THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 20, Issue of May 14, pp. 21038–21045, 2004

Printed in U.S.A.

Non-fibrillar Components of Amyloid Deposits Mediate the Self-association and Tangling of Amyloid Fibrils*

Received for publication, December 22, 2003, and in revised form, February 18, 2004
Published, JBC Papers in Press, March 18, 2004, DOI 10.1074/jbc.M314008200

Christopher A. MacRaild‡, Cameron R. Stewart‡, Yee-Foong Mok‡, Menachem J. Gunzburg‡, Matthew A. Perugini‡, Lynne J. Lawrence§, Viyada Tirtaatmadja¶, Justin J. Cooper-White¶, and Geoffrey J. Howlett‡‖

From the ‡Russell Grimwade School of Biochemistry and Molecular Biology, and ¶Particulate Fluids Processing Centre, Department of Chemical and Biomolecular Engineering, University of Melbourne, Victoria 3010, Australia and §Commonwealth Scientific and Industrial Research Organization, Parkville, Victoria 3052, Australia

Amyloid deposits are proteinaceous extra-cellular aggregates associated with a diverse range of disease states. These deposits are composed predominantly of amyloid fibrils, the unbranched, β-sheet rich structures that result from the misfolding and subsequent aggregation of many proteins. In addition, amyloid deposits contain a number of non-fibrillar components that interact with amyloid fibrils and are incorporated into the deposits in their native folded state. The influence of a number of the non-fibrillar components in amyloid-related diseases is well established; however, the mechanisms underlying these effects are poorly understood. Here we describe the effect of two of the most important non-fibrillar components, serum amyloid P component and apolipoprotein E, upon the solution behavior of amyloid fibrils in an *in vitro* model system. Using analytical ultracentrifugation, electron microscopy, and rheological measurements, we demonstrate that these non-fibrillar components cause soluble fibrils to condense into localized fibrillar aggregates with a greatly enhanced local density of fibril entanglements. These results suggest a possible mechanism for the observed role of non-fibrillar components as mediators of amyloid deposition and deposit stability.

The self-association of proteins into amyloid fibrils and the further aggregation of these fibrils into insoluble deposits is implicated in a diverse range of diseases, including Alzheimer's and other neurodegenerative diseases (1), type 2 diabetes (2), and a number of systemic amyloidoses (3). Over twenty proteins are known to form amyloid fibrils *in vivo*, and the deposits formed by each of these proteins are associated with a distinct disease state (4). Despite the lack of any similarity in primary sequence or native structure among these precursors, the underlying structures and histological properties of the deposits are remarkably similar. The bulk of the amyloid deposit is the amyloid fibrils themselves: linear unbranched assemblies of the specific precursor protein with a core cross-β structure (5). Deposits also contain a number of non-fibrillar components, the identity of which is essentially independent of the precursor protein comprising the amyloid fibrils. These non-fibrillar components include the protein serum amyloid P component (SAP),[1] apolipoprotein E (apoE), as well as other proteins, glycosaminoglycans, and proteoglycans.

The ubiquity and specificity of the non-fibrillar components of amyloid deposits is such that radiolabeled SAP is used clinically as a quantitative tracer of amyloid deposits (3). Furthermore, findings that SAP stabilizes *in vitro* amyloid deposits from phagocytic and proteolytic degradation has prompted the design of inhibitors of the SAP-amyloid interaction. One such molecule is under clinical trial against systemic amyloidosis (6). Despite this growing clinical interest in the effects of non-fibrillar components of amyloid deposits, structural details of their interactions with amyloid fibrils and deposits are entirely lacking, as are details of the mechanism by which they protect amyloid deposits from degradation.

In vitro studies of the mechanism of amyloid fibril formation by a number of proteins have shown it to be a complex process, often involving a number of intermediate species and occasional off-pathway aggregates. Although the relationship between this process and the pathogenic mechanisms of the amyloid diseases are unclear and somewhat controversial, the distinct biological properties of the different assembly states of amyloid fibrils are likely to be significant. For example, several unrelated proteins, including some that are not known to form amyloid *in vivo*, are cytotoxic as pre- or proto-fibrillar aggregates early in the amyloid formation process but not as mature fibrils (7–10). These findings have been taken as evidence that all amyloid fibrils share a common cytotoxic mechanism (7), and much recent research into the etiology of the neurodegenerative amyloidoses has focused upon this mechanism as an important factor. In the case of the systemic amyloidoses, however, the major cause seems to be direct impairment of organ function by the large amounts of insoluble material deposited (11).

We have recently developed methodology for sedimentation velocity analysis of amyloid fibrils and demonstrated the utility of this approach as a probe of higher-level association of the fibrils (12). Here we extend that work to consider the effect of non-fibrillar components of amyloid deposits upon the association state of amyloid fibrils. As a model system for this investigation, we have chosen the fibrils formed by human apolipoprotein C-II (apoC-II). Apolipoproteins are over-represented among proteins known to form amyloid *in vivo* (13), and apoC-II has been proposed as a good model for their behavior

* This work was supported by the Australian Research Council and the National Health and Medical Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Russell Grimwade School of Biochemistry and Molecular Biology, University of Melbourne, Victoria 3010, Australia. Tel.: 613-8344-7632; Fax: 613-9347-7730; E-mail: ghowlett@unimelb.edu.au.

[1] The abbreviations used are: SAP, serum amyloid P component; apoE, apolipoprotein E; apoE3/4, apolipoprotein E3/4; apoC-II, apolipoprotein C-II.

(14). Recent evidence implicates apoC-II amyloid fibrils in the activation of macrophages during the development of atherosclerosis (15). Furthermore, apoC-II fibrils display a simple and homogenous morphology that has been characterized in detail, and which, importantly, is not complicated by the presence of appreciable amounts of non-fibrillar aggregates (14). This allows the hydrodynamic properties of apoC-II fibrils to be interpreted in terms of their association state (12). By exploiting this property, we demonstrate an increase in fibril entanglement mediated by the non-fibrillar components apoE and SAP. The capacity of these proteins to modulate the association state of amyloid fibrils may explain their ubiquity in amyloid deposits and provide insight into the mechanisms of fibril deposition *in vivo*.

MATERIALS AND METHODS

Materials—ApoC-II was expressed (16) and purified (14) as described previously. The purified protein ran as a single band on SDS-PAGE. The purified protein stock was stored at a concentration of 40 mg/ml in 6.5 M guanidine hydrochloride, 10 mM Tris, pH 8.0 at 4 °C.

