Side Chain-dependent Stacking Modulates Tau Filament Structure*

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The misfolding of proteins into highly ordered fibrils with similar physical properties is a hallmark of many degenerative diseases. Here, we use the microtubule associated protein tau as a model system to investigate the role of amino acid side chains in the formation of such fibrils. We identify a region (positions 272–289) in the tau protein that, in the fibrillar state, either forms part of a core of parallel, in-register, β-strands, or remains unfolded. Single point mutations are sufficient to control this conformational switch with disease mutants G272V and ΔK280 (found in familial forms of dementia) inducing a folded state. Through systematic mutagenesis we derive a propensity scale for individual amino acids to form fibrils with parallel, in-register, β-strands. This scale should not only apply to tau fibrils but generally to all fibrils with same strand arrangement.

The deposition of proteins into extracellular plaques and intracellular inclusions is a key pathological hallmark of numerous degenerative diseases affecting the brain and peripheral tissues (1, 2). These deposits are comprised of amyloids and “amylloid-like” fibrils, respectively. All fibrils are made up of β-strands that run perpendicular to the fiber axis and are spaced ~4.8 Å apart from each other (3). This arrangement is referred to as a cross-β structure and can be formed in different ways, as individual β-strands can be arranged either parallel (4–12) or antiparallel (13–16) with respect to each other. Although charge, hydrophobicity and β-sheet propensity are important factors that regulate formation of all types of fibrils (17), different requirements are likely to exist for parallel and antiparallel arrangements (18). Fibrils with parallel β-strands are frequently arranged in-register and give rise to structures in which a new molecule is incorporated every 4.8 Å. Such a structural arrangement has been identified in many different fibrils, including those from amyloid-β (4–7), α-synuclein (8), tau (9), IAPP (10), and a fragment of the yeast prion protein Ure2p (11). In all of these fibrils, same residues from different molecules are in direct contact. The degree to which these contacts will be stabilizing should vary for different amino acids, but exactly how these interactions depend on side chain identity and how sequence modulates these interactions remains poorly understood. Sequence analysis does not provide any conserved motifs, as parallel, in-register structures can be formed by proteins with little or no sequence homology. Here, we examine the role of side chain interactions and protein sequence in a parallel, in-register fibril and derive an experimental scale for the propensity of different amino acids to form parallel, in-register structures.

Our studies were performed using fibrils generated from tau, a microtubule-associated protein that is mainly found in the axons and dendrites of nerve cells. Mutations in the tau gene have been shown to cause a hereditary form of dementia referred to as frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (19). Altogether, tau fibrils (typically referred to as filaments) are found in over 20 neurodegenerative diseases (20). Although originally controversial, recent evidence clearly demonstrates that tau fibrils have a cross-β structure (21, 22). The core region in these fibrils coincides with a repeat region (23, 24) that normally mediates microtubule binding (25, 26). In the large isoform of tau (441 amino acids), this region is composed of four conserved repeats (27, 28) (Fig. 1A). A particularly important role in fibril formation has been attributed to the third repeat (29, 30), which is most hydrophobic and forms fibrils on its own (31).

Using site-directed spin labeling and electron paramagnetic resonance (EPR) spectroscopy, we recently showed that 18 consecutive residues (positions 303–320, henceforth referred to as S3) in the third repeat and its N-terminal boundary were tightly packed and formed a parallel, in-register structure wherein same residues from different molecules came into contact (9). The introduction of spin label in this stretch was well tolerated and did not compromise filament stability. In the current EPR study, we set out to investigate an equivalent set of mutants (positions 272–289, henceforth referred to as S2) in the preceding second repeat and its boundary (Fig. 1A). Smaller isoforms of tau lack this repeat, yet are fully capable of forming fibrils (see for example (32). Here we find that S2 can take up two structurally very different forms in the fibril. S2 can either form a separate domain of parallel, in-register structure or remain unfolded. This marginal stability allowed us to assess the ability of different amino acids

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2 The abbreviations used are: EPR, electron paramagnetic resonance; Pipes, 1,4-piperazinediethanesulfonic acid; EM, electron microscopy.
to form parallel-stacked structures, and to evaluate the roles of amino acid composition and sequence on tau fibril structure. Our findings are likely to be important for other fibrils with parallel, in-register structures.

