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Fluorescence Anisotropy: A Method for Early Detection of Alzheimer β-Peptide (Aβ) Aggregation

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Time-resolved anisotropy measurements (TRAMS) have been used to study the aggregation of the β-amyloid (Aβ) peptide which is suspected of playing a central role in the pathogenesis of Alzheimer's Disease (AD). The experiments, which employ small quantities of fluorescently-labelled Aβ, in addition to the untagged peptide, have shown that the sensitive TRAMS technique detects the presence of preformed "seed" particles in freshly prepared solutions of Aβ. More importantly, as 100 μM solutions of Aβ containing tagged Aβ at a concentration level of either 0.5 or 1 μM are incubated, the TRAMS prove capable of detection of the peptide aggregation process through the appearance of a continuously increasing "residual anisotropy" within the time-resolved fluorescence data. The method detects Aβ aggregation in its earliest stages, well before complexation becomes apparent in more conventional methods such as the thioflavin T fluorescence assay. The TRAMS approach promises to provide a most attractive route for establishment of a high-throughput procedure for the early detection of the presence of amyloid aggregates in the screening of biological samples.

Key Words: **Alzheimer's disease; amyloid; Aβ; fluorescein; time-resolved fluorescence anisotropy.**

Alzheimer's disease (AD), which leads to dementia and eventual death, is characterized by the presence of neurofibrillary tangles and senile plaques in the brain. The latter lesions are composed of amyloid fibrils surrounded by dystrophic and dying neurons. The principal component of the highly insoluble amyloid fibrils is β-amyloid (Aβ), a 39–43 residue peptide derived from proteolytic processing of the larger amyloid precursor protein. Accumulating evidence supports the hypothesis that Aβ fibrillogenesis is a seminal pathogenic event in AD (for reviews see (1, 2)). One of the supporting pieces of evidence for this "amyloid hypothesis" is the fact that Aβ is toxic to neurons, both *in vivo* and *in vitro* (2). The development of neurotoxicity appears to be connected with the formation of aggregates of Aβ, but the precise molecular organization of the neurotoxic form of Aβ and the mechanism of toxicity have not been defined, although the generation of hydrogen peroxide may play a role in toxicity (3, 4). Recent attention has focussed on the suggestion that small, nonfibrillar oligomers or "protofibrils" may represent a particularly toxic species (5–7). Inhibiting or reversing the early stages of Aβ oligomerization is thus seen an a possible therapeutic strategy for AD, and developing sensitive methods for the detection of early oligomeric forms of Aβ is an important objective. Various techniques have been employed to monitor aggregation of Aβ and other fibril-forming proteins and peptides (8–11), but most of these methods lack the sensitivity required to detect aggregation in its very early stages. Fluorescence methods provide both sensitivity and selectivity, and have a distinct advantage over other techniques in that it should prove possible to detect the binding of a fluorescently-labelled protein to a preformed, nontagged protein aggregate, which could have important diagnostic implications (12). In this paper, we elaborate upon our earlier preliminary reports (13, 14) that time-resolved anisotropy measurements (TRAMS) can detect Aβ aggregation in its earliest stages and represent, arguably, one of the most attractive routes for establishing high-throughput amyloid aggregation assays.

Abbreviations used: AD, Alzheimer's disease; Aβ, β-amyloid peptide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; TRAMS, time-resolved anisotropy measurements; FRET, fluorescence resonance energy t.

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MATERIALS AND METHODS

Peptides. The nonlabelled Aβ 1-40 peptide was synthesized on a Milligen 9050 peptide synthesizer starting from Val-PEG-PS resins and using Fmoc N-protection, as described previously (15). Synthetic

0006-291X/01 $35.00

Aβ 1-40 fluo-peptide, bearing a fluorescein tag, was obtained from New England Nuclear Life Science Products (Boston, MA).

