The Structural Basis of Monoclonal Antibody Alz50's Selectivity for Alzheimer's Disease Pathology*

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Alz50 is an IgM-class monoclonal antibody that stains the fibrillar pathology (dystrophic neurites, neurofibrillary tangles, and neuritplagues) commonly observed in postmortem histological analysis of Alzheimer's disease (AD)1 brain (1, 2). Because of these properties, it has emerged as an important tool for gauging the temporal and spatial severity of Alzheimer's disease pathology (3, 4). The major components of the fibrillar pathology are straight and paired helical filaments (PHF) (5), which themselves are comprised largely of hyperphosphorylated forms of the microtubule-associated protein tau (6–10). Previous studies have shown that Alz50 reacts with tau and that its epitope is located at the N terminus (11–13) in a region conserved in all known splice variants of human tau (14). Indeed, Alz50 has been shown to react with tau proteins isolated from normal brain (15), recombinants (12), and PHFs (8) by Western analysis. Nonetheless, the ability of Alz50 to label distinct populations of neurons in normal human brain (16), fetal brain (17), and early stage neurofibrillary degeneration (4, 18) suggests that Alz50 selectively recognizes a distinct subset of tau proteins.

To place the many observations on Alz50 immunocytochemistry into a structural context, we reinvestigated its epitope selectivity in vitro. The results suggest that individual tau monomers adopt a specific conformation preceding or during filament formation that is selectively recognized by Alz50.

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The results suggest that tau adopts a distinct conformation when polymerized into filaments and that this conformation is recognized selectively by Alz50.
**The Alz50 Epitope**

**TABLE I**

| Mutant | Method | Sites or sequence | Resultant sequence |
|--------|--------|------------------|--------------------|
| Δ430-441 | S | NheI | ... A320+S* (stop) |
| Δ395-427 | S | BsrGl/NheI | ... Y294+L320 |
| Δ357-393 | S | PvuMI/BsrGl | ... S266+Y294 |
| Δ345-441 | S | HindIII | ... L344+ N356 (stop) |
| Δ333-441 | S | PFM/NheI | ... P332+A*-S* (stop) |
| Δ321-441 | S | BstEI/NheI | ... T319+S320 (stop) |
| Δ283-441 | O | CAGATAAATAAAGACTGAAAGATTCCAAG | ... L320 (stop) |
| Δ231-240 | O | GCAGTGTCGCGAGCCGGCGGCAGCAG | ... K330+S340 |
| Δ214-245 | O | GTTCCTCGCCAAGGTATGCCAAGATG | ... S250+V266 |
| Δ256-270 | O | CAGACCTGAAAGATGAGCAGCAAGGAG | ... N255+G271 |
| Δ271-282 | O | AAGGCCACCCGGCGATTCTTACGACTACC | ... P270+D283 |
| Δ222-301/344 | S | Pvu1/PvuMI | ... R221+V*-E*-G250+ ... -L344-N* (stop) |
| Δ211-256 | S | SfiI/PvuMI | ... S160+L270 |
| Δ163-255 | S | SfiI/PstI | ... A162+S256 |
| Δ162-244 | S | SfiI/PstI | ... Q162+S256 |
| Δ162-209 | S | SfiI/BsrGl | ... G161+H*-S210 |
| Δ155-393 | S | SacII/BsrGl | ... P154+S210 |
| Δ155-355 | S | SacII/PvuMI | ... P154+S256 |
| Δ155-244 | S | SacII/PstI | ... P154+T240 |
| Δ155-209 | S | SacII/BsrGl | ... P154+S210 |
| Δ155-163 | S | SacII/SfiI | ... P154+G250 |
| Δ122-427 | S | PmlII/NheI | ... H211+S250 |
| Δ103-324 | S | PvuII/HindIII | ... T302+G256 |
| Δ103-155 | S | PvuII/SacII | ... T302+G*+G256 |
| Δ84-393 | S | SacII/BsrGl | ... G33+V294 |
| Δ84-354 | S | SacII/PvuMI | ... G33+S256 |
| Δ84-161 | S | SacII/SfiI | ... G33+G210 |
| Δ19-121 | S | BstUII | ... Y18+T212 |
| Δ19-88 | S | BstUI/SacI | ... Y18+V*+S210 |
| Δ9-155 | S | BstUI/SacI | ... F9+G*+G256 |
| Δ2-18 | S | CGGCCACCCATATTGGGGTGTTGCGG | ... M*+G210 |

*a Deletions were created by oligonucleotide-directed mutagenesis (O) or by restriction digests (S).