EXPERIMENTAL PROCEDURES

Constructs—A pET-28 vector containing the large isoform of human tau, with its two cysteines replaced by serines (9), served as a template for the construction of all single cysteine mutants (positions 272–289, positions 296–298). Mutagenesis was performed using the QuikChange protocol from Stratagene. Five of the single cysteine mutants (positions 277, 283, 284, 287, and 296) were used as templates for creating the following double mutants: I277C/V275K, I277C/I278K, I277C/K280V, D283C/V275K, D283C/I278K, D283C/K280V, L284C/V275K, L284C/I278K, L284C/K280V, L284C/G272V, L284C/ΔK280, V287C/V275K, V287C/I278K, V287C/K280V, and N296C/K280V. An additional cysteine mutant (position 306, described in Ref. 9) served as a template for the following control mutants: V306C/V275K, V306C/I278K, V306C/K280V, V306C/G272V, and V306C/ΔK280. The accuracy of all sequences was verified by DNA sequencing.

Protein Expression and Purification—Protein expression and purification were performed essentially as described previously (9). Briefly, proteins expressed in the Escherichia coli strain BL21 (DE3) were pelleted and resuspended in extraction buffer (50 mM NaCl, 20 mM Pipes, pH 6.5, 1 mM EDTA, 50 mM 2-mercaptoethanol). Frozen pellets were heated for 20 min at 80 °C and further treated by sonication. Bacterial debris was pelleted by centrifugation, and soluble tau was precipitated from supernatant with ammonium sulfate (60%, m/V). Precipitates were collected by centrifugation, and soluble tau was precipitated from supernatant with ammonium sulfate (60%, m/V). Precipitates of the single cysteine mutants (positions 272–289 in the tau filament) gave rise to three sharp lines, indicative of high mobility in the EPR spectrum. Toward this end, we used the large isoform of tau for the following experiments: I277C/V275K, I277C/I278K, I277C/K280V, D283C/V275K, D283C/I278K, D283C/K280V, L284C/V275K, L284C/I278K, L284C/K280V, L284C/G272V, L284C/ΔK280, V287C/V275K, V287C/I278K, V287C/K280V, and N296C/K280V. An additional cysteine mutant (position 306, described in Ref. 9) served as a template for the following control mutants: V306C/V275K, V306C/I278K, V306C/K280V, V306C/G272V, and V306C/ΔK280. The accuracy of all sequences was verified by DNA sequencing.

Spin Labeling and Filament Assembly—Tau pellets (~4–6 mg) were solubilized in 200 µl of 6 M guanidinium chloride, and incubated for 1–2 h at room temperature with a 10-fold molar excess of spin label [1-oxy-2,2,5,5-tetramethyl-pyrroline-3-methyl]methanethiosulfonate (Toronto Research Chemicals, Downsview, Canada) or with a 1:3 mixture of spin label and diamagnetic analog (33). The disulfide cross-linking of the spin label resulted in the side chain R1 (Fig. 1B). The properties of this side chain have been extensively characterized, and its minimal structural perturbation and high structural sensitivity are well established (34, 35). After labeling, all samples were loaded onto PD-10 columns (Amersham Biosciences), and proteins were collected as 1.5-ml fractions (elution buffer: 100 mM NaCl and 10 mM Hepes, pH 7.4). Protein concentrations were determined by absorbance measurement at 276 nm. For this purpose, protein aliquots of 100 µl were adjusted to 6 M guanidinium chloride.

Spin-labeled tau proteins (free of denaturant and excess label) were mixed with heparin (Mw ∼ 3,000, at a tau/heparin molar ratio of 4:1) and brought to a final protein concentration of 40 µM (volume, 1 ml). Samples were incubated for 8 days, stirring at room temperature. Filaments were sedimented for 30 min at 160,000 × g, and pellets were washed once with elution buffer.