SAP was purified from human plasma (kindly provided by the Australian Red Cross Blood Service) by affinity chromatography on single-stranded calf thymus DNA-agarose (Amersham Biosciences). 5 ml of DNA-agarose was washed thoroughly with 10 mM Tris, 1 M NaCl, 25 mM Ca^{2+}, pH 7.4, and then with TNC buffer (10 mM Tris, 150 mM NaCl, 25 mM Ca^{2+}, pH 7.4). The washed resin was resuspended in 5 ml of TNC buffer and then mixed with 250 ml of serum and stirred at room temperature for 1 h. The resin was removed by filtration, poured into a glass chromatography column, and washed with TNC buffer until a steady baseline $A_{280\ nm}$ was attained. SAP was eluted from the resin with 10 mM Tris, 150 mM NaCl, 10 mM EDTA, pH 7.4, and then dialysed against this buffer and concentrated to ~1 mg/ml by ultrafiltration. Purity of the preparation was confirmed by SDS-PAGE. Human ApoE3 and E4 isoforms were expressed and purified as described previously (17).

Amyloid Fibril Preparation—ApoC-II was prepared by direct dilution from the stock into the experimental buffer (10 mM Tris, 150 mM NaCl, pH 7.4, with 10 mM EDTA or 2 mM Ca^{2+}, as appropriate) to a final concentration of between 0.2–1 mg/ml apoC-II. ApoC-II freshly prepared in this manner consists almost entirely of monomers (14). Amyloid fibrils were allowed to form by incubation of freshly prepared apoC-II at room temperature for 3–7 days, depending upon the apoC-II concentration.

Sedimentation Velocity Analysis—Samples were analyzed using an XL-A analytical ultracentrifuge (Beckman Coulter, Fullerton, CA) equipped with an AnTi60 rotor at 20 °C. Protein samples (300–400 μl) were added to double-sector epon-filled centerpieces, with refolding buffer in the reference compartment. Radial absorbance data were acquired at wavelengths of 280 or 292 nm, radial increments of 0.002 cm in continuous scanning mode, and rotor speeds of between 4,000–10,000 rpm. Scans were taken at 5- or 10-min intervals. The sedimenting boundaries were fitted to a model describing the sedimentation of a distribution of sedimentation coefficients with no diffusion (ls-g*(s)) using SEDFIT (18). Data were fitted using a Tikhonov-Phillips regularization parameter of $p = 0.95$, and 200 sedimentation coefficient increments in the range of 20–3000 S, depending upon the sample. Weight average sedimentation coefficients were calculated from the resulting ls-g*(s) distributions using the algorithm included for this purpose in SEDFIT (19). To correct for residual monomeric apoC-II or free non-fibrillar components, which sediment negligibly at the rotor speeds used, we fitted a time-independent background contribution to the overall radial absorbance.

Bead Modeling and Determination of Sedimentation Coefficients—The procedure for constructing bead models of apoC-II amyloid fibrils has been described in detail previously (12). Briefly, the fibrils are treated as worm-like chains of n linear segments, each of length l and zero mass. Spherical beads of radius $l/2$ and mass m are positioned at the vertices of the chain, where l and m are selected such that the average volume per unit length and density of the model match that of hydrated apoC-II fibrils. The orientation of each chain segment with respect to the previous one is randomly determined within a normal distribution, such that the persistence length of the chain is 36 nm, matching that measured for apoC-II fibrils.

Protein bound to the apoC-II fibrils was treated in the following way: additional beads were added to the model at regular distances along the fibril, displaced from the fibril axis by a distance l. For computational reasons, these beads must also have radius $l/2$ and mass m,[2] corresponding to a total mass of ~180 kDa, ~50% more than the mass of the SAP pentamer.

In this manner, ensembles of bead models were constructed at each of 7 lengths (44 nm, 87 nm, 170 nm, 440 nm, 870 nm, 1.7 μm, and 5.2 μm), and additional beads representing bound protein were added at regular intervals to give added mass fractions of 0.02, 0.1, 0.5, 1, and 2. The added mass fraction is given by the total mass of the additional beads divided by the mass of the fibril prior to the addition of the additional beads. A total of 100 bead models were constructed for each length/added mass combination, and the sedimentation coefficient of each model was calculated using HYDRO (20). As in our previous method, fibril flexibility was treated by averaging the sedimentation coefficient over each ensemble of models of identical composition but randomly varied conformation (12).

Electron Microscopy—Copper grids (400 mesh) were coated with thin carbon film and glow-discharged in nitrogen. Samples of apoC-II fibrils, with or without non-fibrillar components, were applied to the grid and allowed to equilibrate for 30 s. The samples were washed twice with refolding buffer followed by staining with 3–4 drops of 2% potassium phosphotungstate at pH 6.0–7.0. The grids were air-dried and examined using a JEOL 2000FX transmission electron microscope (Brooksvale, NSW, Australia) operating at 120 kV. Micrographs were recorded at nominal magnifications of ×40,000.

Rheology—Aliquots of 1.0 mg/ml apoC-II amyloid fibrils were incubated at room temperature with 0.3 mg/ml SAP and 2 mM Ca^{2+} for 24 h before measurement. To obtain accurate viscoelastic profiles, solvent viscosities were raised prior to measurement by the addition of 40% (w/w) glycerol. The addition of glycerol caused no change in the observed sedimentation behavior of the system after correction for the change in solvent viscosity, confirming that glycerol does not affect the structure or interactions of the fibrils. Samples were then mixed by gentle inversion 10 times, placed on a slowly rocking platform for 30 min, and allowed to stand for 15 min. This procedure was adopted to avoid more vigorous agitation, which had been observed to disrupt the underlying gel-like structure of the fibril solution. Measurements were made at 20 °C using an ARES rheometer (Rheometric Scientific, Piscataway, NJ) using a cone-and-plate geometry with a 50-mm plate diameter and a 42-μm gap. Dynamic Strain sweeps were performed to elucidate the linear viscoelastic region, and dynamic frequency tests were done within this region with 2 or 5% constant strain. Steady-shear rate tests were performed with a 10-s measurement delay and 30-s measurement time to ensure that steady state was achieved.