*b Pairs of restriction sites were selected that, when digested and religated, would conserve the htau40 reading frame. pT7C-htau40 was cleaved with the selected endonucleases, blunt-ended with either Klenow fragment of DNA polymerase I (for 5’ overhangs) or T4 DNA polymerase (for 3’ overhangs), or both. Resultant fragments were ligated and used to transform MM294 cells.

*c Asterisks represent residues not normally part of htau40 that were introduced by mutagenesis.

*d The Δ222-301/344 and Δ345-441 were gifts of G. Jicha and P. Davies. Chimera Δ222-301/344 was created by ligating a SmaI-HindIII fragment encoding to the third microtubule repeat (Gly302–Leu344) to a SmaI-HindIII fragment from Tau-5. Each antibody returned linear Scatchard plots and bound monomeric tau and PHFs with nearly identical affinity. These results are consistent with Tau-5 binding a tau epitope that is presented nearly identically in monomeric and PHF-tau. In the final construct, the two segments are connected via a two residue linker (Val–Glu). Bacterial expression of this and all other constructs was confirmed by SDS-polyacrylamide gel electrophoresis.

**RESULTS**

**Alz50 Reacts Preferentially with PHF-tau**—To quantify the binding selectivities of a panel of anti-tau monoclonal antibodies, equilibrium binding experiments with both monomeric tau (htau40) and authentic affinity-purified PHFs were performed as described under “Experimental Procedures.” On the basis of SDS-polyacrylamide gel electrophoresis, all protein reagents used in binding experiments were of high purity (Fig. 1). The analysis began with four anti-tau IgGs known to bind continuous epitopes on tau independently of the state of tau phosphorylation: Tau-5 (22), 5E2 (23), Tau46.1 (24), and TG5 (see below). Results for Tau-5 binding to both htau40 and PHFs are illustrated in Scatchard format in Fig. 2. The resultant Scatchard plots are linear, indicating that the Tau-5 antibody interacts with tau via a single noncooperative binding site (37). Furthermore, each plot revealed nearly complete fractional occupancy at saturating concentrations of analyte (tau), proving that essentially all antibody binding sites are accounted for and that the Tau-5 antibodies are fully active. The binding constants ($K_a$) calculated from these plots and summarized in Table II show that Tau-5 binds both monomeric htau40 and PHFs with nearly identical affinity. These results are consistent with Tau-5 binding a tau epitope that is presented nearly identically in monomeric and PHF-tau.

Binding data for Tau46.1, 5E2, and TG5 are also summarized in Table II. Although these antibodies bind tau with differing affinities, each retains the characteristics summarized above for Tau-5. Each antibody returned linear Scatchard plots and bound monomeric tau and PHFs with nearly identical affinities ($K_a$) and with essentially full occupancy of available antibody binding sites. Thus, antibodies that bind tau through simple continuous epitopes, such as Tau-5, Tau46.1, 5E2, and 5E2, bound PHFs with nearly identical affinity. The differences in $K_a$ for PHF binding reflect the differences in their carbohydrate composition and folding, which is consistent with the carbohydrate composition of the site of the epitope for these antibodies. The differences in $K_a$ for PHF binding further reflect the differences in carbohydrate composition and folding, which is consistent with the carbohydrate composition of the site of the epitope for these antibodies.
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results are depicted as Scatchard plots in which (25). Tau-5 binds both forms of tau with high affinity and complete bound analyte and stained with Coomassie Blue.Resolved by SDS-polyacrylamide gel electrophoresis (11% acrylamide), htau40 were purified as described under "Experimental Procedures," and stained in the Tau-5 and Alz50 lanes correspond to heavy and light immunoglobulin chains. The purity of Tau-5 is typical of all IgGs used in this study.

