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Thioflavine T interaction with synthetic Alzheimer's disease β-amyloid peptides: Detection of amyloid aggregation in solution

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Abstract

Thioflavine T (ThT) associates rapidly with aggregated fibrils of the synthetic β/A4-derived peptides β(1–28) and β(1–40), giving rise to a new excitation (ex) (absorption) maximum at 450 nm and enhanced emission (em) at 482 nm, as opposed to the 385 nm (ex) and 445 nm (em) of the free dye. This change is dependent on the aggregated state as monomeric or dimeric peptides do not react, and guanidine dissociation of aggregates destroys the signal. There was no effect of high salt concentrations. Binding to the β(1–40) is of lower affinity, K_d 2 μM, while it saturates with a K_d of 0.54 μM for β(1–28). Insulin fibrils converted to a β-sheet conformation fluoresce intensely with ThT. A variety of polyhydroxy, polyanionic, or polycationic materials fail to interact or impede interaction with the amyloid peptides. This fluorometric technique should allow the kinetic elucidation of the amyloid fibril assembly process as well as the testing of agents that might modulate their assembly or disassembly.

Keywords: β/A4; dye fluorescence; pH dependence

Amyloid was defined by Virchow (1855) on the basis of a blue staining reaction with iodine (starch-like) followed by treatment with acid. ThS and ThT, (structure in Fig. 1A) characteristically stain amyloid-like deposits in a number of pathophysiological conditions (Vassar and Culling, 1959; Kelényi, 1967). Along with Congo red they are believed to specifically interact in some unknown way with the crossed-β-sheet structure common to amyloid structures comprised of different materials. Naiki et al. (1989, 1990, 1991) have described the interaction of these dyes with amyloid proteins derived from serum amyloid A protein and apoAII to give enhanced fluorescence emission. The work described in this manuscript documents a similar phenomenon for interactions with aggregated species of synthetic peptides (Fig. 1B) whose sequences are derived from a protein (β/A4) isolated from deposits found in the brains of patients with Alzheimer's disease (Glenner & Wong, 1984; Masters et al., 1985). This neurodegenerative disorder is characterized by the deposition of inappropriately processed proteins in senile plaques and neurofibrillary tangles surrounded by damaged neurons (reviewed in Joachim & Selkoe, 1992). While the precise etiology of the disease does not prescribe a primary causative role for the β/A4 of the senile plaques, these deposits also contain elements of the complement cascade (McGeer et al., 1991) and could therefore act as a nidus for the immune response with concomitant neuronal injury. Agents that could delay or reverse the formation of such associations might be of therapeutic benefit. An assay system that detects the aggregated peptide but not the oligomeric or monomeric forms could identify agents that perturb the equilibrium. It would also potentially be useful for cell culture investigations or assays of tissues from animal model systems for aggregation.

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Abbreviations: ThT, thioflavine T; ThS, thioflavin S; β/A4, residues 1–42 of the Alzheimer's disease-associated peptide; K_{app}, apparent K_d; B_{max}, maximal binding (fluorescence change); ex, excitation; em, emission; OD, optical density.

Results

The histologic amyloid stains ThT (Fig. 1A) and ThS were assessed for their suitability as probes of the aggregated state of β/A4-derived synthetic peptides. When added to

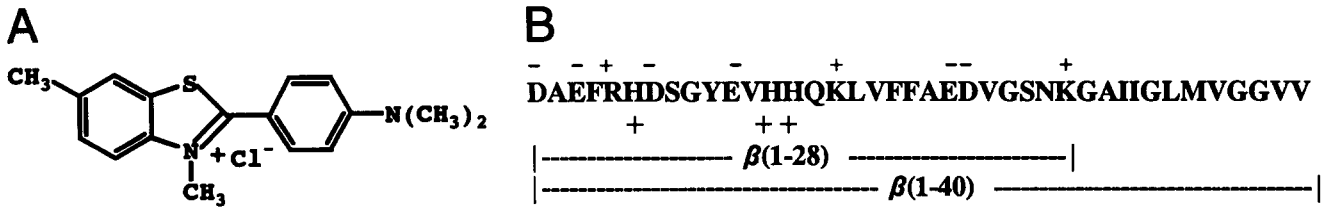

Fig. 1. Structures of synthetic β/A4 peptides and thioflavine T (ThT). **A:** ThT. **B:** β(1–28) and β(1–40) peptides. The charges indicated on the amino acid residues are those expected at pH 6.