Fluorescence spectroscopy and anisotropy measurements. The fluo-peptide was initially dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in order to remove any preformed aggregates, and stored frozen at −80°C in small working aliquots. These were thawed immediately before use, and the HFIP was evaporated by centrifugation under vacuum. The fluo-peptide was then dissolved in a very small volume of 0.1 M acetic acid, before the addition of 100 mM Tris buffer, pH 7.4, to give a final concentration of 1–2 μM fluo-peptide (nonaggregation control). In the aggregation experiments, an equal volume of nonlabelled Aβ 1-40, freshly dissolved in distilled water, was added to the nonaggregation control to give a mixture which was either 0.5 or 1 μM with respect to fluo-peptide and 100 μM in non-labelled peptide, in 50 mM Tris buffer, pH 7.4. Time-resolved anisotropy measurements were taken after incubation of the control or aggregating peptides for various time periods at 37°C. Fluorescence spectra were obtained using a Perkin Elmer LS50 spectrometer. Time-resolved anisotropy measurements (TRAMS) were carried out on a specially modified Edinburgh Instruments 199 time-correlated single photon counter. The excitation source used was an IBH nanoLED 05 light emitting diode (LED) with a repetition rate of 1 MHz and a FWHM of 1.1 ns. Vertically polarized light was used to excite the sample and fluorescence intensities transmitted by a polarizer were analyzed in planes parallel, $I_{\parallel}(t)$, and perpendicular, $I_{\perp}(t)$, to that of the polarized excitation. A "toggling procedure" was employed in the collection of $I_{\parallel}(t)$ and $I_{\perp}(t)$ wherein the orientation of the analyzer was sequentially altered while memory quarters in the multichannel analyzer were switched simultaneously.

Negative stain electron microscopy (EM). One microliter samples of nonlabelled Aβ 1-40 (100 μM in 50 mM Tris buffer, pH 7.4) were removed after incubation at 37°C, mixed with 4 μl of 2 mM octylglucoside and 5 μl of 1% uranyl acetate, applied to formvar/carbon coated grids, and left for 10 min. Excess fluid was blotted off, and the grid was air dried before examination in a Jeol JEM-1010 electron microscope.

Thioflavin T assays. Samples (15 μl) of nonlabelled Aβ 1-40 (100 μM in 50 mM Tris buffer, pH 7.4) were removed after incubation at 37°C and added to 2 ml of 10 μM thioflavin T in 50 mM Glycine/NaOH, pH 9.0. Duplicate samples were scanned three times in a Perkin Elmer LS50 fluorescence spectrometer before and immediately after the addition of peptide (16). Results are for excitation at 450 nm and emission at 482 nm.

RESULTS AND DISCUSSION

Fluorescence anisotropy can be used to determine the rate at which a fluorescent species tumbles in solution. Since the rate of reorientation of a particle is a function of its size, fluorescence anisotropy measurements, particularly time-resolved experiments, offer a very sensitive and informative means of interrogating interactions between molecules.

A time-resolved anisotropy experiment uses pulses of polarized light for excitation of the fluorescent sample. The probability of absorption of this radiation by a molecule depends upon the orientation of its transition vector with respect to the plane of polarization of the incident excitation. Consequently, molecules are photoselected according to the extents to which their absorption axes are aligned parallel to the plane of polarization at the moment of incidence. The resultant

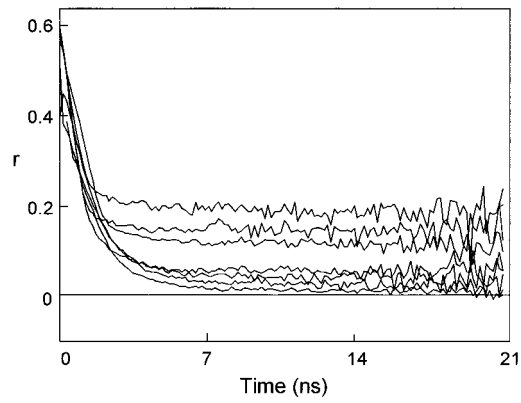

FIG. 1. Anisotropy decay of Aβ 1-40 (100 μM normal peptide + 1 μM fluo-peptide) following incubation for various times at 37°C. The traces in order of increased '*r*' values after anisotropy decay are due to −1 μM fluo-peptide only (*r* decays rapidly to zero) and then 1 μM fluo-peptide plus 100 μM normal peptide after incubation for 0, 1, 24, 72, 96, and 120 h. (Note: These are "raw" anisotropy decay curves and contain some "contamination" from scattered light).