RESULTS

Our initial investigations focused on the sedimentation behavior of 0.3 mg/ml apoC-II fibrils in the presence of varying concentrations of non-fibrillar components of amyloid deposits (Figs. 1 and 2). In all cases, the fibrils were observed to sediment significantly faster in the presence of non-fibrillar components at concentrations between 0.05–0.3 mg/ml. The most striking effect was seen in the presence of 0.3 mg/ml SAP, in which the majority of the sample optical density had reached the bottom of the cell within a 5-min centrifugation at 3000 rpm (data not shown). It is not possible to determine a sedimentation coefficient distribution for such rapidly sedimenting material; however, the observed behavior implies maximum apparent sedimentation coefficients greater than 10^4 S. In contrast, SAP at the lower concentration of 0.1 mg/ml causes a more modest increase in sedimentation coefficient, with the weight average sedimentation coefficient increasing from 120 S for the apoC-II fibrils alone to 171 S in the presence of SAP. When apoE3 is added to the fibrils at a concentration of 0.025 mg/ml, an increase in average sedimentation coefficient of slightly smaller magnitude is observed. This effect is seen to increase systematically with apoE concentration over the range 0.025–0.075 mg/ml (Fig. 2) to a maximum weight average sedimentation coefficient of 220 S at 0.075 mg/ml apoE. The effect of apoE4 on the sedimentation coefficient of apoC-II fibrils is indistinguishable from that of apoE3.

[2] O. Byron, personal communication.

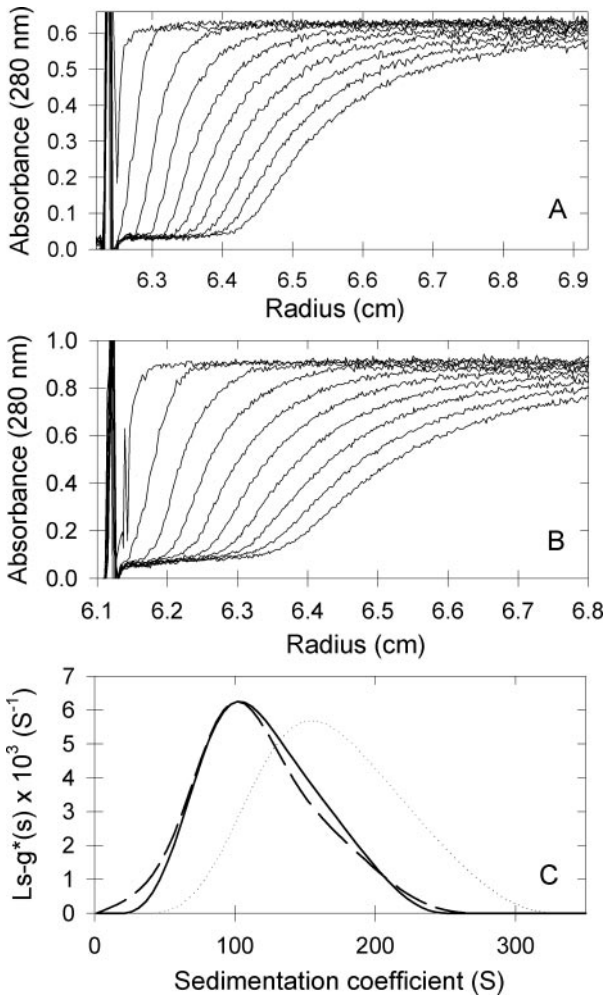

FIG. 1. Sedimentation velocity of apoC-II amyloid fibrils (0.3 mg/ml) in the absence (*A*) and presence (*B*) of 0.1 mg/ml SAP. Rotor speed was 8000 rpm, with radial absorbance scans collected at 10-min intervals. For clarity, every second scan was omitted from the figure. *C*, ls-g*(s) sedimentation coefficient distribution for apoC-II fibrils (0.3 mg/ml) alone (*solid line*), in the presence of 0.1 mg/ml SAP and 10 mM EDTA (*dashed line*), and in the presence of 0.1 mg/ml SAP and 2 mM Ca^{2+} (*dotted line*).

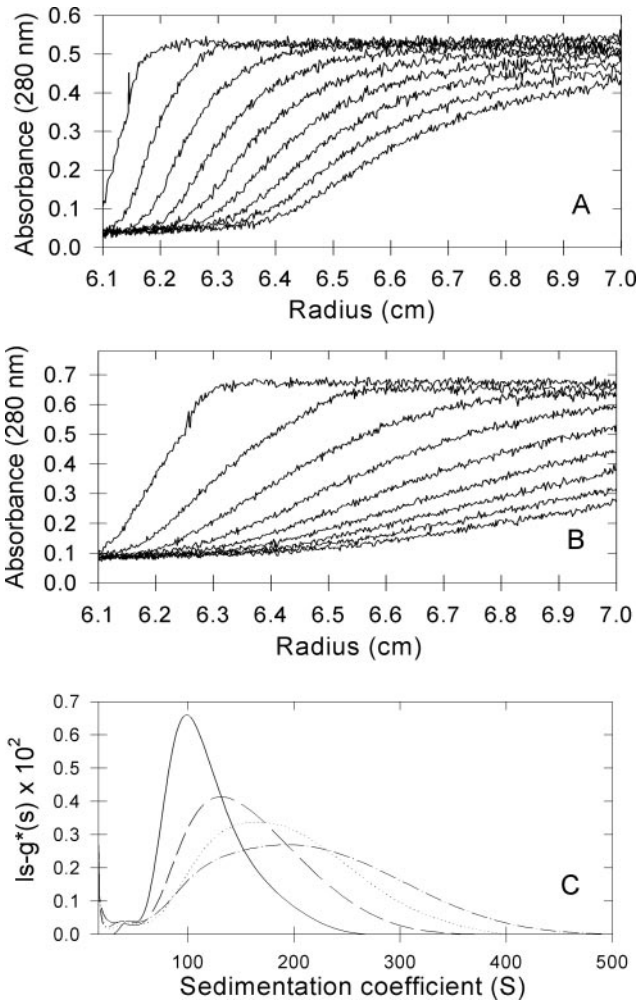

FIG. 2. Sedimentation velocity of apoC-II amyloid fibrils (0.3 mg/ml) in the absence (*A*) and presence (*B*) of 0.075 mg/ml ApoE3. Rotor speed was 8000 rpm, with radial absorbance scans collected at 10-min intervals. For clarity, every second scan was omitted from the figure. *C*, ls-g*(s) sedimentation coefficient of apoC-II fibrils in the absence (*solid line*), and the presence of apoE3 at 0.025 mg/ml (*dashed line*), 0.05 mg/ml (*dotted line*), and 0.075 mg/ml (*dash-dot line*).

