Macromolecular Crowding Accelerates Amyloid Formation by Human Apolipoprotein C-II*

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Human apolipoprotein C-II (apoC-II) slowly forms amyloid fibers in lipid-free solutions at physiological pH and salt concentrations (Hatters, D. M., MacPhee, C. E., Lawrence, L. J., Sawyer, W. H., and Howlett, G. J. (2000) Biochemistry 39, 8276–8283). Measurements of the time dependence of solution turbidity, thioflavin T reactivity, and the amount of sedimentable aggregate reveal that the rate and extent of amyloid formation are significantly increased by the addition of an inert polymer, dextran T10, at concentrations exceeding 20 g/liter. High dextran concentrations do not alter the secondary structure of the protein, fiber morphology, or the thioflavin T and Congo Red binding capacity of apoC-II amyloid. Analytical ultracentrifugation studies show that monomeric apoC-II does not associate significantly with dextran. The observed dependence of the overall rate of amyloid formation on dextran concentration may be accounted for quantitatively by a simple model for non-specific volume exclusion. The model predicts that an increase in the fractional volume occupancy of macromolecules in a physiological fluid can nonspecifically accelerate the formation of amyloid fibers by any amyloidogenic protein.

The specific self-association of proteins to form amyloid fibrils is a characteristic of a number of pathologies, including Alzheimer, Parkinson, and Creutzfeldt-Jakob diseases (1). This process involves slow nucleation coupled to self-association steps, which constitute an alternative folding pathway to those leading to the native state (2, 3). Amyloid formation is promoted by destabilization of the native state through events such as mutation or truncation. For example, certain mutants of lysozyme that exhibit molten globule characteristics also form amyloid (4) whereas apomyoglobin forms amyloid under partially denaturing conditions (5).

Human apolipoprotein C-II (apoC-II) (M, = 8915, 79 residues) is normally a component of very low density lipoprotein, where it plays an important physiological role as an activator of lipoprotein lipase (6, 7). When associated with polar lipids (e.g. phospholipids or SDS) apoC-II adopts a primarily α-helical conformation (8–10). Our previous work (11) demonstrates that, in the absence of lipid, human apoC-II self-associates to form twisted ribbon-like fibrils with all of the hallmarks of amyloid, including binding to Congo Red with red-green birefringence under cross-polarized light, binding to thioflavin T, and increased β-structure. In addition, x-ray diffraction patterns of aligned apoC-II fibrils indicate a cross-β-sheet structure. In vitro amyloid formation by apoC-II can be compared with the in vivo deposition of amyloid involving other apolipoproteins such as apoA-I (12, 13), apoA-II (14, 15), apoA-IV (16), apoE (17), and apolipoprotein-like proteins, α-synuclein (18) and serum amyloid A (19). A significant clue to the prevalence of apolipoproteins in amyloid formation is provided by the observation that many apolipoproteins have limited conformational stability or secondary structure in the absence of lipid (20). Destabilized conformations in many proteins promote amyloid formation (3–5, 21). Apolipoprotein derivatives that form amyloid are frequently mutant isoforms or truncated products; for example, amyloid deposition involving apoA-I (12), apoA-II (14), and the C-terminal domain of apoE (17). We propose that these modifications destabilize lipid binding leading to amyloid formation in vivo (20). The well-characterized ability of apoC-II to form amyloid fibrils provides a convenient model to examine in vivo parameters (9, 22) that could control the growth of amyloid fibrils in vivo.

Most biological fluids contain a high total concentration of macromolecules, including proteins, nucleic acids, and carbohydrates, that collectively occupy a high fraction of the fluid volume (23). Volume exclusion or “macromolecular crowding” in such fluids may result in significant alterations of the rates and equilibria of macromolecular associations (23–26). It has been suggested on conceptual grounds that volume exclusion in vivo could modulate the rate and extent of amyloid formation in vivo (27, 28). In the present study we investigate the potential effects of macromolecular crowding on amyloid formation using lipid-free apoC-II as a model. We find that the rate and extent of amyloid formation by apoC-II is substantially enhanced by the addition of an inert polymer, dextran T10, and demonstrate experimentally that this effect is not due to association between dextran and apoC-II. The magnitude of the observed effect upon the rate of amyloid formation may be accounted for quantitatively by a simple model for volume exclusion.