fluorescence is polarized, its degree of anisotropy reflecting, at a given time after excitation, the extent to which molecular randomization has occurred through microBrownian motion. The rate of rotational motion of the fluorescent species can be quantified, through the decay of emission anisotropy, $r(t)$, in terms of a correlation time τ_c. For a spherical rotor, the anisotropy decay may be expressed as

$$r(t) = r_o \exp(-t/\tau_c), \qquad [1]$$

where r_o is the intrinsic anisotropy, a characteristic property of a given fluorophore. The larger the volume of the particle (and its associated solvent sheath), the longer it takes to reorient in solution, and the greater the value of its τ_c.

A commercially available, fluorescently labelled form of synthetic Aβ(1–40) was used in our anisotropy experiments. This Aβ variant bears a fluorescein marker attached to a cysteine residue substituted at position 7 of the amino acid sequence. The fluo-peptide was treated with 1,1,1,3,3,3,-hexafluoro-2-propanol (HFIP) to disperse any pre-formed aggregates (17). Figure 1 shows that the anisotropy of the fluorescence from a dilute solution (2 μM) of fluo-peptide on its own, immediately after solubilization (Tris buffer, pH 7.4), decayed rapidly to zero ($\tau_c \sim 1$ ns), indicating that the fluo-peptide molecules tumble rapidly in solution at 37°C. Incubation of this fluo-peptide solution for periods of up to 6 days resulted in no significant change in the anisotropy decay, indicating that no detectable self-association or aggregation of the fluo-peptide occurs under these conditions. This is to be expected because the threshhold concentration for nucleation-dependent aggregation of Aβ 1-40 is in the range 10–40 μM (18),

and pretreatment of Aβ with HFIP significantly retards the aggregation process (17). Glabe and coworkers (19) using analogous fluorescent Aβ variants and fluorescence resonance energy transfer, FRET, have concluded that under conditions similar to those used here, the peptide exists as stable dimers. Indeed, using the equation

$$\tau_c = \frac{\eta V}{RT}, \qquad [2]$$

(where η is solvent viscosity, V is molar volume, R is gas constant, and T is absolute temperature) we estimate that the rotor (if assumed to be a spherical entity) has a radius of the order of 1.5 nm, which is reasonable for a dimeric form of the peptide, and is consistent with the hydrodynamic radius of 1.8 $\pm$ 0.2 nm for dimeric Aβ calculated from quasielastic light-scattering data (20).

On addition of unlabelled Aβ (1–40) to freshly dissolved fluo-peptide (to give a solution of 100 μM unlabelled peptide and 1 μM fluo-peptide), we observed that the anisotropy, r, no longer attained zero within the time range over which the fluorescence could be detected (Fig. 1). These changes in the appearance of the anisotropy decays are similar to those which we have observed upon mixing varying amounts of 2 intercomplexing, synthetic macromolecules {poly(acrylic acid) and poly(dimethylacrylamide)} one being fluorescently tagged (21, 22). In the latter case, the changes in the anisotropy decay profiles reflected the inhibition, as aggregation occurred, of the motion of the chain segments of the two water-soluble polymers, each of which exists in the form of a flexible coil, in dilute aqueous solution. The complex formed, however has a very rigid structure, characterized by a very long rotational correlation time, in solution, which is presumably that for molecular reorientation of the entire species (21, 22). In the case of the peptide mixture, it is clear that, as soon as mixing is effected, some of the fluo-peptide becomes incorporated into a larger, more slowly relaxing species. It would appear that a fraction of the fluo-peptide immediately complexes with small amounts of preformed aggregate (or seed) present in the 100 μM solution of unlabelled peptide. These aggregates must be large enough for the emission from the bound fraction of the fluo-peptide to suffer no depolarization during the time (ca. 30 ns) for which fluorescence is observable, their presence being characterized by the appearance of a "residual anisotropy" in the time-resolved decay profiles. Samples of normal peptide taken at "time zero" for negative stain electron microscopy (EM) (see Fig. 2a) revealed the presence of globular structures 8–24 nm in diameter, and slightly larger ellipsoids with dimensions of 16–28 by 20–32 nm (similar to those described by Nybo *et al.* (23)). Rotation of particles of these dimensions would be characterized by τ_c values greatly in excess of the fluorescein's excited state lifetime. Consequently, either (or both) of these species could represent the preformed seed present in the unlabelled peptide to which the fluo-peptide immediately becomes attached.