suspensions of β(1–40) or β(1–28) (structures in Fig. 1B), previously exposed to conditions causing aggregation, pH 5, 0.5 mg/mL peptide (Burdick et al., 1992), both ThT and ThS exhibited enhanced fluorescence emission at 482 nm. No effect on dye fluorescence was noted if the peptide was diluted directly from water (pH 2) at 5 μg/mL into dye-containing buffers. ThS emission is stimulated by excitation at 385 nm, which is unchanged from that of the free dye in solution (data not shown). ThT, by contrast, undergoes a change in excitation spectrum in the presence of aggregated β(1–40) or β(1–28), with a new peak appearing at 450 nm that was not seen for the free dye (Fig. 2). These differences in absorption characteristics are most likely due to the different modes of interaction of the two dyes with the protein structure. While derived from the same general chemical structure, 2-(*p*-aminophenyl)-6-methylbenzthiazole (Venkataraman, 1952), these dyes differ in charge under the conditions of binding (pH between 5 and 9), with ThT being positively charged, and ThS, a sulfonated derivative of ThT, being negatively charged at these pHs. ThS binding may be similar to that of Congo red, a sulfonated azo dye also used to characterize amyloid structures. The explanation for the new excitation peak corresponding to a new absorption peak in the ThT dye-amyloid peptide species is not clear. The classical metachromatic shift occurring on dye stacking is a blue shift rather than the red shift observed here. ThT was selected for further investigation of its interactions with amyloid peptides. The new excitation peak of the bound dye exhibited less interference with free dye than the unshifted absorbance of ThS.

Binding of ThT to the aggregated amyloid peptides occurs within seconds, too rapidly to be quantitated in a standard fluorescence assay. Stopped-flow experiments are in progress to try to extract information about dye binding from the pre-steady-state kinetics. The fluorescence change is related to peptide concentration and is dependent upon the total dye concentration, shown in Figure 3A,B for several dye concentrations. A double reciprocal plot of the ThT concentration dependence (Fig. 3C) yields apparent K_d's for ThT fluorescence enhancement of 0.54 and 2 μM for the β(1–28) and β(1–40) peptides, respectively. The B_{max}'s are similar for equal weights of peptide although the difference in their molecular weight would predict a β(1–28)/β(1–40) intensity ratio of 0.75 on a molar basis for equifluorescent numbers of ThT bound per molecule of peptide. The number of dye molecules bound per mole of peptide is not known.

Formation of the fluorescent ThT-peptide complex requires aggregate formation

Several pieces of information indicate that the fluorescence enhancement of ThT depends on the structure of