Two lines of evidence point to the specificity of these effects: (*i*) SAP has no effect on the sedimentation behavior of apoC-II fibrils in the absence of calcium (Fig. 1*C*), and the effect in the presence of calcium is entirely reversible with the addition of an excess of EDTA; and (*ii*) serum albumin, a protein found at high concentrations in serum but not in amyloid deposits, does not elicit an effect on the sedimentation behavior of apoC-II fibrils under similar conditions and at concentrations up to 0.14 mg/ml.[3]

Under the rotor speeds selected for these experiments, neither monomeric apoC-II nor free non-fibrillar components sediment appreciably. On the other hand, non-fibrillar components that are bound to the apoC-II fibrils will sediment with the fibrils. Thus, if the amount of non-sedimenting material is determined in the presence and absence of non-fibrillar component, the difference between these two values will yield an estimate of the amount of free non-fibrillar component in each sample. In this way, we estimate the amount of non-fibrillar component bound to the fibrils. At an SAP concentration of 0.1 mg/ml, 0.27 g of SAP is bound per gram of fibril, corresponding to an approximate stoichiometry of one SAP pentamer per 50 apoC-II monomers. ApoE at 0.075 mg/ml binds at a density of 0.15 g of apoE per g of apoC-II, corresponding to a stoichiometry of one apoE monomer per 40 apoC-II molecules.

To determine whether the resulting increase in sedimentation coefficient can be accounted for in terms of the increased mass of the fibrils due to binding of non-fibrillar components, we have modified our previous methodology for the analysis of sedimentation coefficients of flexible fibrils (12). Bead models of the apoC-II fibrils are constructed as before, with the addition of extra mass and volume corresponding to the bound non-fibrillar component. This additional mass is added to the model fibril as discrete spherical units positioned regularly along the fibril. This distribution of additional mass is expected to model the frictional effects of the binding of globular proteins at discrete positions along the fibril. We have also predicted the change in sedimentation coefficient associated with an increase in fibril mass using an approximate analytical solution for the frictional properties of worm-like chains (21). This model treats the additional mass as uniformly distributed by increasing the radius of the cylindrical fibril and, as such, gives the maximum possible change in sedimentation coefficient for a given mass increase, because uniform distribution minimizes the frictional contribution of the additional volume.

The results of both of these approaches give qualitatively

[3] C. L. L. Pham, G. J. Howlett, unpublished data.

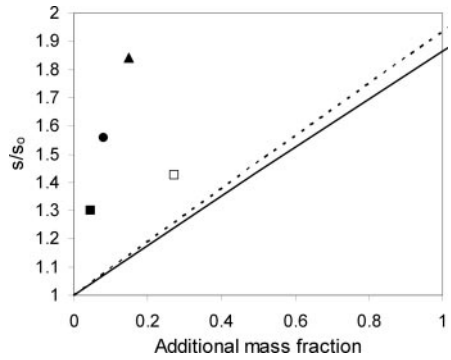

FIG. 3. **Changes in sedimentation coefficient caused by binding of non-fibrillar components to apoC-II amyloid fibrils.** The change in sedimentation coefficient (s/s_o) is plotted against the mass fraction of bound non-fibrillar component for apoE3 (*black symbols*), SAP (*white symbol*), the bead model (*solid line*), and the analytical model of Yamakawa and Fujii (21) (*dashed line*).

similar pictures of the changes to the sedimentation coefficient because of the binding of inert mass to apoC-II fibrils. In each case, the change in sedimentation coefficient, expressed as the ratio of the sedimentation coefficient of the fibril with additional mass bound to the sedimentation coefficient in the absence of additional mass (s/s_o), is found to be approximately linear with respect to the amount of mass bound for fibrils of fixed length. For fibrils with a fixed amount of additional mass (as a fraction of initial fibril mass), s/s_o is essentially invariant with fibril length for fibrils longer than 500 nm.

The results from these models are compared with the experimental data in Fig. 3. The experimental results are plotted as s/s_o *versus* the mass fraction of additional protein bound to the fibrils, with s and s_o values calculated as weight averages from the sedimentation coefficient distributions in Figs. 1*C* and 2*C*, and the mass of bound protein determined from the non-sedimenting optical density, as described above. Similarly, s/s_o is plotted against the mass of additional protein bound to the fibrils for the two model approaches at a fibril length of 870 nm. It is clear from Fig. 3 that the observed changes in sedimentation coefficient are not consistent with our models, which are based upon a simple increase in fibril mass and volume because of the binding of non-fibrillar components. This is evident not only from the fact that the observed changes are much larger than predicted by any of the models; it is also suggested from the dramatic concentration dependence observed for the SAP-induced effect, by which SAP at 0.3 mg/ml induces increases in the sedimentation coefficient of at least two orders of magnitude. This behavior is in clear contrast to the predictions of our models, in which the relationship between s/s_o and mass bound is approximately linear and has a maximal gradient of 1. As such, the observed behavior suggests that the effect of SAP on the sedimentation behavior of apoC-II fibrils is due to higher order interactions between SAP and the fibrils rather than simple one-to-one binding. Although the effect of apoE at the concentrations studied is less dramatic than the effect of SAP at 0.3 mg/ml, the apoE-induced changes in sedimentation coefficient exceed those predicted theoretically by more than a factor of five. Thus, it is likely that both SAP and apoE promote higher order interactions between apoC-II fibrils.

To explore the structural basis for the observed changes in sedimentation coefficient and thus to shed light on the interaction of non-fibrillar components of amyloid deposits, we examined apoC-II fibrils in the presence and absence of SAP (Fig. 4) and apoE (Fig. 5) by electron microscopy. In the absence of non-fibrillar components, apoC-II fibrils can be seen as the long, twisted ribbons we have characterized in detail elsewhere (14, 22), distributed in a uniform and apparently random fashion over the electron microscopy grid. In the presence of SAP and calcium, the fibrous morphology of the apoC-II remains unchanged, and small ring-like structures can be seen bound along the fibrils. Identical structures are seen when solutions of SAP alone are imaged under identical conditions, and the shape and size of these structures is consistent with that of the SAP pentamer (23). The distribution of the fibrils in the presence of SAP and calcium is markedly different from that in the absence of SAP, however, with the SAP-bound fibrils displaying a less uniform, more "clumped" distribution. Similarly, the presence of apoE has no effect on the fibrillar morphology of apoC-II, but instead it results in the apparent clumping or bundling of the fibrils.

This observation raises the possibility that SAP and apoE change the conformation or flexibility of the fibrils, and that these changes might have an impact on the observed sedimentation behavior. To address this question, we calculated sedimentation coefficients from the analytical approximation of Yamakawa and Fujii (21) for fibrils of varying persistence length. This revealed that changes to the persistence length by a factor of two produced corresponding changes in the sedimentation coefficient of less than 25%. Thus, any small change in persistence length that may be caused by SAP is not likely to contribute greatly to the observed change in sedimentation coefficient.