Like the antibody binding sites (see text for details).

Alz50 bound recombinant tau (\(K_d \approx 20 \text{ nM}\)) with nearly complete saturation of available binding sites. Yet Alz50 differed by displaying a clear selectivity for PHF-tau, which it bound with an observed affinity of 0.32 nM (Fig. 2; Table II). Because this value approximates the concentration of antibody binding sites in the assay, it must be considered an upper limit for \(K_d\) (38, 39), so its true affinity for PHF may be even greater. The results indicate that the interaction of Alz50 with PHF-tau is nearly 2 orders of magnitude greater affinity than its interaction with recombinant monomeric tau. In addition, the stoichiometry of binding is reduced to \(\sim 0.5\) PHF-tau molecules/antibody binding site, perhaps reflecting steric hindrance between the decavalent Alz50 (an IgM) and the PHFs (which average 20 nm in width and 0.25 \(\mu\)m in length) (26).

The behavior of Alz50 was paralleled by that of Tau-2, an IgG raised against bovine tau (21). Tau-2 is known to react poorly with recombinant human tau (40) but strongly with the fibrillar pathology found in AD brains (41). As summarized in Table II, Tau-2 also is selective for the PHF conformation of human tau, which it binds with \(>10\)-fold higher affinity than it binds recombinant htau40. Similar behavior also is exhibited by MC1, a new IgG raised against PHF (25). These results suggest that Alz50, Tau-2, and MC1 form a second class of anti-tau antibody that reacts preferentially with tau epitopes when they are present in the context of PHF.

Alz50 Recognizes a Conformational Epitope on tau—The affinity data confirms that nonphosphorylated recombinant tau contains the sequences necessary for Alz50 binding but suggests that unlike Tau46.1, 5E2, and Tau-5, factors other than primary structure mediate the high affinity interaction between PHF-tau and Alz50. To examine this possibility, the ability of anti-tau antibodies to bind either htau40 or htau40 denatured via boiling in dilute SDS was assessed. As shown in Fig. 3, antibodies that recognize continuous epitopes on tau, such as Tau46.1, react similarly with htau40 regardless of whether this analyte was subjected to SDS-mediated denaturation. Alz50 staining, however, is nearly destroyed by this treatment. Similar results were obtained when PHF-tau was substituted for htau40 as analyte. In this case, however, total Tau46.1 reactivity seems to increase after PHF denaturation (Fig. 4). Because the binding affinity of Tau46.1 for tau is insensitive to denaturation (Fig. 3), this increase in Tau46.1 reactivity probably results from more tau being accessible to it. These results suggest that a portion of tau molecules packed into PHFs are inaccessible to antibody until released by denaturation. Despite the increase in accessible tau, PHF denaturation still leads to a dramatic decrease in Alz50 reactivity (Fig. 4). Together these data suggest that the Alz50 epitope on human tau and PHF is denaturation sensitive, and therefore probably conformational in nature.

Epitope-mapping Experiments—To determine the amino acid sequences on tau that serve as epitopes, a recombinant
after denaturation. Sensitive tau molecules, Alz50 reactivity toward PHF drops dramatically upon PHF denaturation, suggesting that a third of the tau molecules packed into PHFs are inaccessible to antibody until the terminal 18 residues of tau are reduced dramatically relative to that of Tau46.1, an antibody that recognizes a continuous epitope on tau.

To validate this mapping approach, the library was first screened with two antibodies whose epitopes on tau are known: Tau-5, Tau-46.1, and TG5 to interact with deletion mutants of htau40 was assessed as described under "Experimental Procedures." The regions deleted are shown graphically in white for each mutant. A schematic of htau40 is shown for comparison, including the positions of alternatively spliced exons 2 and 3 (e2 and e3) (62) and the four microtubule binding repeats (m1–m4). These results confirm that Tau-1, Tau-5, Tau-46.1, and TG5 bind continuous epitopes within the tau molecule.