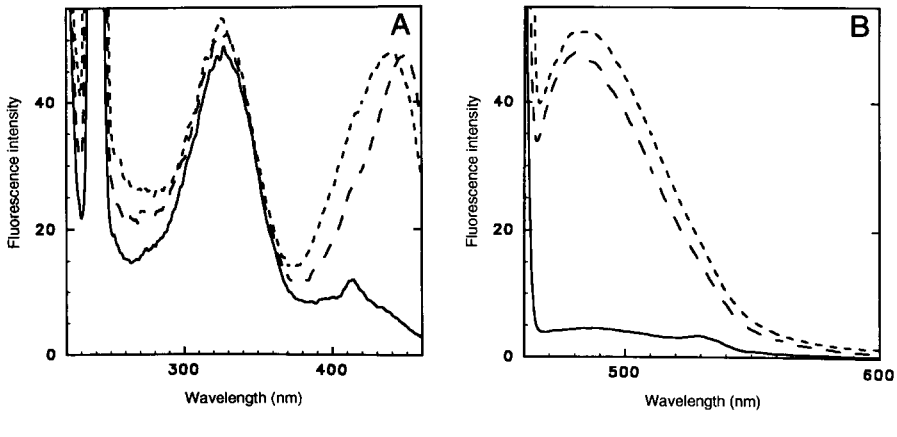

Fig. 2. Fluorescence spectra of ThT in the presence and in the absence of aggregated β(1–28) and β(1–40) amyloid peptides. **A:** Excitation spectra: 3 μM ThT in 50 mM potassium phosphate buffer, pH 6.0, in the absence (solid line) of peptide, and in the presence of equifluorescent quantities of preaggregated β(1–28), 5 μg/mL (short dashed line), or β(1–40), 10 μg/mL (long dashed line) peptide. Excitation bandpass = 5 nm; λ_{em} = 482 nm, bandpass = 10 nm. There was no contribution noticeable from the peptide alone. **B:** Emission spectra recorded under the same conditions as the excitation spectra with the λ_{ex} fixed at 450 nm, bandpass of 5 nm.