Upon incubation of the mixture of labelled plus unlabelled peptide, the contribution from the residual anisotropy, r_∞, to the overall anisotropy decay profile increased progressively with time (Fig. 1). After 1–7 h incubation, the peptide was seen by EM (Figs. 2b and 2c) to assemble into larger chain-like groups, which became fibrillar in structure, and displayed elements resembling so-called "protofibrils" (6, 7, 20) and eventually (after >24 h) "mature" amyloid fibrils (Fig. 2d). However, the increases in r_∞ were apparent even within the first 30 min of incubation. In contrast, conventional thioflavin T fluorescence assays (16) on separate samples of 100 μM nonlabelled Aβ 1-40, incubated under similar buffer and temperature conditions, proved incapable of detecting peptide aggregation at times of incubation which were less than 7 h. Thioflavin T is a reagent, the fluorescence from which is enhanced when it adsorbs on to a hydrophobic surface (16). It is commonly used to detect the presence of aggregates formed from amyloidogenic peptides. As noted above, significant changes in thioflavin T fluorescence above background levels ($P < 0.05$) occurred only after 7 h incubation of the peptide (Fig. 3) in trials for comparison with the TRAMS experiments.

The time-resolved anisotropy of the fluorescence observed from the fluo-peptide was found to be well described by a function of the form,

$$r(t) = r_1 \exp(-t/\tau_{c_1}) + r_\infty. \qquad [3]$$

In this expression, τ_{c_1} is the correlation time for Aβ rotational relaxation and $r_o = r_1 + r_\infty$. Use of this model function resulted in the consistent recovery of a component, τ_{c_1}, of the order of 0.7–1.0 ns. In addition, the value of r_∞ increased with the incubation time of the system until the latter approached ca. 2000 h, by which time abundant fibrillar structures could be detected by EM. Thereafter, the relative contribution of r_∞ to the overall anisotropy decay of $r(t)$ decreased, presumably because, at this stage, the aggregated peptide rapidly fell out of suspension.

The lifetime, τ_f, of the excited state of the fluorescein label (ca. 4 ns) was unaffected by the formation of complexes with the unlabelled peptide throughout the course of the incubation experiments. In addition, replacement of the fluo-peptide by an equivalent concentration of free fluorescein did not result in the development of a measurable anisotropy in the fluorophore's emission in an incubating mixture over a 48 h period. Thus, the effects produced by addition of the unlabelled peptide to solutions of its fluo-peptide analogue are not