Table III

| Antibody | Mapped previously? | Reference no. | This study* |
|----------|---------------------|---------------|-------------|
| Tau-46.1 | Yes: Ser404–Leu441  | 24            | Leu426–Leu441 |
| Tau-5    | Yes: Pro162–Gly207  | 24, 42, 43, 44 | Pro162–Gly210 |
| Tau-5    | No                  | Ser210–Arg230  |
| TG5      | No                  | Ser210–Arg230  |

* Data are from Fig. 5.

For Tau46.1, the segment Leu426–Leu441 (Fig. 5) identified by deletion mapping lies within the 38-residue region Ser404–Leu441 identified previously (24) but improves the resolution of the epitope to just 14 residues.

Deletion mapping was extended to two previously uncharacterized antibodies as well: Tau-5, an IgG raised against bovine tau (22), and TG5, an IgG raised against PHF (25). The results, again illustrated graphically in Fig. 5 and summarized in Table III, reveal that both antibodies recognize essential residues located within the segment Ser210–Arg230. In this respect Tau-5 and TG5 closely resemble not only 5E2 (Ser214–Pro233) (24) but also AT120 (Pro218–Lys224), an antibody raised against PHF that is useful in premortem diagnosis of AD (45, 46). Thus, four monoclonal antibodies (Tau-5, TG5, AT120, and 5E2) raised independently from two different tau antigens (bovine tau and human PHF) all bind within the same ~20-residue segment of tau. In addition to demonstrating the utility of deletion mapping, these data suggest that the region Ser210–Arg230 is both highly antigenic and accessible on the surface of at least some of the tau molecules aggregated into PHFs.

Identification of the Alz50 Epitope on tau—The amino acid sequences necessary for Alz50 binding were determined by screening the deletion library described above. The results, shown graphically in Fig. 6, reveal that Alz50 binding is mediated by two segments on tau. The first lies within the N-terminal 18 residues of tau and corresponds to the epitope identified previously (13, 14). It is essential for Alz50 binding...
The Alz50 Epitope

because any mutant that contains a deletion in this region cannot bind Alz50. The second epitope consists of sequences located within the microtubule repeat region. As shown in Fig. 6, deletion of the entire microtubule repeat region, such as in mutant Δ155–393, results in >90% loss of Alz50 reactivity. Smaller deletions that leave only a portion of one repeat, such as mutant Δ210–355, show the same loss of reactivity. These results suggest that Alz50 is sensitive to the conformation of tau because the epitope it recognizes is discontinuous. The full epitope is comprised of sequences located in the N-terminal and microtubule binding regions of tau.

The microtubule repeat region of htau40 consists of four ~31-residue repeats arranged in tandem, with pairwise identities that range from 55–60% (47). When conservative substitutions are taken into account (conservative substitutions are defined in Ref. 48), the repeats share as much as 70% similarity. To determine whether the four repeats of htau40 contribute to Alz50 binding individually or in aggregate, mutants containing deletions spanning the microtubule repeat region were assayed for Alz50 reactivity as described above. The results show that mutants containing just three repeats retain full Alz50 reactivity (Fig. 7). This is most clearly seen with mutant Δ345–441 (which retains repeats m1, m2, and m3 but not m4) and the naturally occurring tau isoform htau23 (which retains repeats m1, m3, and m4 but not m2). Partial deletion of the first repeat leaving m2, m3, and m4 is also fully reactive (e.g. Δ241–255 and Δ256–270). Sequential deletion of C-terminal residues to leave two (Δ333–441 and Δ321–441) or just one (Δ283–441) repeat are similarly reactive with Alz50. These results suggest that the minimal reactive unit of the second Alz50 epitope is a single microtubule binding repeat. To confirm this assignment, a chimera was constructed containing the full sequence of the third microtubule binding repeat fused to the N-terminal 221 residues of htau40 and analyzed for reactivity with Alz50. Like Δ283–441, this mutant also retains full Alz50 reactivity. These results confirm that the presence of a single microtubule repeat is sufficient to enhance the binding affinity of Alz50 for tau.