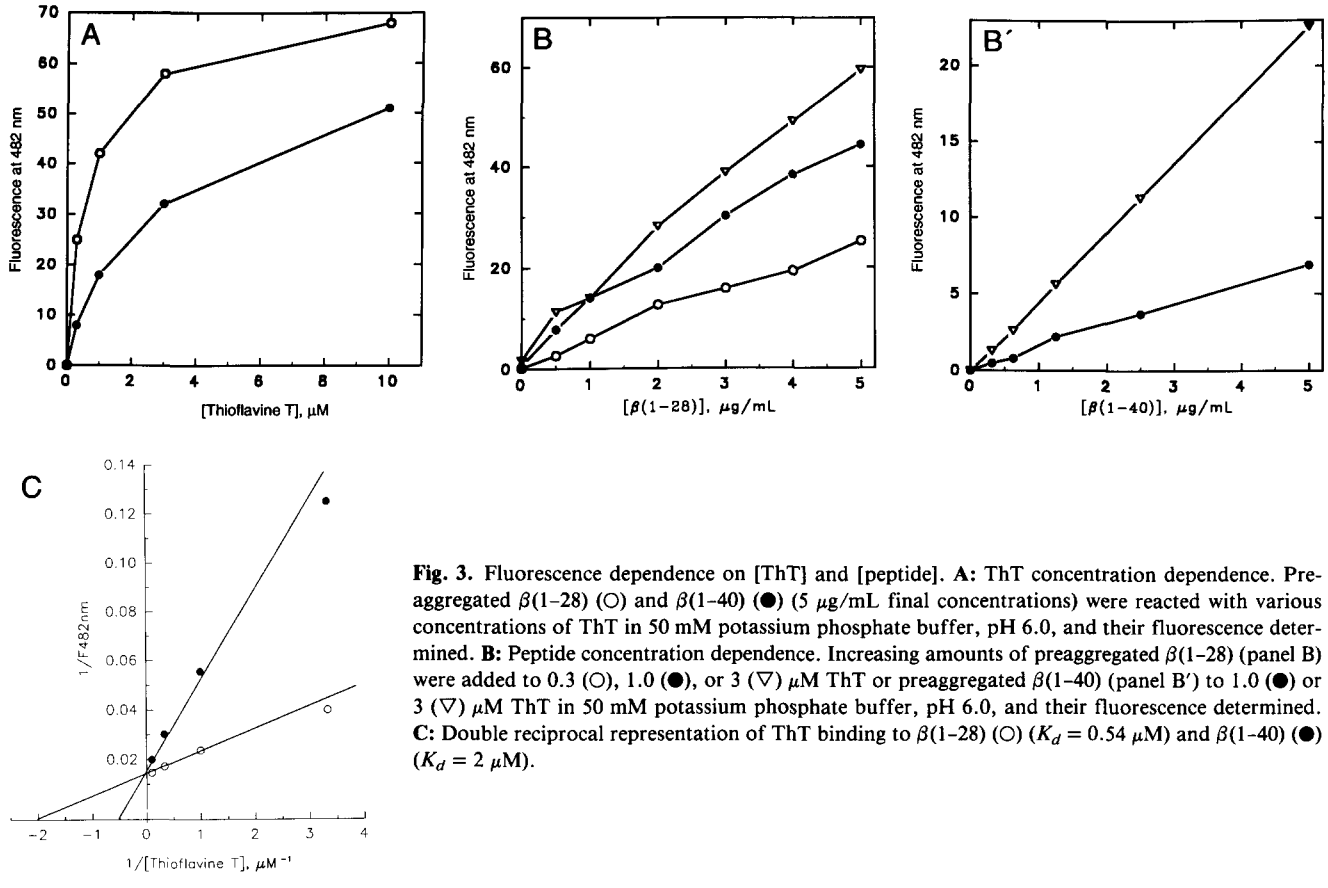

Fig. 3. Fluorescence dependence on [ThT] and [peptide]. **A:** ThT concentration dependence. Preaggregated β(1-28) (○) and β(1-40) (●) (5 μg/mL final concentrations) were reacted with various concentrations of ThT in 50 mM potassium phosphate buffer, pH 6.0, and their fluorescence determined. **B:** Peptide concentration dependence. Increasing amounts of preaggregated β(1-28) (panel B) were added to 0.3 (○), 1.0 (●), or 3 (▽) μM ThT or preaggregated β(1-40) (panel B') to 1.0 (●) or 3 (▽) μM ThT in 50 mM potassium phosphate buffer, pH 6.0, and their fluorescence determined. **C:** Double reciprocal representation of ThT binding to β(1-28) (○) (K_d = 0.54 μM) and β(1-40) (●) (K_d = 2 μM).

the aggregated state of the amyloid peptides. A number of studies (Halverson et al., 1990; Fraser et al., 1991; Burdick et al., 1992) have correlated the sedimentability and electron microscopic appearance of amyloid fibrils for several synthetic β/A4 peptides. A more limited series of experiments confirms the sedimentation characteristics for β(1-28) and extends them to β(1-40). Because ThT did not appear to interact with the peptides at pH 5 or below (data not shown), β/A4 peptides were pretreated at 0.5 mg/mL with various pH buffers, and fluorescence was subsequently measured by rapidly diluting aliquots to 5 μg/mL in 25 mM sodium phosphate containing 10 μM ThT at pH 6. Figure 4 illustrates that ThT fluorescence primarily correlates with the formation of sedimentable aggregates of both peptides. β(1-28) required centrifugation at 100,000 × *g* in order to obtain consistent results between experiments. Higher *g* forces did not affect the results for β(1-40). Monomers or dimers, chromatographically separated by sizing on Superose 12 (OD 214 nm, solid line, Fig. 5), are not sedimentable under the conditions used. When diluted directly into ThT-containing solutions at pH 6 (hatched bars, Fig. 5), at peptide concentrations sufficient to produce a signal with aggregated material, these species yield no fluorescence change (open circles, Fig. 5). Thus, the ThT is most probably interacting with an oligomeric species. In saying this, it should be noted that there is a population of material for both β(1-28) and β(1-40) produced at pH 4, which fluoresces in dilute solution in the presence of ThT at pH 6, which is poorly sedimentable. The quaternary structure of this material remains to be characterized.