EXPERIMENTAL PROCEDURES

apoC-II Preparation—apoC-II was expressed and purified as described previously (11, 29) and stored as a stock in 5 M GdnHCl at 30

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g/liter. The protein was refolded by directly diluting to 0.7 g/liter in 100 mM sodium phosphate, 0.1% sodium azide, pH 7.4. The protein was immediately diluted to the final concentration required. For the crowding experiments, solutions of freshly prepared apoC-II were added to dextran T10, and the samples were monitored using fluorescamine nucleation is the rate-limiting step (9, 22). Centrifugation under these conditions makes the supernatant after centrifugation was monitored using fluorescamine 7.4, in the absence or presence of 100 mM sodium phosphate, 0.1% sodium azide, pH 7.4. The samples were rapidly mixed, and bubbles were removed by centrifugation (10 s at 15,000 \( \times g \)).

Turbitity Assays—Freshly prepared apoC-II solutions were placed into 1-cm path length acryl cuvettes. The absorbances of the covered samples were monitored at 400 nm at 20 \(^\circ\)C (Cary-5, Varian, Clayton, Australia). The time evolution of the samples before the first measurement took 10–15 min.

Centrifugation Assays—Aliquots from a stock solution (200 \( \mu \)l of apoC-II were sedimented in polycarbonate tubes at 350,000 \( \times g \) for 20 min in a Beckman TLS-100 ultracentrifuge (Beckman/Coullter, Fullerton, CA) at 20 \(^\circ\)C. Our previous studies show that, during amyloid formation, apoC-II exists as a bimodal population of monomers (\( s \approx 1 \)) and large aggregates (\( s > 50 \)) at any given time point suggesting nucleation is the rate-limiting step (9, 22). Centrifugation under these conditions pellets aggregate, but not monomer, and thus provides a means to monitor aggregation. The concentration of apoC-II in the supernatant after centrifugation was monitored using fluoromeric reactivity with primary amine groups (30). This involved rapidly mixing supernatant after centrifugation was monitored using fluorescamine 1S fluorescamine in acetonitrile). The fluorescence was measured using an f_{\text{rms}} fluorescence plate-reader (Molecular Devices, Sunnyvale, CA) with a 350 nm/460 nm excitation/emission filter set.

Thioflavin T Reactivity—Aliquots of 25 \( \mu \)l of apoC-II (0.4 g/liter) were added to final solution volumes of 200 \( \mu \)l containing 100 mM sodium phosphate, 0.1% (w/v) sodium azide, 5 \( \mu \)M thioflavin T, pH 7.4. The fluorescence was monitored using an f_{\text{rms}} fluorescence plate-reader with a 444 nm/485 nm excitation/emission filter set.

Congo Red Assay—Sample volumes of 75 \( \mu \)l were prepared containing 5 \( \mu \)g Congo Red, 100 mM sodium phosphate, 0.1% sodium azide, pH 7.4, in the absence or presence of 100 \( \mu \)g of apoC-II. Absorbance spectra were recorded from 600 nm to 300 nm in 1-cm path length acryl cuvettes, and absorbance contributions due to protein alone were deducted from the relevant spectra.

**pH and Conductivity Measurements**—The conductivities of solutions containing 0–200 mM sodium phosphate, pH 7.4, at 20 \(^\circ\)C were measured to make a standard curve. Conductivities were measured with a Philips PW 9501/01 conductivity meter (Philips, New York, NY) using a PFP5313 probe at a frequency of 2000 Hz and cell constant of 0.3–1. The conductance of solutions containing dextran T10 (0–150 g/liter) or apoC-II (0.4 g/liter) in 100 mM sodium phosphate, pH 7.4, was monitored under identical conditions. The pH of solutions containing 0–150 mM dextran or apoC-II (0.4 g/liter) in 100 mM sodium phosphate buffer, pre-made at pH 7.4, was measured using an Actimom Model 107 pH meter (Melbourne, Australia).