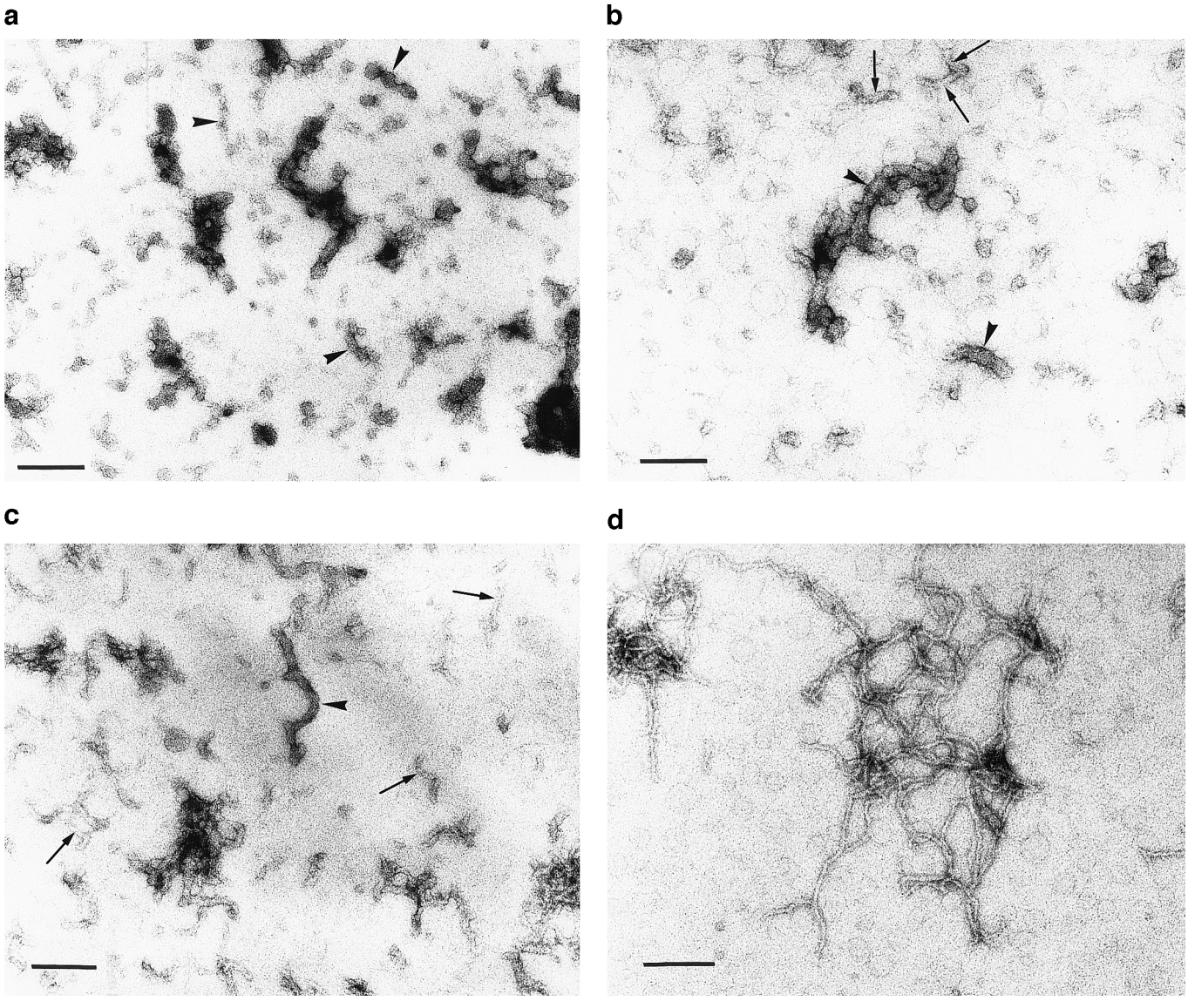

FIG. 2. Aβ 1-40 aggregates observed by negative stain electron microscopy. A 100 μM solution of peptide was incubated at 37°C for various time periods. (a) Time 0 h. Small globular or ellipsoid structures are seen to from groups or chains (arrowheads). (b) Time 1 h. The structures take on a more linear form (arrowheads). A few protofilaments are observed (arrows). (c) Time 3 h. Linear structures (arrowhead) appear more fibrillar, and greater numbers of protofilaments (arrows) are seen. (d) Time 24 h. Numerous amyloid fibrils are seen, having an approximate diameter of 8 nm. Prefibrillar structures and protofilaments are less evident. Scale bar 100 nm.

driven by any tendency of the fluorescein dye itself to interact with the Aβ peptide aggregates.