It is noteworthy that the pH dependence for fluorescence enhancement of ThT differs from that observed by Naiki et al. (1989) who find that ThT fluorescence for murine senile amyloid protein is low at pH 6 and peaks between pH 9 and 10. Thus, the fluorescence change seen for ThT binding to β(1-28) and β(1-40) reflects a protein structural change and not a change in the fluorescent properties of the dye or dye-dye interactions in solution. Exposure to pH 4-6 at a peptide concentration of 0.5 mg/mL (preaggregation) is required for a fluorescence change to occur. The concentration dependence for aggregation of β(1-28) and β(1-40) as reported by fluorescence of ThT and by sedimentability are similar, ≥0.5 mg/mL, and are consistent with that reported for similar peptides (Burdick et al., 1992) (data not shown). Further evidence comes from an experiment, detailed in Figure 6, wherein aggregated β(1-40) treated with 8 M guanidine-hydrochloride to disaggregate the peptide reduces the expected fluorescence enhancement to near

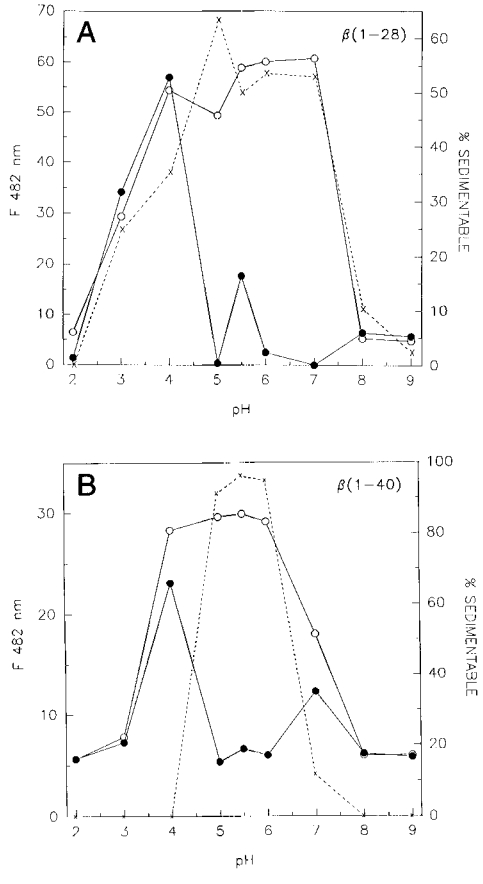

Fig. 4. ThT fluorescence follows the pH stability of the aggregated state of β(1-40). **A:** β(1-28) peptide was aggregated in 25 mM buffers ranging in pH from 2 through 9 at 0.5 mg/mL for 6 h. The amount of peptide present was measured with fluorescamine and the ThT fluorescence at pH 6 determined on aliquots before and on the supernatant after centrifugation at 16,000 × *g* at room temperature for 15 min. ○, ThT fluorescence before centrifugation; ●, ThT fluorescence after centrifugation; ×—×, % of peptide sedimentable at 16,000 × *g*. **B:** β(1-40) peptide was aggregated under the same conditions as for the β(1-40) peptide except that the centrifugation was performed at 100,000 × *g*. The symbols are the same as for panel A.

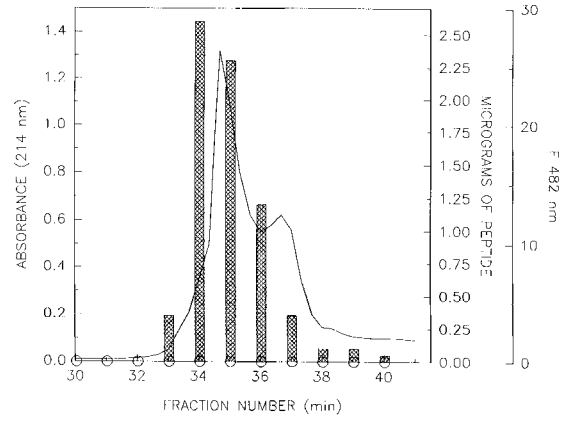

Fig. 5. Monomeric and dimeric β(1-28) do not induce ThT fluorescence. β(1-28) dissolved in water was chromatographed on a Superose 12 column equilibrated with 0.5 M NaF and the effluent monitored at 214 nm. Solid line, OD 214 nm; hatched bars, amount of peptide measured by fluorescamine added from fraction to ThT assay; ○, ThT fluorescence induced by the indicated amount of unaggregated peptide.

control level. Nonchaotropic salts such as NaF (0.5 M), sodium phosphate (0.5 M), or NaCl (2 M) had no effect on ThT binding to the two preaggregated peptides. The mild chaotrope, KCl, was equally ineffective at up to 2 M. This again is in distinction to inhibition of ThT binding to murine senile amyloid fibrils by salt, which is in that case related to fibril elongation (Naiki et al., 1991).

