the interior of the model, like the interiors of all parallel β-helical
domains (41, 42), is densely filled with side chain mass). Both models also presume that some of the outward-projecting side chains will also be packed into similar, adjacent elements to build up the lateral packing required to form fibrils from protofilaments. Importantly, neither published model addresses the orientation of these extended chains, that is, whether particular amino acid side chains in these segments are inward- or outward-projecting. Our Cys accessibility studies were designed to address this issue, as well as the environments of other Aβ amino acid side chains in the fibril.

We utilized Cys mutants in two ways. First, by growing fibrils from single Cys mutants under reducing conditions, we could use chemical reactivity of the free Cys sulfhydryl group to probe the solvent accessibility of that side chain in the fibril. Second, by modifying peptides at the monomer level, then growing fibrils and assessing the equilibrium positions of the fibril formation reaction, we could determine the compatibility of the modified Cys side chain with the chemical environment within the fibril.

Fibril Growth and Characterization—The ability to reliably grow amyloid fibrils from mutant Aβ-(1–40) peptides is a requirement for all the experiments described in this report. All peptides were purified, disaggregated, and subjected to amyloid growth conditions, including seeding with wild type Aβ-(1–40) fibrils, as described under “Materials and Methods.” Typical kinetics results for free Cys mutants, grown in the presence of the non-alkylatable reducing agent TCEP, are displayed in Fig. 2a, which shows a variety of lag times and final ThT levels. Mutants with Cys at positions 20, 21, 25–30, and 38 exhibited fibril formation kinetics similar to wild type. Mutants with Cys at positions 24, 31, 32, and 36 showed longer lag times, whereas those at positions 17, 18, 34, and 35 exhibited unusually low ThT signals. The D23C mutant peptide proved to be highly insoluble and therefore could not be used to

FIGURE 1. Aβ-(1–40) peptide and fibril structures. a, the amino acid sequence of Aβ-(1–40), with the mutants obtained as single Cys replacements indicated by red letters. b–d, structural models of the core unit of the amyloid protofilament based on analysis of fibrils grown without (b and d) or with (c) agitation. b, ribbon diagram of a parallel β-helix-derived model based on proline mutagenesis (26) and threading analysis (39). c, hairpin model based on solid state NMR data (40). d, schematic drawing of the intrapeptide side chain interactions in a cross-section of the amyloid filament, showing side chains predicted to be inward-pointing, based on accessibility (this work) and disulfide mutant analysis (30), and outward-pointing side chains predicted to be involved in packing between filaments (red bars) based on Cys accessibility experiments (see “Results”). The figure in b and c was adapted from figures in Refs. 39 and 40.

FIGURE 2. Kinetics of amyloid fibril growth for selected Cys mutants of Aβ-(1–40) modified in various ways. a, Aβ-(1–40) wild type (○) and single Cys mutants A21C (■), I31C (●), I32C (○), and L34C (▲). b and c, various Cys derivatives at positions 21 (b) and 34 (c). ▲, free Cys; ■, carboxymethyl-Cys; ●, methyl-Cys.
grow fibrils. As discussed below, low ThT levels do not necessarily indicate the absence of fibril formation since there are large mutational effects on ThT signals of amyloid fibrils on a weight-normalized basis. The final equilibrium position of each fibril formation reaction was confirmed and quantified by HPLC analysis of the centrifugation supernatant of a reaction aliquot ("Materials and Methods"). The reduced state was confirmed by subsequent HPLC analysis and/or by reactivity with the probe alkylation agent, iodoacetamide.

Representative electron micrographs of aggregation products are shown in Fig. 3. In the work described in this report, we set out to make fibrils from 75 different peptides (25 Cys mutants, each in three chemical states at the Cys residue). All except the Cys-23 mutant could be grown into an aggregate from soluble monomeric peptide. The vast majority (88%) of these aggregated products resembled WT amyloid fibrils, with 64% being indistinguishable from WT fibrils and another 24% being very similar. In addition, three of these peptides (L17C, V18C, and K28C) made filamentous aggregates that are particularly short, and another two peptides (G29C and the carboxymethyl-Cys derivative of A21C) produced aggregates that appeared to be a mixture of fibrils and other structures. Representative images of these different classes of structure are shown in Fig. 3.