The Reaction of Alz50 with tau Is Consistent with an Intramolecular Mechanism of Action—In some studies, PHFs appear as two hemifilaments wound helically around one another (5, 49), suggesting they consist of a multidimensional lattice of tau monomers, each in contact with several nearest neighbors (50). Yet, when PHFs are prepared for microscopy by freeze-drying and vertical platinum-carbon replication, no ordered substructure is resolved (51, 52), suggesting that tau monomers are arranged randomly within the PHF. Thus, Alz50 may interact preferentially with PHF-tau because individual tau monomers adopt an ordered structure, thereby stabilizing an intramolecular epitope (i.e. the epitope consists of different regions on a single tau polypeptide) or because amorphous aggregation of tau facilitates an intermolecular reaction (i.e. the epitope spans two regions of neighboring tau monomers).

Similarly, the interaction of Alz50 with recombinant tau may be mediated by one or multiple tau molecules. To determine whether the interaction of Alz50 with recombinant tau was intra- or intermolecular, equal amounts of purified tau deletion mutants that lacked complementary portions of the Alz50 epitope were mixed, spotted on nitrocellulose paper, and examined for restoration of Alz50 binding. The mutants employed for this experiment were Δ2–18, an N-terminal deletion containing an intact microtubule repeat region, and Δ211–356, which has an intact N terminus but a defective microtubule repeat region. The results confirmed that deletion of either component of the Alz50 epitope eliminates or greatly reduces Alz50 binding relative to wild type (Figs. 6 and 7). Mixing of Δ2–18 and Δ211–356 before incubation with antibody, however, did not further augment Alz50 binding or restore it to wild-type levels. These data suggest that reaction of Alz50 with recombinant tau mutants cannot be reconstituted by transcoplementation of adjacent molecules, which is consistent with an intramolecular reaction mechanism.

DISCUSSION

Because Alz50 was raised against a crude Alzheimer brain homogenate (1), the precise nature of its immunogen has remained elusive. Although initial characterization suggested that Alz50 bound a novel Alzheimer’s disease-associated protein (ADAP) (1, 53), subsequent work proved that it reacted with tau (12, 14), the principal component of neurofibrillary tangles (10). Nonetheless, the basis of its selective immunohistochemical properties remained unclear. The results presented here suggest that Alz50 is selective for neurofibrillary pathology in part because it binds a conformational epitope on tau that is stabilized or prevalent in PHFs. Thus, although most anti-tau antibodies stain the fibrillar pathology, Alz50 is among the most robust. Selective binding may also underlie the efficiency of PHF immunopurification using P42, an IgG class switch of Alz50 (26).

![Fig. 6. The Alz50 epitope is discontinuous.](image-url)

![Fig. 7. Alz50 and the microtubule repeat region of tau.](image-url)
A model of Alz50 selectivity is illustrated in Fig. 8. It predicts that the N terminus of PHF-tau is in close association with the microtubule repeat region. Together, the two regions comprise the Alz50 epitope. Because of an emphasis on N-terminal deletion or expression screening methods better suited for analysis of continuous epitopes (12, 26), earlier mapping strategies successfully identified the first but not the second component of the Alz50 epitope. The reaction of Alz50 with human tau isoforms in adult and fetal brain is consistent with the conservation of the epitope in all known splice variants of the tau gene (11). Although the presence of a single repeat promotes Alz50 binding, it is not clear which of the four repeats present in htau40 actually forms the epitope when the tau molecule is packed into the PHF. It is conceivable that, due to the flexibility of the tau molecule and the conserved nature of each microtubule repeat, that individual tau molecules within the PHF form the epitope from different repeats.