Stability of the fluorescent aggregate

The stability of the ThT fluorescence also depends on pH in a fashion similar to sedimentability for the different peptides. The β(1-40) amyloid-ThT fluorescent aggregate in dilute solution containing ThT is relatively stable as reflected by a very slow fluorescence change at pH >7 (data not shown). The β(1-28) peptide, by contrast, demonstrates a distinct time-dependent loss of fluorescent signal shown in Figure 7. A similar differential stability was previously observed by Burdick et al. (1992) for β(1-42) and β(1-28). The half-time of the initial rate of fluorescence loss for β(1-28) decreases with increasing pH, with the reaction becoming biphasic above pH 6. It is unclear whether this reflects a decrease in the number of dye molecules bound or a change in fluorescence yield of the bound dye molecules. The basis for this instability will require further study.

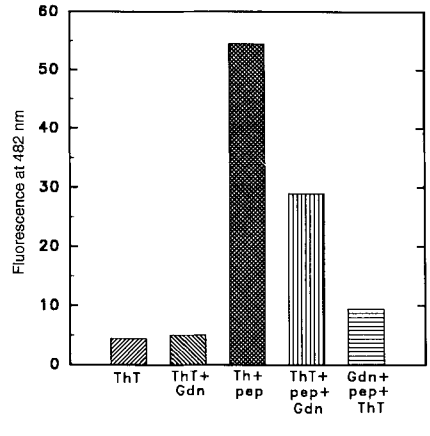

Fig. 6. Requirement for β(1-40) aggregation for ThT fluorescence. β(1-40) (0.5 mg/mL) was aggregated at pH 5 in 0.1 M acetate buffer in the presence or absence of 8 M guanidine and the diluted to 5 μg/mL (100-fold) into 1 mL of 50 mM potassium phosphate buffer, pH 6.0, containing 3 μM ThT. Controls included the addition of the same concentration of guanidine-HCl (80 mM final) to ThT alone and adding 5 μg/mL aggregated β(1-40) to 3 μM ThT + 80 mM guanidine-HCl.

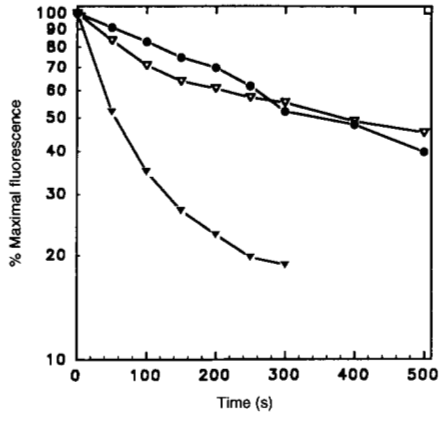

Fig. 7. Time course of ThT/β(1–28) complex fluorescence decrease as a function of pH. Preaggregated β(1–28) (0.5 mg/mL) was diluted to 5 μg/mL into potassium phosphate buffers of different pH containing 3 μM ThT. The fluorescence was monitored as a function of time. ●, pH 6.0; ▽, pH 7.0; ▼, pH 8.0.