To relate this observation to the solution properties and interactions of the fibrils, we performed oscillatory rheological measurements on solutions of apoC-II fibrils in the absence and presence of SAP. These measurements yielded the viscoelastic spectra shown in Fig. 6. In the absence of SAP, apoC-II fibrils behave as a very weak physical gel, with both the storage modulus, G′, and the loss modulus, G″, showing only a weak dependence upon frequency over the range measured and with the value of G′ exceeding that of G″ over the whole range of frequencies explored. Such behavior is consistent with a model of the fibril solution as a network of interacting fibrils that spans the entire solution volume. As indicated by the large value of G′, this transient network is able to efficiently store the applied stress through conformational deformations that subsequently relax. The frequency at which G′ and G″ are equal in magnitude is a measure of the timescale of this mechanical stress relaxation in the sample. In the case of apoC-II fibrils alone, this value lies outside of the experimentally accessible frequency range, suggesting relaxation times of at least several seconds. The viscoelastic response of apoC-II fibrils in the presence of SAP is markedly different to that of the fibrils alone, with both G′ and G″ showing a decrease of more than an order of magnitude and significantly increased frequency dependence. Furthermore, the crossover of the loss and storage curves is shifted to higher frequencies ($\sim 30\ s^{-1}$), indicating relaxation times of the order of 30 ms. The increased rate of mechanical stress relaxation is an indication of a more localized network structure as compared with the extensive, gel-like network formed in the absence of SAP. The value of the loss modulus, G″, now exceeds that of G′ over much of the experimental frequency range, indicating that the applied mechanical stress is not efficiently stored but rather is dissipated through frictional interactions with the solvent.

To further probe this effect, we have measured the steady-shear viscosity of apoC-II fibril solutions over a range of strain rates in the presence and absence of SAP (Fig. 7). The degree to which the solution viscosity decreases with increasing strain rate (the degree of shear thinning) is a measure of the susceptibility of the solution to mechanical shear. In the absence of SAP, apoC-II fibrils are highly shear-thinning, with the viscosity dropping almost an order of magnitude over two decades of

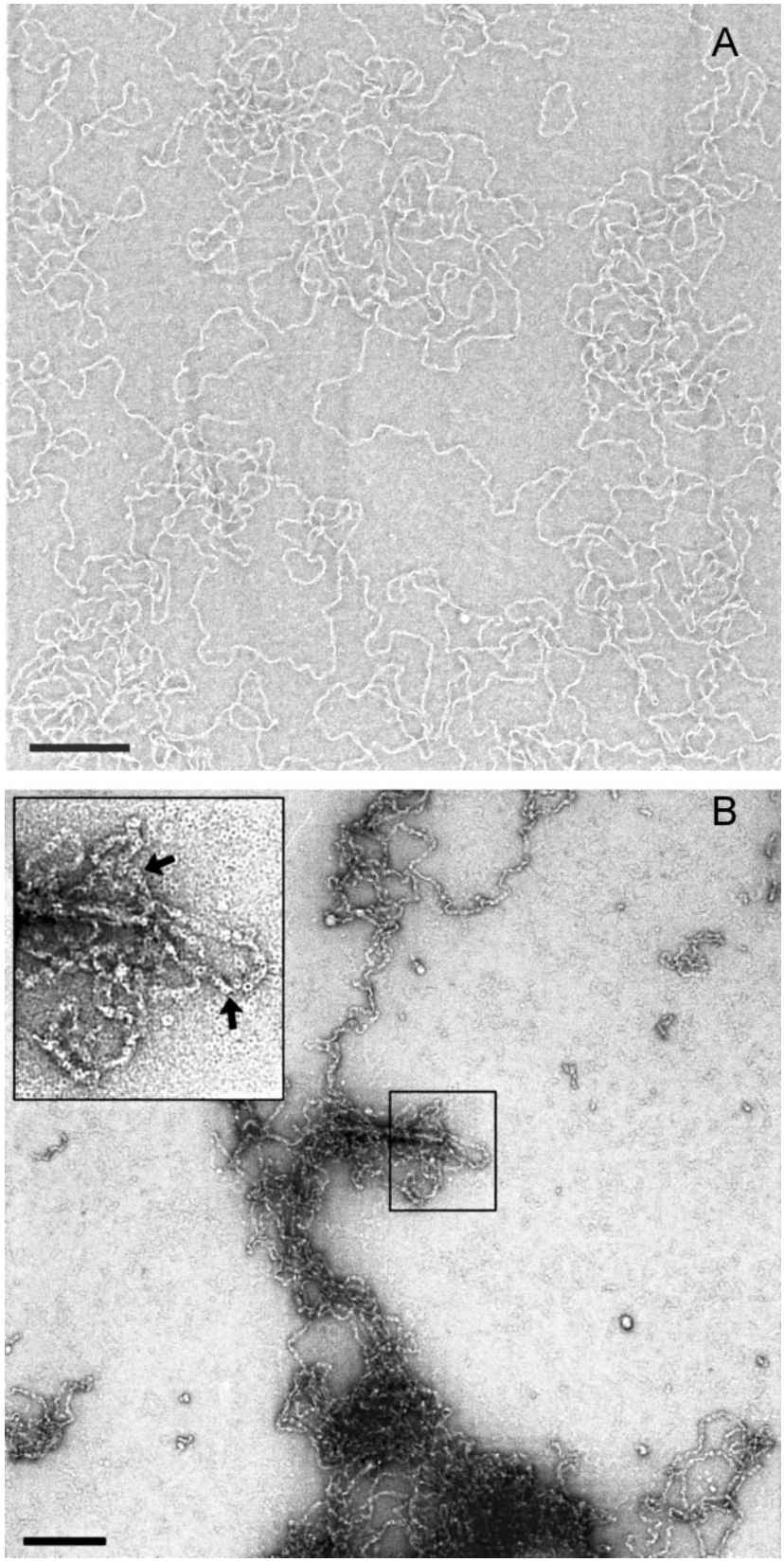

FIG. 4. **Negatively stained electron micrographs of apo C-II fibrils formed at 0.3 mg/ml before (*A*) and after the addition of 0.1 mg/ml SAP (*B*).** *B*, *inset*, magnified view of the marked region in the center of the figure. *Arrows* highlight SAP molecules bound to the fibril. *Bar*, 200 nm.

strain rate to a value only slightly higher than that of the solvent. This indicates that the network of fibril-fibril interactions in the absence of SAP is weak and readily broken down by the applied mechanical shear. The viscosity of the solution in the presence of SAP at low strain rates is reduced by a factor of three relative to apoC-II fibrils alone. The extent of shear-thinning is also significantly reduced, indicating that the system of SAP-mediated fibril-fibril interactions is less susceptible to shear.