Because of their relatively low ThT responses (see below), we were particularly concerned whether the three short filamentous aggregates (L17C, V18C, and K28C) were actually fibrils or perhaps were protofibrils. The word protofibril was introduced to apply to distinctive intermediate structures that appear transiently in the early stages of Aβ amyloid fibril formation under native conditions (43, 44). Such Aβ aggregates appear in the EM as short, curvilinear filaments ≤5 nm in diameter and of variable length, normally less than 200 nm (45–48), and exhibit low to negligible ThT fluorescence (28, 45) and substantially fewer backbone amide protons protected from hydrogen-deuterium exchange (25, 28). Given the short lengths and low ThT responses, we were concerned that some of the aggregates formed by Cys mutants might be protofibrils with arrested development; this would not be surprising since it is known that certain mutations can extend the stability of Aβ protofibrils (49). We applied two tests of these short aggregates to determine whether they were fibrils or protofibrils. First, we measured fibril diameters in the EM fields. Amyloid fibrils tend to have diameters in the range of 8–12 nm (16), whereas Aβ protofibrils tend to be in the 5-nm diameter range (45–48). Width ranges (nm) for aggregates of various sequence forms of Aβ-(1–40) were: WT, 7.5–14.3; L17C, 8.5–12.9; V18C, 7.5–11.5; K28C, 6.5–9.5. Thus, all of these short aggregates have diameters consistent with a fibril structure.

The other test we applied was hydrogen-deuterium exchange by mass spectrometry (23, 24, 26). Hydrogen-deuterium exchange is a particu-
Cysteine Mutagenesis of Amyloid Fibrils

Other data in Fig. 4 address aspects of different models of Aβ-(1–40) fibrils. Residues 24–27 are all accessible to alkylation within the fibril. This is inconsistent with their being in a β-sheet, where one or both faces are inaccessible, and is more consistent with their being in some kind of irregular structure. Residue 38 is also alkylatable, which is inconsistent with it being in an extension of the 31–36 β-sheet since it would be expected to be on the buried face of the extended chain but which is consistent with it being in a less ordered, non-H-bonded structure. Residues 29 and 30 are accessible, consistent with their being in a turn. Residue 22 is buried, which is inconsistent with being located in a simple turn. It is interesting that in these amyloid fibrils grown under unaltered conditions, a Cys residue at position 28 is inaccessible to alkylation. This would be expected if this residue were buried in an internal salt bridge. Such an interaction has been characterized within Aβ-(1–40) fibrils grown under agitated conditions but apparently is not present in a clearly defined way in quiescent fibrils (33) grown under conditions similar to those used here.

The explanation for partial modification of some side chains is not clear. These partial modifications are not due to insufficient reaction times. In cases in which partial modification was observed, reaction times were extended and later time points were analyzed, but the percentage of modification of the Cys residue changed minimally or not at all. Longer extended reaction times could not be done routinely since incubation for times longer than 1 h typically led to multiple modification of Aβ; this is not surprising since iodoacetamide can also react with His, Lys, and Met residues, as well as α-amino groups (50). For example, mutants H6C, S26C, N27C, V24C, and I32C, which exhibit a range from 0 to 51% modification after 1 h, were exposed to further reaction time up to 4 h. This led to observation of minimal change in the singly modified peak, as compared with the 1-h result, and formation of an additional mass ion corresponding to doubly modified material at 12, 10, 17, 0, and 0%, respectively, of the total Aβ recovered. Assuming that the doubly labeled Aβ, like the singly labeled Aβ, is modified at Cys, then the change in total percentage of molecules modified at Cys, by extending reaction time from 1 to 4 h, was: H6C, from 51 to 52%; S26C, from 28 to 32%; N27C, from 38 to 45%; V24C, from 27 to 32%; I32C, from 0 to 0%. Thus, partial modification appeared to be due to multiple environments. Whether these consist of subtle microenvironment differences in a largely uniform fibril preparation, or more major differences indicative of multiple conformers of fibrils, is not clear. With respect to other chemical probes, some fibril preparations previously have been shown to exhibit protein environments that behave uniformly in some parts of the structure and variably in other parts (21, 22, 51).