Specificity of the ThT fluorescence enhancement

The fluorescence enhancement of ThT by aggregated amyloid peptides is quite specific. Dye fluorescence in the presence or absence of aggregated β(1–40) or β(1–28) is unaffected by high concentrations (100 μg/mL, μM concentrations) of a number of molecules mimicking histologic amyloidophilic dye-binding sites. These include sulfated polysaccharides such as heparin (4–6 kDa), heparin sulfate (7,500 kDa), dextran sulfate; polyols such as polyethylene glycol (15–20 kDa) and Ficoll 400; polycationic polymers and peptides such as polyethyleneimine and substance P; and a protein known to bind small drug molecules in hydrophobic pockets, bovine serum albumin (data not shown). Table 1 shows that turkey ovalbumin induced a slight fluorescence enhancement of the dye (2.3-fold by comparison with 22-fold enhancement with β(1–28) and 5.3-fold for β(1–40)). The ovalbumin concentration used represents $\frac{1}{20}$ of the mass of peptide (one-half the molar amount).

Table 1. *Thioflavine T fluorescence with β-sheet-containing proteins and peptides*

Additive	Fluorescence (482 nm)	-Fold
25 mM potassium phosphate, pH 6.0	0	—
3 μM ThT	3	1.0
3 μM ThT + 100 μg/mL bovine albumin	3.6	1.2
3 μM ThT + 100 μg/mL turkey ovalbumin	6.8	2.3
3 μM ThT + 5 μg/mL β(1–40)	16	5.3
3 μM ThT + 5 μg/mL β(1–28)	65	22
3 μM ThT + 5 μg/mL β-sheet insulin	172	57

A common structural motif in amyloid-like proteins is the β-pleated sheet. Amyloidophilic dyes such as Congo red, ThS, and ThT are thought to interact with this structural element in some as yet undefined way. By heating in acid, insulin can be induced to assume a β-sheet conformation (Burke & Rougevie, 1972), which binds Congo red with the characteristic amyloid-like green birefringence (Klunk et al., 1989). ThT likewise interacts with insulin β-sheet fibrils (Table 1), displaying fluorescent properties indistinguishable from the β/A4 peptides (data not shown). It is likely that both the β-sheet structure and the aggregated state provide the environment to stabilize the long-wavelength ThT fluorescent complex, regardless of the identity of the participating peptides. Recently, new technology has provided evidence that amyloidogenic peptides may be characterized by unusual structures partially, but not wholly accounted for by those seen in crystalline or oriented samples (Lansbury, 1992).

Discussion

The thiazine dyes ThS and ThT have been used for many years as histologic agents for the detection of amyloid protein deposits (Vassar & Culling, 1959; Kelényi, 1967). Their peculiar changes in optical properties upon association with the β-pleated sheet structures formed in common by the amyloids are shared with the direct cotton azo dye Congo red and its congeners. This interaction is not a function of their normal tinctorial properties, as similar dyes in a chemical series often do not selectively associate with amyloid. The direct cotton dyes that are thought to interact with the multiple cellulose hydroxyls do not, in general, stain amyloid selectively.

From the experiments with β(1–28) and β(1–40) it is probable that ThT is not associating with the hydrophobic portion of the amyloid peptide, residues 29–40. Similar fluorescence of the dye (similar B_{max}) is observed for the aggregated forms of the two peptides, although the apparent K_m's differ for formation of the fluorescent ThT species. The hydrophobic residues (29–40) could contribute to the apparent stability of the fibrils at pH >5, as suggested by the results of Burdick et al. (1992) and here by the lack of a fluorescence decrease for β(1–40) with time in contrast to the slow fluorescence decrease observed for β(1–28) (Fig. 7). They may also contribute to the kinetics of the fibril formation, perhaps in a nucleation event, but this remains to be demonstrated.