EPR Measurements and Analysis—Pellets were taken up in quartz capillaries (Vitro Com Inc., New Jersey) and measured at X-band frequency in a Bruker EMX spectrometer fitted with an ER 4119HS resonator. The scan width in all experiments was 150 G. All spectra were normalized to the same amount of spins. Single line EPR spectra were generated by subtracting a simulated fast motional spectrum from the experimental composites. This fast motional spectrum was computed from the theoretical stick pattern of an electron interacting with 1 proton on the ring, 12 equivalent protons in the α-methyl groups, and 6 equivalent 13C nuclei at their natural abundance. Groups of stick spectra corresponding to the 14N hyperfine states were individually convoluted with a Lorentzian line of adjustable width (for more detail, see Ref. 36). The simulated spectrum had a shape as that of the experimentally obtained spectra for positions 401–405 in the random coil region of the filament (9). In fact, these latter spectra could be used interchangeably to generate the single line spectra, as might be expected in view of random coil structure at sites 272–289 in the tau filament. Spectra of free spin label and unpolymerized tau, however, were sufficiently different in line shape and could not be used for subtraction (for a comparison of spectra, see supplemental Fig. S1).

Negative Stain Electron Microscopy (EM)—400-mesh carbon-coated copper grids were placed for 1 min on 10–15 µl drops of tau filaments (4 µM), for 40 s on 10–15 µl drops of 2% uranyl acetate (freshly prepared), and for 5 s on 10–15 µl drops of EM grade water. The grids were air-dried on filter paper and images were taken on a JOEL JEM1200EX microscope at 80 kV.

RESULTS

Two Independent Conformational States of S2—Our previous study demonstrated that S3 forms a parallel, in-register β-sheet structure in the filament (9). As shown in Fig. 1A, S2 is similar in sequence to S3, but harbors one additional lysine residue. Because such an additional charged residue could potentially be destabilizing, we first sought to determine whether S2 was still capable of forming a parallel, in-register structure. Toward this end, we used the large isoform of tau for a nitroxide scan in which each residue in S2 (positions 272–289) was replaced with the spin label R1 (Fig. 1B), one amino acid at a time. To monitor the structural changes in S2 upon filament formation, EPR spectra were obtained for spin-labeled tau in the monomeric and filamentous states.

EPR spectra for all 18 derivatives in the soluble, monomeric state gave rise to three sharp lines, indicative of high mobility that is characteristic of a random coil structure (data not shown). These data agreed closely with our previous EPR data for S3 (9), as well as with the notion that soluble, monomeric tau is predominantly unfolded (37).
Tau filaments were grown in the presence of heparin (32, 38) under constant agitation. After 8 days, the filaments were harvested and carefully washed from any remaining unfibrillized material or free spin label (see “Experimental Procedures”). Filament formation was monitored by negative stain electron microscopy. All 18 derivatives formed wild-type-like filaments, as shown for representative examples I278R1 and K281R1 (Fig. 2A). The EPR spectra for the resulting spin-labeled tau filaments are depicted in Fig. 2B. In contrast to the single line, exchange-narrowed EPR spectra previously obtained for sites in S3 (9), the EPR spectra for sites in S2 were much more heterogeneous, varying widely in amplitude and line shape. This heterogeneity was the consequence of varying amounts of two different spectral components that were identified in all spectra. These components were resolved by the subtraction-simulation procedure described under “Experimental Procedures.” The first component is characterized by three sharp and narrowly spaced spectral lines that are of high amplitude. This component is very similar to the previously published spectra for sites outside the core region of the filament, and is clearly distinct from spectra of free spin label or unpolymerized tau (supplemental Fig. S1). Furthermore, the spectral component could not be observed in the supernatants of sedimented fibrils indicating that it cannot arise from an equilibrium of fibrils with monomers. The spectral component was particularly prominent for sites at which isoleucines or valines were substituted (I277R1, I278R1, V275R1, V287R1). In contrast, it was much less pronounced at other sites, especially when charged residues were replaced (e.g. K280R1, K281R1, and D283R1). Despite these strong site-to-site variations, all spectra indicated the presence of at least some amount of unfolded structure.