Thermodynamic Experiments—The alkylation of the Cys sulphydryl group is one of the most efficient and highly selective chemical modifications that can be done on polypeptides. A variety of alkyllating agents of various chemical structures are available. We chose two of these reagents, iodoacetic acid and methyl iodide, to modify the sulphydryl groups of the Cys mutant peptides to produce additional Aβ analogs with different chemical characteristics in the side chains. The reaction conditions are mild, and the products are easily purified from unreacted starting material and trace side products by HPLC (“Materials and Methods”). We used these chemically derived mutant peptides, plus the unmodified Cys mutants, to probe the effect of side chain chemistry at various Aβ-(1–40) sequence positions on the thermodynamics of fibril formation.

Fig. 2, b and c, show the fibril formation reactions of two sets of Cys derivatives, at residues 21 and 34, under standard conditions, as monitored by ThT fluorescence (36). As with most other fibril formation reactions in this work, these were seeded with 0.1% weight percentage...
Cysteine Mutagenesis of Amyloid Fibrils

![Graph showing free energy comparisons of amyloid fibril formation for Aβ-(1–40) point mutants containing Cys in various chemical states. Cross-hatched bars, free Cys; filled bars, carboxymethyl-Cys; open bars, methyl-Cys. a, ΔΔG values for fibril formation by mutant Aβ-(1–40) peptides, plotted with respect to WT. The ΔG for wild type peptide, based on the C₅₀ of 0.86 μM, is −8.6 kcal/mol. More positive ΔG values correspond to less favorable fibril formation. b, ΔΔG values comparing alkylated mutants with free Cys. The asterisk at position 23 indicates no data due to inability to solubilize peptide.](image)

To present the data in a different way, we recalculted the data shown in Fig. 5a as ΔΔG values comparing the stability of the fibrils from an alkylated mutant to the stability of the corresponding free Cys derivative at each residue position. The data (Fig. 5b) show how individual environments within the amyloid fibril tolerate a negatively charged group, as compared with a hydrophobic group, added to the Cys side chain sulfhydryl. In this formalism, as expected for a hydrophobic packed core, negatively charged substitutions at most positions are destabilizing, whereas hydrophobic replacements at the same positions are most often somewhat stabilizing. The most dramatic effect in Fig. 5b is the ability of both Cys modifications at position 22, relative to the free Cys peptide, to enhance the stability of fibrils. Position 23 is not represented in this figure because it was not possible to grow fibrils from the free Cys peptide; however, the ΔΔG values for the Cys derivatives at position 23 are similar to the position 22 ones in having essentially no effect on stability relative to WT. These data are consistent with previous assignments of a turn position to these positions based on proline mutagenesis (26, 39).

One difficulty with interpreting the data from the modified Cys peptides is that the substitutions are inevitably being tested, not only for the compatibility of their hydrophobic/hydrophilic character but also for their bulk. Although the densely packed regions of the amyloid fibril may have somewhat more flexibility to accommodate additional side chain bulk, compared with typical globular proteins, there are also presumably limits to fibril plasticity. This might explain situations such as position 33, where both alkylated forms of Cys destabilize the fibril relative to the free Cys derivative. In a sense, the relevant difference at position 33 (which is a Gly in the WT) is the relative ease with which it accommodates the Cys replacement, as seen in Fig. 5a. Besides in the N and C termini, those mutations for which the substitution of the relatively bulky, hydrophobic Cys destabilizes fibrils as compared with WT by less than 0.5 kcal/mol are K16C, F20C, G26C, K28C, A30C, and G33C (Fig. 5a). Of these, the modest effects at positions 20, 26, and 30 are understandable simply in terms of their surface exposure (Fig. 4) and presumed ability to accommodate some additional bulk.