Although the Alz50 epitope could result from either intra- or intermolecular interactions, the inability to reconstitute it from mixed deletion mutants, its appearance before neurofibrillary tangle formation in postmortem brain sections (4), and its existence on monomeric recombinant tau as seen on Western blots suggest that the mechanism of interaction is intramolecular. Presumably, monomeric tau can adopt the Alz50 conformation due to its flexibility (54), but the energy required to do so is reflected in a higher $K_d$ value.

It is important to emphasize that Alz50 is selective but not specific for tau conformation. As shown here, selectivity can be demonstrated at low concentrations of antibody and tau. High concentrations of either reagent can drive the reaction and mask the selectivity (13). Thus, Alz50 reacts with recombinant tau on Western blots after denaturation in SDS-polyacrylamide gels (12) and can be blocked by incubation with high concentrations of N-terminal peptide (14). The antibody also can cross-react with proteins unrelated to tau, including bovine serum albumin (55) and p125$^{^\text{neo}}$ (56). The selectivity properties of Alz50 probably contributed to the early controversies surrounding the relationship between PHFs and tau (1, 10, 53).

Early reports suggested that the Alz50 epitope was phosphorylation-sensitive (57). Although it is clear that phosphorylation is not required for epitope recognition, it may contribute to high affinity binding of Alz50 by stabilizing certain conformations of tau. Indeed, the development of Alz50 immunoreactivity parallels that of hyperphosphorylated tau as measured by the AT8 antibody during onset of AD (3). Both AT8 and Alz50 immunoreactivity seem to precede the occurrence of neurofibrillary tangles. In addition, phosphorylation may promote the assembly of tau monomers into straight and paired helical filaments, which then locks tau into the conformation selectively bound by Alz50 (58, 59).

The first antibody selective for tau conformation characterized was Tau-2, which recognizes a continuous epitope ~16 residues in length located in the N-terminal third of the molecule. It has been suggested that as a result of differences in the structures of bovine and human tau in this region, Tau-2 reacts poorly with human tau until it adopts a conformation similar to that of bovine tau (40). Presumably, this conformation is adopted by human tau in PHFs, so that like Alz50, Tau-2 is useful in immunohistochemical studies of AD (41). On the basis of affinity measurements, we predict that MC1 will share histochemical selectivity with Alz50 and Tau-2.

The results presented herein suggest that the many anti-tau antibodies raised to date can be organized into at least three categories. The first, exemplified by Tau-5, consists of reagents that bind continuous epitopes on tau independently of tau conformation or state of posttranslational modification. Antibodies of this class have proved valuable as capture antibodies for tau-based immunoassays (44, 45). The second class, exemplified by Alz50, selectively binds specific conformations of tau. These antibodies can identify populations of tau adopting filament-like conformations before PHFs are sufficiently developed to view microscopically (4). The third class of antibody, exemplified by AT8, is dependent on tau phosphorylation for binding (44). These agents are particularly useful for selectively detecting hyperphosphorylated forms of tau that accumulate in neurofibrillary tangles (58, 59). We predict that a potential fourth class of antibody, simultaneously selective for both tau conformation and phosphorylation state, will provide the most sensitive probes for neurofibrillary tangles yet discovered.

The existence of conformation-selective antibodies described herein provides additional evidence that tau, a protein that contains little ordered structure when isolated in monomeric form, is capable of adopting a more organized structure under certain circumstances. For example, in AD and other neurodegenerative diseases, tau assembles into two regular repeating pathological structures termed paired helical and straight filaments. When so assembled, tau acquires the ability to bind thioflavine-S (54), a fluorescent dye that preferentially interacts with proteins that adopt an amyloid conformation (60). Furthermore, when bound to fatty acids, soluble tau acquires the ability to activate phospholipase C-$\gamma$ in vitro (61). This suggests that tau can adopt specific conformations in solution as well as upon polymerization.

We conclude that progressive modification and polymerization of tau proteins into filaments is preceded or accompanied by conformational changes that can be identified and quantified by a class of conformation-selective monoclonal antibody exemplified by Alz50. Currently, we are applying this approach to other anti-PHF/tau antibodies to correlate molecular structure with early events in the development of the fibrillar pathology.

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