The second component was already quite apparent in some spectra (e.g. K280R1, K281R1, and D283R1), and was clearly revealed by subtracting out the sharp component from the EPR spectra in Fig. 2B (see under “Experimental Procedures”). The spectral line shapes were very similar to each other and exhibited clear evidence of exchange narrowing (Fig. 3). Such exchange narrowing is the consequence of a loss of hyperfine structure (i.e. absence of outer lines) and results in single line EPR spectra. Exchange narrowing is rarely observed in site-directed spin labeling because it requires a highly specific and
organized structure that allows a large number of spin labels to be in van-der-Waals contact at the same time (9). However, in our previous studies on tau filaments, we also observed exchange-narrowed spectra at consecutive single-labeled sites in S3. These data indicated a pronounced parallel, in-register arrangement in which same residues were stacked at a distance of 4.8 Å along the fibril axis (9). Similarly, the occurrence of exchange narrowing at consecutive sites in S2 suggested that this region, like S3, could adopt a highly ordered parallel, in-register structural state. This notion was further supported by spin dilution experiments, which showed that the exchange-narrowed component was of low mobility (supplemental Fig. S2).

Thus, analysis of the S2 spectra revealed varying amounts of two very different spectral components: one component with a highly unfolded and dynamic structure, and the other with an unusually ordered, highly stacked structure. These structures cannot be simultaneously taken up by a single tau molecule, as a given residue cannot be folded and unfolded at the same time. Rather, they require the existence of two separate structures (as shown in Fig. 4A) that are present in varying amounts.

To determine why the relative amounts of these structural states varied for the different derivatives, we performed more quantitative analyses of the respective spectral components for each site. This analysis was performed in two independent ways: (a) by plotting the experimentally observable spectral amplitudes, and (b) by quantitative analysis of the spectral subtractions described above. The rationale for the former was based on the observation that all spectra are made up of two distinctively different spectral components that vary by approximately one order of magnitude (Fig. 4A, bottom). Thus, the signal amplitude increases linearly with increasing amounts of the high amplitude mobile state. Systematic analysis revealed that the signal amplitudes strongly depended on the nature of the amino acid side chain that had been replaced, regardless of their position in sequence. Substitutions of the charged residues (aspartate and lysine) invariably resulted in very small amplitudes (Fig. 4B, left), whereas the highest amplitudes were observed when hydrophobic, β-branched residues (isoleucine and valine) were replaced (Fig. 4B, right).

The next highest amplitudes were observed for sites at which Leu was replaced by R1. Although Leu is also hydrophobic, it is not β-branched and has a lower propensity than Ile and Val for forming β-structures. Thus,
the elimination of any charged residue strongly promoted formation of the ordered, parallel state of S2, whereas the replacement of residues with high β-sheet propensity, such as Ile and Val, was destabilizing.

These conclusions were further supported by quantitative analysis of the aforementioned spectral subtractions, which gave direct estimates of the relative amounts of the ordered and disordered states. As shown in Fig. 4C, these results closely followed the trend observed in the amplitude plot and reestablished that charge substitution strongly favored the ordered state, while loss of the β-branched Ile and Val was highly destabilizing. Taken together, these data strongly suggest that the two highly different structural states of S2 are of similar energy, since single amino acid substitutions had a significant impact on the relative populations (also see below). Because the relative populations were simply dependent on the nature of the amino acids that had been replaced, the values presented in Fig. 4, B and C likely reflect an intrinsic propensity of a given amino acid to stabilize the folded state of S2.

**Mutations in S2 Have Long Range Stability Effects on S2, but Not S3—**Next, we sought to obtain independent support for the two-state nature of S2, and to test whether we could use the propensity scale depicted in Fig. 4 to predict how additional changes in amino acid composition could affect the folding of S2. We reasoned that, if the marginally stable S2 region folds up cooperatively, mutations at one site in S2 should have long range effects on other distant sites in that region. To test this notion, we developed a reporter/guest system. Reporter sites are sites at which spin labels were introduced. The EPR signal from this spin-labeled site was then used to determine how additional mutations at a guest site influenced the relative proportions of the folded and unfolded states.