The observed changes in the viscoelastic properties of apoC-II fibril solutions mediated by SAP are consistent with a loss of the solution-spanning network of fibril-fibril interactions seen in the absence of SAP. Instead, we see a shift to a colloid-like behavior characterized by more localized networks

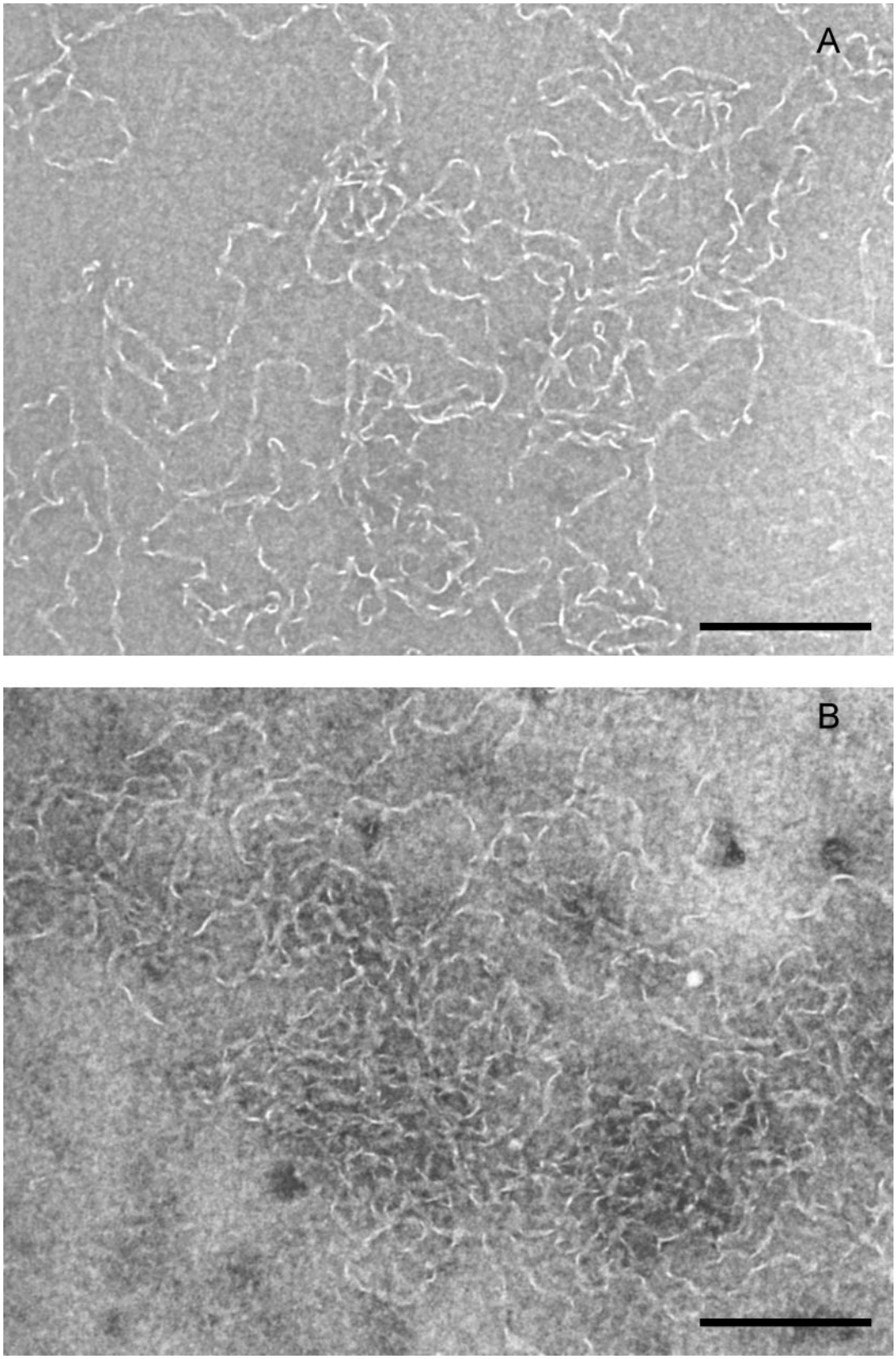

FIG. 5. **Negatively stained electron micrographs of apo C-II fibrils formed at 0.4 mg/ml before (*A*) and after the addition of 0.05 mg/ml apoE3 (*B*).** *Bar*, 200 nm.

of fibril entanglements similar to microgel-like domains. This change in solution structure can be understood as follows: in the absence of non-fibrillar components, apoC-II fibrils are distributed evenly throughout the solution and are extensively cross-linked by transient interactions mediated by non-covalent associations between fibrils. The fibril solution has gel-like properties because of the resulting entangled and cross-linked network which spans the entire solution volume. The addition of SAP to this solution results in an increase in the number of fibril-fibril interactions, and this increase necessarily results in

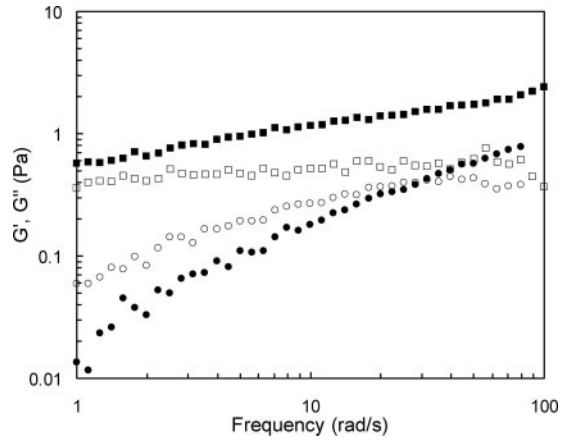

FIG. 6. **Mechanical spectrum of 1 mg/ml apoC-II amyloid fibrils in 10 mM Tris, 150 mM NaCl, 2 mM Ca^{2+}, 40% (w/w) glycerol, pH 7.4 in the presence (*circles*) and absence (*squares*) of 0.1 mg/ml SAP.** The storage modulus (G') is shown as black symbols, and the loss modulus (G'') is shown as white symbols.