Tomski and Murphy (1992) have studied the kinetics of synthetic β-amyloid peptide aggregation by classical and quasielastic light scattering. They derive a model for β/A4 aggregation and filament assembly, which admits interpretation of the data obtained with ThT. The pH and ionic strength dependencies of the reaction monitored by their method are indistinguishable from those determined by other means. Unlike other techniques, light scattering is sensitive to the sizes of the macromolecular species

formed in a reaction. The mechanism of assembly suggested by the kinetics is consistent with literature data. In this model, soluble β(1–40) exists as dimers in dilute solution and at pH <3 (Hilbich et al., 1991). At higher pHs and concentrations it is interpreted by Tomski and Murphy (1992) to rapidly form tetramers of dimers (octamers), which can be thought of as protofilaments. These octamers add relatively slowly to protofilament ends yielding long thin 5-nm filaments, which can associate laterally to form ribbons. All of these structures are observed in electron micrographs of naturally occurring amyloid fibrils and are similar or slightly smaller than synthetic amyloid fibrils (Kirschner et al., 1987; Fraser et al., 1991; Hilbich et al., 1991). The kinetics of the peptide aggregation process monitored by the light scattering technique suggest that in such a reaction scheme ThT would probably bind to an oligomeric form such as the octamer since the fluorescence enhancement is observed within seconds after aggregation is initiated, and it is not observed to be effected by the subsequent slower addition of octamers at filament ends. This would be distinct from the mode of binding of this dye to murine senile amyloid fibrils formed from apoAII, a component of serum high density lipoprotein, in a pathogenic mouse amyloidosis (Naiki et al., 1991). In that system the fluorescence of ThT increases as the filaments add at the ends. The physical basis for the fluorescence changes is unknown for either amyloid system. The development of reporter molecules selective for the individual macromolecular states of amyloid assemblies would provide the basis to discover agents that would interfere with initiation and extension as well as agents that could promote dissolution of preformed fibrils.

Materials and methods

ThT (C.I.49010; Basic Yellow 1), ThS (C.I.49005; Direct Yellow 7) were from Aldrich. Fraction V bovine serum albumin, heparin (4–6 kDa molecular weight), type VII heparin sulfate (molecular weight 7,500, fast electrophoretic fraction), type III glycogen, turkey ovalbumin, bovine insulin, polyethylene glycol (15–20 kDa), polyethyleneimine, dextran sulfate, and fluorescamine were obtained from Sigma. Guanidine-HCl was ultraPure grade from BRL/GIBCO. Synthetic β/A4 amyloid peptides were obtained from commercial sources β(1–40) (BACHEM) (two lots), β(1–28) (Multiple Peptide Systems, San Diego, California). Purity was evaluated by C-18 reverse-phase high performance liquid chromatography, and identity by amino acid composition and mass spectroscopic (molecular ion) analysis. They were stored as lyophilized trifluoroacetate salts in powder form at −20 °C under nitrogen. In agreement with previous work (Kelényi, 1967), ThT showed a major yellow fluorescent spot with a very minor slowly migrating blue component upon silica gel thin-layer chromatography in the three different solvent systems of Kelényi (1967). Peptide concentrations were measured by fluorescamine in 0.15 M sodium phosphate, pH 8.5 (Böhlen et al., 1973). Under these conditions no difference in reactivity was noted between aggregated and unaggregated peptides.

In accord with the observations of Burdick et al. (1992), the peptides dissolved readily in deionized water as the trifluoroacetate salts at concentrations exceeding 5 mg/mL, yielding a solution pH of 2. Aggregation was induced by raising the pH to 5.0 in 0.1 M sodium acetate buffer at a peptide concentration of 0.5 mg/mL. Dye binding assays were performed at 5 μg/mL peptide, diluted from the 0.5-mg/mL stock into 1 mL of buffer of a chosen pH, pH 6 for routine measurements, containing the dye in a cuvette followed by inversion of the Parafilm-sealed cuvette several times to mix the solution. No visible aggregates were observed. Fluorescence measurements were made in semimicro quartz cuvettes (Hellmas) with a 1-cm excitation light path containing 1 mL (or 0.5 mL for fluorescamine) of unstirred solution using a Shimadzu RF-5000U fluorimeter equipped with a 150-W xenon light source. Temperature was maintained at 25 °C by a thermostatted cell holder. Measurements with ThT utilized λ_{ex} = 450 nm, 5 nm bandpass; λ_{em} = 482 nm, 10 nm bandpass. These settings minimized light scattering contributions to the observed signal.

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