**Weight-normalized Thioflavin T Signals**—ThT is often used to follow amyloid formation reactions (36). For any amyloidogenic protein, the time-dependent ThT increase is a good measure of the time required to reach the reaction end point. ThT cannot be used to accurately determine the position of the fibril-monomer equilibrium, however, since it cannot sense the amount of unpolymerized monomer remaining at equilibrium. In addition, ThT cannot be used to compare the degree of completeness of fibril formation for different amyloidogenic peptides, even peptides that are highly related structurally. This is because different amyloid fibrils can generate significantly different fluorescence yields in response to ThT. This is illustrated in Fig. 6. ThT yields were measured, and the fluorescence values were normalized based on independent determination of the amount of peptide in the reaction that had converted to amyloid (that is, the fibril component in the C, determina-
Cysteine Mutagenesis of Amyloid Fibrils

FIGURE 6. Comparisons of weight-normalized thioflavin T fluorescence signals for amyloid fibrils of Aβ-(1–40) point mutants containing Cys in various chemical states. Cross-hatched bars, free Cys; filled bars, carboxymethyl-Cys; open bars, methyl-Cys.

Although this argument is admittedly speculative, the basic arrangement arising from this logic was subsequently confirmed using further cysteine mutagenesis (30). Postulating that residue 20 is outward-facing leads directly to the corollary that residues 17 and 19 must be inward-facing. Based on this hypothesis, residue 17 was chosen as an anchor for a series of double Cys mutant cross-linking experiments. Thus, double Cys mutants were constructed in which each mutant contains Cys-17 plus 1 additional Cys within the 34–36 segment. Each double Cys mutant was used in a variety of experiments, which uniformly show that residues 17 and 34 within the same Aβ peptide molecule are packed together within Aβ-(1–40) fibrils grown in PBS at 37 °C under quiescent conditions (30). Since some degree of interaction between the 17 and 36 side chains was also indicated in these experiments, Fig. 1d is drawn to suggest a possible staggered orientation such that 17 resides between 34 and 36 in the filament interior. Later experiments further confirm the schematic model of Fig. 1d, in showing that side chains 19 and 32 from the same peptide molecule are also within disulfide cross-linking distance in the packed interior of these quiescent fibrils.5

Of the other accessibility data, some were expected, and some were puzzling and surprising. It is surprising that residue 30 is so accessible to alkylation; no other residue, except Cys-4 in the presumably completely flexible N terminus, is completely alkylated in these experiments. The accessibility of Cys-29 and Cys-30 is consistent with these residues being in a turn or loop, as suggested by the models in Fig. 1. The accessibility of residue 38 is consistent with data showing that the C terminus, like the N terminus, is relatively unstructured (20, 26, 27). That residues 24–27 are all partially accessible to alkylation is less consistent with their being involved in a third β-extended chain segment in the quiescent Aβ-(1–40) amyloid fibrils (26, 39) and more consistent with their being in more irregular structure (27),3 as suggested previously for Aβ-(1–40) fibrils grown under agitated conditions (40).

Residues 22 and 23 remain structurally enigmatic. These were predicted to be in a turn based on Pro mutagenesis (26), and this assignment is also consistent with their lack of response to modified Cys mutations (Fig. 5) and Ala mutations.3 However, the backbone amide hydrogens of these residues are strongly protected from exchange, and the side chain of Cys-22 is completely protected from alkylation; no other residue, except Cys-4 in the presumably completely flexible N terminus, is completely alkylated in these experiments. The accessibility of Cys-29 and Cys-30 is consistent with these residues being in a turn or loop, as suggested by the models in Fig. 1. The accessibility of residue 38 is consistent with data showing that the C terminus, like the N terminus, is relatively unstructured (20, 26, 27). That residues 24–27 are all partially accessible to alkylation is less consistent with their being involved in a third β-extended chain segment in the quiescent Aβ-(1–40) amyloid fibrils (26, 39) and more consistent with their being in more irregular structure (27),3 as suggested previously for Aβ-(1–40) fibrils grown under agitated conditions (40).

Residues 22 and 23 remain structurally enigmatic. These were predicted to be in a turn based on Pro mutagenesis (26), and this assignment is also consistent with their lack of response to modified Cys mutations (Fig. 5) and Ala mutations.3 However, the backbone amide hydrogens of these residues are strongly protected from exchange, and the side chain of Cys-22 is completely protected from alkylation. (Cys-23 cannot be tested because it cannot grow amyloid fibril.)