We constructed a total of 15 double mutants that harbored spin labels either at selected sites in S2 (residue 283, 284, 287, or 277) or in S3 (residue 306). For each of these reporter sites, two types of additional substitutions were made at guest sites. In one set of mutants, the β-branched residues were replaced with lysine (V275K or I278K), while in another set of mutants, the charged lysine was replaced with valine (K280V).

The first row in Fig. 5 shows the results of the D283R1 reporter substitution, which, in the absence of any additional guest mutations, gave rise to a highly ordered state (Fig. 2B and Fig. 5, upper left). Interestingly, substitution of either β-branched residue with Lys caused a significant increase in the sharp lines, demonstrating that these guest site mutations strongly reduced the stability of S2 and increased the amount of the unfolded state. The K280V mutation was made to stabilize S2, but using D283R1, little additional stabilization could be detected since this reporter mutation alone was sufficient to induce the folding of S2. Next, we investigated three reporter sites that, by themselves, did not result in a highly folded state of S2 (L284R1, V287R1, and I277R1). For each of these sites, the additional V275K and I278K guest site substitutions further increased the amount of the unfolded state of S2 (albeit less pronounced for the already highly dynamic V277R1 derivative). Importantly, the K280V guest site substitution induced the formation of a stable folded structure of S2 for all of the sites tested. To test whether mutations in S2 could affect the stability of S3, we chose the β-branched residue Val306 as the reporter site for the S3 structure. As shown in the bottom row of Fig. 5, the spectra for V306R1 were invariable regardless of any guest site mutation in S2, indicating an independent parallel, in-register structure for S3 under all conditions.

Collectively, these data confirm that S3 is a stable core region of tau filaments that can fold regardless of the structural state of S2. In contrast, S2 is only a marginally stable, cooperatively folding unit. Its stability depends on the delicate balance of residues that promote the formation of stacked, parallel, in-register structures with residues that are charged or that otherwise destabilize the parallel, in-register structure.

**Linker Region—**Having established that S3 and S2 fold as independent domains, the question arose as to whether residues connecting the two stretches also take up a parallel, in-register structure or whether they represent a more flexible linker region. To address this question, we generated three additional derivatives harboring spin labels at sites between S2 and S3 (N296R1, I297R1, and K298R1). EPR spectra of filaments formed from these derivatives (Fig. 6) were distinctively different from those observed at any site in S2 or S3. None of the spectra contained the sharp lines that are indicative of a highly unfolded region; rather, the spectra contained multiple compo-
nents with mobilities ranging from moderately mobile to immobile. Although some spin-spin interaction might well be present at all three sites, no exchange narrowing could be detected, demonstrating that this region did not form a perfectly stacked, parallel, in-register structure. As a further control, we generated the N296R1/K280V double mutant to test what effect the more complete ordering of S2 had on the structure of the region between S2 and S3. This additional substitution, however, caused only minor spectral changes and failed to induce the formation of a highly stacked, parallel, in-register structure at position 296. Thus, we conclude that the region around residues 296–298 is part of a moderately flexible linker region that is structurally distinct from that of S2 or S3.