Under the incubation conditions adopted here, only two types of fluorescent species are detectable in the anisotropy data; the "uncomplexed" Aβ dimer and large aggregates. In general, for a system containing two species of differing dimension but having a common fluorescence lifetime, τ_f, the resultant emission anisotropy would exhibit a dual exponential decay law, i.e.,

$$R(t) = r_1\exp(-t/\tau_{c_1}) + r_2\exp(-t/\tau_{c_2}). \qquad [4]$$

If, as in the current case, one of the species is so large that $\tau_{c_2} \gg \tau_f$, the second term appears to be constant, at r_2 ($= r_\infty$). For diagnostic purposes this is a bonus: aggregation is detectable, at the very earliest times, through the appearance and growth of this residual anisotropy. For studies of the kinetics and mechanism of peptide oligomerization and assembly, on the other hand, use of a much longer-lived luminescent marker would be advantageous, since this would allow the rotational relaxation of the large aggregates to be quantified. The present observations show that nucleation of a growth centre is followed by rapid peptide

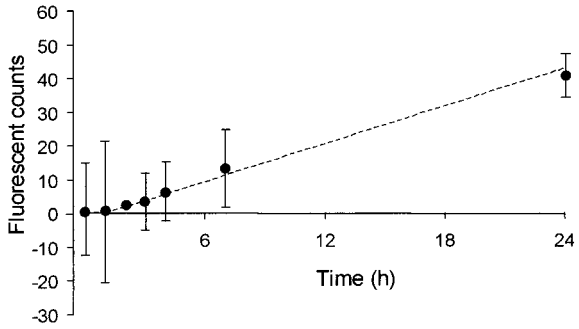

FIG. 3. Aβ 1-40 aggregation as monitored by a standard thioflavin T fluorometric assay. Graph shows the mean effect of peptide addition (from incubation of 100 μM peptide at 37°C) to thioflavin T, with 95% confidence limits, at each time point.

complexation to form aggregates of significant size (at least 25 nm across), which coexist with Aβ dimers in the incubating mixture.

Fluorescence anisotropy has clear advantages over other techniques for detecting and monitoring the early stages of amyloid oligomerization. Past studies on the kinetics of Aβ aggregation have utilized techniques such as turbidity, thioflavin T binding, sedimentation, Congo red binding, and HPLC (8–11, 16, 18). These methods provide information on the appearance of high molecular weight aggregates, or the disappearance of soluble peptide, but early intermediates in the oligomerization process cannot be detected. Quasielastic light scattering spectroscopy (QLS) has been used to monitor the various phases of Aβ aggregation (20, 24), but cannot be used to study early intermediates of oligomerization of Aβ when they are in equilibrium with high molecular weight forms. Recently, size-exclusion gel filtration has been also used to study Aβ oligomerization (6, 7, 20). However, this method detects only the formation of a high molecular weight gel-excluded fraction, shown to contain protofibrils, but no early intermediates. The anisotropy method can detect oligomerization in its very early stages, and, furthermore, the specificity of the fluorescent tagging should allow the detection of preformed aggregates in biological samples. In this respect, the potential of the approach has already been demonstrated by analysis of samples of cerebrospinal fluid from patients with Alzheimer's disease by means of fluorescence correlation spectroscopy (12). The principal advantage of fluorescence anisotropy over fluorescence correlation spectroscopy is that the former method should be much simpler to adapt for high-throughput analysis. This is also where the fluorescence anisotropy approach scores over FRET, because a high-throughput drug screening or diagnostic assay based on fluorescence anisotropy would use smaller amounts of fluorescently-tagged peptide so that the assay would be cheaper to run.

Elucidation of the molecular events that initiate oligomerization, is crucial to understanding the pathology and developing potential therapeutics for AD. This study provides a new strategy for detecting the early stages of oligomerization, and for studying the molecular mechanisms, kinetics and inhibition of this process.

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