Sedimentation Velocity Analysis—Samples of apoC-II (400 \( \mu \)l), preincubated overnight in the absence and presence of 100 g/liter dextran, were analyzed using the XL-A analytical ultracentrifuge (Beckman/ Coullter). Samples were sedimented at 4000 rpm with 20-min radial scans using a wavelength of 280 nm. Data were collected using 0.002-cm radial increments in continuous scanning mode. Data were analyzed using log(I)/log((r/a)(s)) analysis (31) with the baseline absorbance set to zero and sedimentation range from 0.1 S to 350 S and 100 sedimentation coefficient increments. A regularization parameter of \( p = 0.68 \) was used. The meniscus position was varied to give the lowest root mean square deviation of the fit. A buffer viscosity of 0.01 poise and density of 1.01 g/ml was calculated from solution composition. In the presence of 100 g/liter dextran T10, a viscosity of 0.02 poise and buffer density of 1.04 g/ml were calculated using the formula provided by the American Polymers Standards Corp. (Mentor, OH) and Amersham Biosciences, Inc. (Uppsala, Sweden).

The sedimentation velocity behavior of freshly prepared apoC-II (0.3 g/liter) in the presence or absence of 2 g/liter dextran T10 was analyzed using a rotor speed of 40,000 rpm and a temperature of 20 \(^\circ\)C. Radial scans were monitored at a wavelength of 280 nm and 10-min intervals. The data were analyzed to obtain molecular masses and sedimentation coefficients using a model for a single sedimenting species (32). The partial specific volume (0.732 ml/g) for apoC-II was calculated from the amino acid composition.

Electron Microscopy—Solutions of apoC-II (0.4 g/liter) were diluted 3-fold in water and applied to freshly glow-discharged carbon-coated copper grids. After 1 min, excess material was removed and the grids were washed twice with 20 \( \mu \)l of water before negatively staining with 2% (w/v) potassium phosphotungstate. The samples were imaged using a JEOL 2000 transmission electron microscope (Peabody, MA) operating at 120 kV.

**Circular Dichroism Spectroscopy**—Spectra for apoC-II solutions (0.3 g/liter) in 1-mm path length cuvettes were acquired using a Jasco J720 spectropolarimeter (Tokyo, Japan) with measurements taken at 0.5-nm wavelength intervals, a 0.5-s integration time, and 1-nm spectral bandwidth. Four sequential scans were recorded before averaging and subtraction of baseline contributions. The data were standardized using the formula \( \theta = \theta_{\text{max}} \), where \( \theta \) is the measured ellipticity, \( M \) is the molecular mass, \( c \) is the concentration (grams/liter), \( n \) is the number of amino acid residues, and \( l \) is path length (millimeters).

**Data Analysis**—The purpose of the present study was not to provide a detailed description of the kinetics of amyloid formation by apoC-II but, rather, to compare the overall rate of amyloid formation under different experimental conditions. This comparison was facilitated by the following empirical description of the time course of the reaction. The time-dependent appearance of turbidity following dilution of GdnHCl was found to be well described by the empirical Hill function, where \( A_{\text{obs}}(t) \) is the turbidity (apparent absorbance) in the long time limit, \( t_{50} \) is the elapsed time at which \( A_{\text{obs}} \) is equal to one-half of \( A_{\text{obs}}(\infty) \), and \( n \) is a cooperativity parameter. The time course of aggregation as measured by centrifugation was found to be well described by the following empirical inverse decay function for the time-dependent fluorescence of remaining non-aggregated protein in the supernatant, where \( F(0) \) is the initial fluorescence of the solution and \( t_{50} \) is the time at which the fluorescence has reached half of its initial value. In both types of assay, \( t_{50} \) is taken as a quantitative measure of the overall rate of the amyloid forming reaction.