Tau Mutants Involved in Familial Forms of Frontotemporal Dementia—Mutations in the tau gene have been implicated in a number of diseases that cause frontotemporal dementia. These mutations could have multiple effects, as they may influence the splicing into different isoforms, the binding to microtubules, or the aggregation into filaments (19). Here, we investigated whether some of these mutations might have a structural effect on tau filament formation. Previous in vitro studies had demonstrated that two naturally occurring mutations located within S2, G272V, and ΔK280, promote tau aggregation (21, 39). Both mutants either introduced a β-branched residue or resulted in the loss of a charged residue in S2. According to the propensity scales in Fig. 4, these mutations would, therefore, be expected to induce folding of S2. On the other hand, the ΔK280 mutant also resulted in an amino acid deletion, which could potentially result in the loss of sequence-dependent interactions. To test the influence of these mutants on S2 folding, we used positions 284 (in S2) and 306 (in S3) as reporter sites and created a set of four double mutants: L284R1/G272V, L284R1/ΔK280, V306R1/G272V, and V306R1/ΔK280. Interestingly, filaments formed from each of these derivatives resulted in spectra with single lines and reduced amplitudes (Fig. 7, second and third columns). Considering that the spectrum of the L284R1 reporter has a significant unfolded component (Fig. 7, first column), the disease mutants therefore strongly promoted formation of the stacked, parallel, in-register form of S2. In contrast, the stacking of S3 remained unchanged. These results demonstrated that the G272V and ΔK280 mutants had a pronounced impact on fibril structure, a feature that could contribute to the pathogenesis of these mutants in frontotemporal dementias. Furthermore, these data underscore the fact that the propensity scales can be used reliably to predict the effect of mutations on the structure of S2 and, in the case of ΔK280, in the presence of an additional deletion.

DISCUSSION

The goal of the present study was to investigate the effect of side chain properties and amino acid sequence on fibril structure and stability. Toward this end, we examined the structure of S2 in the large isoform of tau (441 amino acids) and tested how its structure is affected by mutations.

We found that mutations can have a strong influence on whether S2 is in an ordered or random coil state (Fig. 4A). The ordered state of S2 was characterized by single line, exchange-narrowed EPR spectra that had previously been observed for sites in S3. Such spectra indicate the formation of extensive arrays of spin labels in orbital overlap and provide several important structural constraints. First, the close proximity of same sites in S3 and the ordered state of S2 demonstrates that these regions must be arranged in a parallel, in-register structure. Second, this parallel arrangement cannot be limited to two consecutive strands, as the resulting proximity of only two spin labels would not result in the observed exchange narrowing. Rather, the parallelism must be extensive and must include a large, indefinite number of strands that allow same sites from different molecules to stack on top of each other, as schematically illustrated by black dots in Fig. 4A, III. These constraints are rather incompatible with commonly observed β-sheet structures which contain relatively twisted strands flanked by a loop or turn region, in which case the stacking of multiple sites would be difficult. Although the precise fold is as yet undetermined, we previously noted that β-helices, which have been proposed as potential structures in other fibrils (12, 40), satisfy all currently existing constraints (9). Left-handed and right-handed β-helices allow for stacking of consecutive residues in the strand as well as in the turn regions, and would therefore be in good agreement with the EPR data. Fig. 4A arbitrarily shows an example of a left-handed β-helix. It should be noted that, if tau were to take up a β-helical structure, the structure would be one in which each layer is formed by a new tau molecule (9).

Regardless of the precise structural details, the present study clearly reveals the highly specific stacking interactions along the fibril axis, and provides a well defined structural framework for understanding the marginal stability of S2. Typically, the replacement of a native side chain by R1 is well tolerated and, in the case of the previously studied S3, the tau filament structure was not affected. In the case of the marginally stable S2, however, the introduction of R1 affected the ratio of the unfolded and folded structures. This effect was not sequence specific, but rather depended on the nature of the amino acid that was replaced by R1. Substitution of the charged lysine and aspartate residues invariably led to a predominantly folded form of S2, while substitution of the β-branched residues isoleucine and valine greatly prevented folding. Importantly, the data obtained from the guest/reporter system demonstrate that this effect does not require a specific position within the sequence. The structural effect of losing a charged or β-branched residue at one site can be compensated for by reintroducing the given type of residue at another site (Fig. 5). These observations can easily be rationalized in terms of structure. In a structure wherein same residues from different molecules are stacked on top of each other, the presence of charged residues (e.g. aspartates and
lysines) will invariably lead to charge repulsions. Although fraying of the side chains or the presence of counter charges could reduce this interaction, it is likely that the repulsion would remain destabilizing. Heparin (a polysulfonated glycosaminoglycan), which was used to induce fibril formation, could in principle act as a counter ion and thus influence the stacking of side chains. However, because the elimination of negatively and positively charged residues is equally stabilizing and heparin appears not to be incorporated into the fibril (41), the effects of heparin on side chain stacking appear to be minimal. In contrast to the charged side chains, the \( \beta \)-branched isoleucines and valines have high intrinsic \( \beta \)-sheet propensities (42–45) and can favor stacking given their ability to maximize their hydrophobic contact surface. In \( \beta \)-helices, isoleucines and valines are often found in stacks of multiple residues with nearly identical orientation of side chain atoms (see Refs. 46 and 47 for examples in right- and left-handed \( \beta \)-helices, respectively). It is likely that these interactions, together with the maximized hydrogen-bonded contact surface of the backbone, are important factors that regulate the stability of filaments. Interestingly, in \( \beta \)-helices, the side chain atoms of aligned \( \gamma \)-branched leucenes often have a different orientation (see for example Ref. 46), possibly explaining why, in our study, these residues had a smaller stacking propensity than did \( \beta \)-branched residues. Nevertheless, leucines still had higher stacking propensities than did the polar, uncharged residues, which had structural effects in between those of charged or hydrophobic residues. Collectively, these data suggest that regions with high contents of \( \beta \)-branched residues and few charges are likely to be good formers of fibrils with parallel, stacked structures.