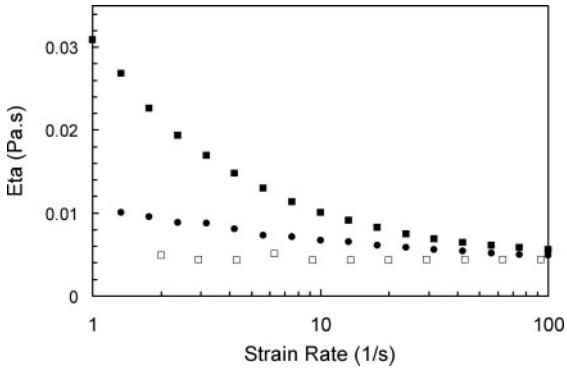

FIG. 7. **Steady-shear viscosity of apoC-II amyloid fibrils (1 mg/ml) in the presence (*circles*) and absence (*black squares*) of 0.1 mg/ml SAP.** The viscosity of the solvent alone (10 mM Tris, 150 mM NaCl, 2 mM Ca^{2+}, 40% (w/w) glycerol, pH 7.4) is also shown (*white squares*).

an increase in the local density of fibrils. As the total amount of fibrillar material is fixed, this increase in local density in some regions of the solution must be matched by a decrease in fibril density over other areas. Thus, we see a condensation of small microgel-like domains within the solution, and a decrease in the fibril density in surrounding regions, resulting in the loss of the solution-spanning network of fibril interactions and, thus, a loss of the gel-like rheological properties in favor of colloid-like behavior. Such a picture of the effect of SAP is confirmed by the electron micrographs of apoC-II fibrils in the presence of SAP, which show the fibrils to form discrete, condensed "bundles" of many fibrils constrained by a locally dense network of fibril entanglements. Furthermore, this change in the nature of fibrillar entanglements offers an explanation for the observed change in the sedimentation behavior of the fibrils, with the localized, dense bundles of fibrils in the presence of SAP likely to sediment considerably faster than the weak and diffuse network of fibrillar interactions present in the absence of SAP.

DISCUSSION

An emerging theme in amyloid research centers around the observation that early intermediates in amyloidogenesis are cytotoxic, whereas mature fibrils and amyloid deposits, the end points in this process, seem not to be (7–10). Such an understanding prompts a rather controversial rethinking of the pathogenic mechanisms underlying amyloid diseases, as well as potential therapeutic strategies for these conditions. To take the extreme position, it raises the possibility that amyloid fibrils and, particularly, amyloid deposits might represent adaptive mechanisms that protect the body from soluble cytotoxic products of protein misfolding and aggregation (24). Traditionally, however, amyloid deposits themselves have been seen as the pathogenic species, and surgical removal of deposits has proved an effective treatment in some cases (25). In the light of this controversy, it is crucial to develop a better understanding of the mechanisms by which fibrils form and aggregate into deposits.

The non-fibrillar components of amyloid deposits are important but poorly understood factors influencing the behavior of amyloid fibrils and deposits *in vivo*. The data presented here identify and characterize a significant change in the solution behavior of amyloid-like fibrils mediated in a general yet specific manner by SAP and apoE, two proteins known to be non-fibrillar components of amyloid deposits *in vivo*. Specifically, these proteins increase the strength and density of local fibril-fibril interactions, causing the formation of more compact and localized fibril networks, in contrast to the weak and dispersed networks formed by the fibrils in the absence of these components. This finding suggests that non-fibrillar components of amyloid deposits may play a role in regulating the aggregation state and solubility of amyloid fibrils by changing the nature of fibril-fibril interactions.

Although the current findings only directly address the interaction of SAP and apoE with the fibrils formed by apoC-II, a number of lines of evidence point to the generality of the interactions of SAP with amyloid fibrils: (*i*) SAP is found ubiquitously associated with amyloid deposits *in vivo*, independently of the identity of the fibril precursor protein (26); (*ii*) SAP binds to all amyloid-like fibrils formed *in vitro* (27); and (*iii*) the effects of SAP as an inhibitor of clearance of amyloid deposits are observed in a range of *in vivo* and *in vitro* experimental systems, again independently of the fibril precursor (6, 28–30). Thus, it is tempting to speculate about the potential of a broader significance for these results in terms of the interaction of SAP with all amyloid fibrils. In particular, a role for SAP as a regulator of the solubility of amyloid fibrils is consistent with the observation that amyloid deposits form more slowly in mice lacking SAP than in wild-type mice when AA amyloidosis is induced in response to chronic inflammation (29, 30). The current observations may also shed light on the capacity of the non-fibrillar components to protect amyloid deposits from degradation (28): the increase in fibrillar entanglements and the resulting increase in local fibril density within deposits is likely to limit the accessibility of fibrils to degradative machinery such as proteases. It is particularly interesting to note that, in addition to its interactions with amyloid fibrils, SAP also binds to proteoglycans of the type that are also seen as components of amyloid deposits (31). Given its pentameric structure, it is likely that SAP is multivalent for its various binding partners, suggesting that SAP might act as a cross-linking agent between condensed amyloid fibrils and these other non-fibrillar components during the formation of the amyloid deposit. Such cross-linking might further increase the density of amyloid deposits and further enhance their resistance to clearance.

Like SAP, apoE is found ubiquitously in all amyloid deposits (32, 33). However, with the important exception of the Aβ deposits associated with Alzheimer's disease, the role of apoE in deposit formation and persistence is very poorly studied; even in the case of Aβ, it remains poorly understood. There is some evidence, however, to suggest that apoE and SAP act via similar mechanisms to influence the formation of amyloid deposits (32, 34). Thus it is of some interest that apoE and SAP elicit similar changes in the solution properties of apoC-II amyloid fibrils, as measured by sedimentation velocity analysis

and electron microscopy. On the other hand, it is surprising to note that the effect of apoE on amyloid fibril entanglements is apparently not isoform-specific. The apoE4 isoform is a major risk factor for early onset Alzheimer's disease, and much research into Aβ-apoE interactions have focused on differences between the apoE2, apoE3, and apoE4 isoforms. The fact that no such isoform-specific behavior was seen in the current study may suggest that the interactions of apoE with apoC-II fibrils are significantly different from those with Aβ. On the other hand, it may suggest that the differential effects of the apoE isoforms on the progression of Alzheimer's disease are not related to differences in the apoE-amyloid interaction itself, but rather arise from other differences in the behavior of the apoE isoforms.

Acknowledgments—We thank Chi Pham for providing unpublished data.