The free energy effects of Cys derivatization provide a wealth of detail on the environments within the folded amyloid fibril that must accommodate these side chains. Overall, there are some trends in these data that are understandable in the context of the current states of the models shown in Fig. 1. Other effects are not so easily understood. These data, and additional data from similar experiments, are expected to be of

3 S. Shivaprasad and R. Wetzel, unpublished observations.
Cysteine Mutagenesis of Amyloid Fibrils

increasing importance in the future to direct and refine further model building.

Acknowledgment—We are grateful to Charles Glabe for the gift of some Cys mutants of Aβ(1–40) that were used to assess feasibility of the approaches described here.

REFERENCES

1. Martin, J. R. (1999) *N. Engl. J. Med.* 340, 1970–1980
2. Merlini, G., and Bellotti, V. (2003) *N. Engl. J. Med.* 349, 583–596
3. Wetzel, R., and Goeddel, D. V. (1983) in *The Peptides: Analysis, Synthesis, Biology* (Meienhofer, J., and Gross, E., eds) Vol 5, pp. 1–64, Academic Press, New York
4. Kane, J. F., and Hartley, D. L. (1991) *Biosci. Technol.* (N. Y.) 12, 121–145
5. Wetzel, R. (1992) in *Biosci. Technol.* (N. Y.) 12, 121–145
6. Wetzel, R. (1994) *Trends Biotechnol.* 12, 193–198
7. Turner, G. C., and Varshavsky, A. (2000) *Science* 289, 2117–2120
8. Hartl, F. U., and Hayer-Hartl, M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 16742–16747
9. Kinoshita, T., and Holtzman, D. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 3983–3988
10. Walsh, D. M., Lomakin, A., Benedek, G. B., Condron, M. M., and Teplow, D. B. (1997) *J. Biol. Chem.* 272, 23346–23372
11. Walsh, D. M., Hartley, D. M., Petre, B. M., Wall, J. S., Simon, M. N., Walz, T., and Teplow, D. B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 948–952
12. LeVine, H. (1999) *Methods Enzymol.* 309, 274–284
13. O’Nuallain, B., Shivasprasad, S., Kheterpal, I., and Wetzel, R. (2005) *Biochemistry* 44, 12709–12718
14. Buchen, T., and Holtzman, D. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 7590–7595
15. Kahana, E., Seilmeier, M., and Holtzman, D. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 11742–11747
16. Walsh, D. M., Selkoe, D. J., and Teplow, D. B. (1999) *J. Biol. Chem.* 274, 25945–25952
17. Harper, J. D., Wong, S. S., Lieber, C. M., and Lansbury, P. T. (1997) *Chem. Biol.* 4, 119–125
18. Walsh, D. M., Lomakin, A., Benedek, G. B., Condron, M. M., Teplow, D. B. (1997) *J. Biol. Chem.* 272, 23346–23372
19. Walsh, D. M., Hartley, D. M., Petre, B. M., Wall, J. S., Simon, M. M., Walz, T., and Lansbury, P. T. (2003) *J. Mol. Biol.* 332, 795–808
20. Walsh, D. M., Lomakin, A., Benedek, G. B., Selkoe, D. J., and Teplow, D. B. (1999) *J. Biol. Chem.* 274, 25945–25952
21. Harper, J. D., Wong, S. S., Lieber, C. M., and Lansbury, P. T., Jr. (1999) *Biochemistry* 38, 8972–8980
22. Kowalewski, T., and Holtzman, D. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 7590–7595
23. Kahana, E., Seilmeier, M., and Holtzman, D. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 11742–11747
24. Walsh, D. M., Selkoe, D. J., and Teplow, D. B. (1999) *J. Biol. Chem.* 274, 25945–25952
25. Harper, J. D., Wong, S. S., Lieber, C. M., and Lansbury, P. T. (1997) *Chem. Biol.* 4, 119–125
26. Walsh, D. M., Lomakin, A., Benedek, G. B., Condron, M. M., and Teplow, D. B. (1997) *J. Biol. Chem.* 272, 23346–23372
27. Whittome, N. A., Mishra, R., Kheterpal, I., Williams, A. D., Wetzel, R., and Sperou, E. H. (2005) *Biochemistry* 44, 4434–4441