How can we explain the relative importance of amino acid composition over that of sequence (even allowing a deletion in the case of the \( \Delta K 280 \) disease mutant), when the stability of \( \beta \)-sheets is strongly dependent on local nearest neighbor interactions (48)? A unique feature of sheets that are formed from parallel, in-register strands is that, for each residue, the immediate neighbors along the axis are always the same (Fig. 8A). This means that, regardless of where in the core region a particular residue is introduced, an important part of the molecular environment (i.e. the stack of same amino acids) will be conserved. These residues are spaced 4.8-Å apart and thus are closer than residues in the \(-2\) and \(+2\) positions, which, depending on the degree of pleating, may be 6.5–7 Å apart. Interactions between sheets, which could modulate stacking propensities (7, 49, 50), appear to be of lesser importance in this study. In fibrils where \( \beta \)-strands are arranged antiparallel along the axis, greater positional dependence for each mutation may be observed since the nearest interstrand neighbors may differ at each site (Fig. 8B). In fact, in such cases, the introduction of charged residues may not always cause destabilization. Work on soluble \( \beta \)-sheet proteins has shown that cross-strand pairs of lysine and glutamate, for example, were far more stabilizing than were pairs of alanine and glutamate or alanine and lysine (48, 51). Thus, nearest neighbor interactions are likely to result in stronger sequence dependence in fibrils with antiparallel strands than in fibrils with parallel, in-register structure.

Recent work suggests that the rate of amyloid fibril formation can be explained by factors such as hydrophobicity, \( \beta \)-sheet propensity and charge (17, 52). The stacking propensities derived in our study are in good agreement with the previously determined aggregation propensities (17, 52) (Fig. 9). While hydrophobicity, charge and \( \beta \)-sheet propensity should influence the stability of fibrils with parallel and antiparallel structure, a much larger variability should be expected for antiparallel structures (53). The remarkable predictability of the effect of mutations on aggregation propensities observed previously might have been aided by the fact that most proteins involved in that study were subsequently shown to have parallel in-register structures.
It is possible to convert the more semiquantitative scale of Fig. 4C into free energies, if one assumes a model in which the two states of S2 are in thermodynamic equilibrium at the time of measurement. Although small, time-dependent changes could be observed at some sites, the equilibrium condition was clearly met when charged or β-branched residues were replaced (data not shown). We can therefore estimate that the relative difference between replacing charged residues or valines and isoleucines is on the order of 1.2 and ~1.9 kcal/mol, respectively. Interestingly, these differences are very similar to the free energies obtained from the corresponding pair-wise interaction studies on soluble proteins (51).

In summary, the present study provides new insights into the domain organization of tau and identifies a molecular switch between two distinct structural states. This switch allows us to investigate the effect of mutations in a well-defined structural context. The ability to correlate structure and stability in tau fibrils should also prove useful for other fibrils with parallel in-register arrangement of β-strands.

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