REFERENCES

1. Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4245–4249
2. Hoppener, J. W., Ahren, B., and Lips, C. J. (2000) *N. Engl. J. Med.* **343,** 411–419
3. Pepys, M. B. (2001) *Philos. Trans. R. Soc. Lond.-Biol. Sci.* **356,** 203–211
4. Westermark, P., Benson, M. D., Buxbaum, J. N., Cohen, A. S., Frangione, B., Ikeda, S., Masters, C. L., Merlini, G., Saraiva, M. J., and Sipe, J. D. (2002) *Amyloid* **9,** 197–200
5. Sunde, M., and Blake, C. C. (1998) *Q. Rev. Biophys.* **31,** 1–39
6. Pepys, M. B., Herbert, J., Hutchinson, W. L., Tennent, G. A., Lachmann, H. J., Gallimore, J. R., Lovat, L. B., Bartfai, T., Alanine, A., Hertel, C., Hoffmann, T., Jakob-Roetne, R., Norcross, R. D., Kemp, J. A., Yamamura, K., Suzuki, M., Taylor, G. W., Murray, S., Thompson, D., Purvis, A., Kolstoe, S., Wood, S. P., and Hawkins, P. N. (2002) *Nature* **417,** 254–259
7. Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C. M., and Stefani, M. (2002) *Nature* **416,** 507–511
8. Conway, K. A., Lee, S. J., Rochet, J. C., Ding, T. T., Williamson, R. E., and Lansbury, P. T., Jr. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 571–576
9. Sousa, M. M., Cardoso, I., Fernandes, R., Guimaraes, A., and Saraiva, M. J. (2001) *Am. J. Pathol.* **159,** 1993–2000
10. Lambert, M. P., Barlow, A. K., Chromy, B. A., Edwards, C., Freed, R., Liosatos, M., Morgan, T. E., Rozovsky, I., Trommer, B., Viola, K. L., Wals, P., Zhang, C., Finch, C. E., Krafft, G. A., and Klein, W. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6448–6453
11. Pepys, M. B. (1995) in *Oxford Textbook of Medicine* (Weatherall, D. J., Ledingham, J. G., and Warrel, D. A., eds) 3rd Ed., pp. 1512–1524, Oxford University Press, Oxford
12. MacRaild, C. A., Hatters, D. M., Lawrence, L. J., and Howlett, G. J. (2003) *Biophys. J.* **84,** 2562–2569
13. Hatters, D. M., and Howlett, G. J. (2002) *Eur. Biophys. J.* **31,** 2–8
14. Hatters, D. M., MacPhee, C. E., Lawrence, L. J., Sawyer, W. H., and Howlett, G. J. (2000) *Biochemistry* **39,** 8276–8283
15. Medeiros, L. A., Khan, T., El Khoury, J. B., Pham, C. L., Hatters, D. M., Howlett, G. J., Lopez, R., O'Brien, K. D., and Moore, K. J. (2003) *J. Biol. Chem.* **279,** 10643–10648
16. Wang, C. S., Downs, D., Dashti, A., and Jackson, K. W. (1996) *Biochim. Biophys. Acta* **1302,** 224–230
17. Saito, H., Dhanasekaran, P., Baldwin, F., Weisgraber, K. H., Lund-Katz, S., and Phillips, M. C. (2001) *J. Biol. Chem.* **276,** 40949–40954
18. Schuck, P., and Rossmanith, P. (2000) *Biopolymers* **54,** 328–341
19. Schuck, P. (2003) *Anal. Biochem.* **320,** 104–124
20. Garcia de la Torre, J., Navarro, S., Lopez Martinez, M. C., Diaz, F. G., and Lopez Cascales, J. J. (1994) *Biophys. J.* **67,** 530–531
21. Yamakawa, H., and Fujii, M. (1973) *Macromolecules* **6,** 407–415
22. Hatters, D. M., MacRaild, C. A., Daniels, D. R., Gosal, W. S., Thomson, N. H., Jones, J. A., Davis, J. J., MacPhee, C. E., Dobson, C. M., and Howlett, G. J. (2003) *Biophys. J.* **85,** 3979–3990
23. Emsley, J., White, H. E., O'Hara, B. P., Oliva, G., Srinivasan, N., Tickle, I. J., Blundell, T. L., Pepys, M. B., and Wood, S. P. (1994) *Nature* **367,** 338–345
24. Caughey, B., and Lansbury, P. T. (2003) *Annu. Rev. Neurosci.* **26,** 267–298
25. O'Doherty, D. P., Neoptolemos, J. P., and Wood, K. F. (1987) *Br. J. Surg.* **74,** 83–88
26. Pepys, M. B., Rademacher, T. W., Amatayakul-Chantler, S., Williams, P., Noble, G. E., Hutchinson, W. L., Hawkins, P. N., Nelson, S. R., Gallimore, J. R., and Herbert, J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 5602–5606
27. Danielsen, B., Sorensen, I. J., Nybo, M., Nielsen, E. H., Kaplan, B., and Svehag, S. E. (1997) *Biochim. Biophys. Acta* **1339,** 73–78
28. Tennent, G. A., Lovat, L. B., and Pepys, M. B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4299–4303
29. Botto, M., Hawkins, P. N., Bickerstaff, M. C., Herbert, J., Bygrave, A. E., McBride, A., Hutchinson, W. L., Tennent, G. A., Walport, M. J., and Pepys, M. B. (1997) *Nat. Med.* **3,** 855–859
30. Togashi, S., Lim, S. K., Kawano, H., Ito, S., Ishihara, T., Okada, Y., Nakano, S., Kinoshita, T., Horie, K., Episkopou, V., Gottesman, M. E., Costantini, F., Shimada, K., and Maeda, S. (1997) *Lab. Invest.* **77,** 525–531
31. Hamazaki, H. (1987) *J. Biol. Chem.* **262,** 1456–1460
32. Wisniewski, T., and Frangione, B. (1992) *Neurosci. Lett.* **135,** 235–238
33. Charge, S. B., Esiri, M. M., Bethune, C. A., Hansen, B. C., and Clark, A. (1996) *J. Pathol.* **179,** 443–447
34. Gallo, G., Wisniewski, T., Choi-Miura, N. H., Ghiso, J., and Frangione, B. (1994) *Am. J. Pathol.* **145,** 526–530

Non-fibrillar Components of Amyloid Deposits Mediate the Self-association and Tangling of Amyloid Fibrils

Christopher A. MacRaild, Cameron R. Stewart, Yee-Foong Mok, Menachem J. Gunzburg, Matthew A. Perugini, Lynne J. Lawrence, Viyada Tirtaatmadja, Justin J. Cooper-White and Geoffrey J. Howlett

J. Biol. Chem. 2004, 279:21038-21045.
doi: 10.1074/jbc.M314008200 originally published online March 18, 2004