_model for the Effect of Excluded Volume on Amyloid Fiber Formation—It is assumed that assembly of amyloid fibrils proceeds via a series of stepwise addition reactions, \( A_{-1} + A \rightarrow A_{+1} \), with forward and backward rate constants \( k_f(i) \) and \( k_b(i) \), respectively. For transition-state rate-limited association reactions in solution, the addition of inert volumeexcluding molecules is expected to affect primarily the association rate constant and leave the dissociation rate constant essentially unperturbed (see limiting case 1 in the Appendix to Ref. 33), where

\( k_b(i) = k_b(i) \Gamma \)  
\( k_f(i) = k_f(i) \)  

where \( k_b(i) \) denotes the respective rate constant in the absence of excluded volume effects, and \( \gamma \) denotes the thermodynamic activity coefficient of the i-mer in solution arising from excluded volume. In a solution containing a low total concentration of apoC-II (<1 g/liter), \( \gamma \) is expected to depend upon the relative sizes and shapes of dextran and all i-mers of apoC-II, and the concentration of dextran, but not upon the concentration of any species of apoC-II.

To estimate the value of \( \gamma \) for each association state of apoC-II, it is necessary to postulate a structural model for both the i-mer and for the crowding macromolecule, in which each species is represented by a rigid convex particle with dimensions similar to those of the corresponding molecule (34). In the present instance, dextran is modeled as a random array of hard cylindrical rods with an effective radius \( r_{\text{eff}} \) and specific exclusion volume of \( v_{\text{excl}} \), in accordance with the available volume theory of Ogston (35). The monomer of apoC-II, which is largely unstructured (9, 11), is modeled as an effective sphere with a radius \( r_i \). The fibrous i-mer is modeled as a spherocylinder \( ^3 \) with cylindrical radius \( r_{\text{cyl}} \) and cylindrical length \( l = \Delta l \), where \( \Delta \) is a constant of proportionality.

\(^3\) A cylinder capped on each end by a hemisphere.
According to the excluded volume models of Ogston (35) and Giddings et al. (36),

\[
sphere: \ln \gamma_i = \left(1 + \frac{r_i}{r_{dex}}\right)^2 \nu_{dex} w_{dex} \quad \text{(Eq. 6)}
\]

spherocylindrical i-mer: \( \ln \gamma_i = \left(1 + \frac{r_i}{r_{dex}}\right)^2 \left(1 + \frac{\Delta r + 2r_i}{2r_{dex}}\right) \nu_{dex} w_{dex} \quad \text{(Eq. 7)} \)

where \( w_{dex} \) denotes the w/v concentration of dextran. Combination of Equations 3, 6, and 7 yields, for constant \( r_{dex} \),

\[
\Gamma_i = \Gamma = \exp\left[\left(1 + \frac{r_i}{r_{dex}}\right)^2 - \left(1 + \frac{r_i}{r_{dex}}\right) \frac{\Delta}{2r_{dex}}\right] \nu_{dex} w_{dex} \quad \text{(Eq. 8)}
\]

At this level of approximation, the nonideal correction factor to the rate constant is thus independent of the size of the oligomer and may be regarded as a common multiplier of all forward rate constants, independent of the magnitude of the individual rate constant. It follows that the overall rate of the reaction should scale with the magnitude of \( \Gamma \), and that any characteristic reaction time, such as \( t_{50} \), should scale inversely with \( \Gamma \),

\[
\frac{d \log t_{50}}{dc_{dex}} = -\frac{1}{2.303} \frac{d \ln \Gamma}{dc_{dex}} \quad \text{(Eq. 9)}
\]

The dependence of \( t_{50} \) upon dextran concentration is obtained by combining Equations 8 and 9,

\[
\log t_{50} = \log t_{50}^0 - \frac{1}{2.303} \left[\left(1 + \frac{r_i}{r_{dex}}\right)^2 - \left(1 + \frac{r_i}{r_{dex}}\right) \frac{\Delta}{2r_{dex}}\right] \nu_{dex} w_{dex} \quad \text{(Eq. 10)}
\]

**RESULTS**

**Aggregate Solubility**

The time dependence of turbidity, measured for different apoC-II concentrations in the absence of added dextran, is plotted in Fig. 1A. At the conclusion of each turbidity measurement (\( t = 5300 \) min), the sample was centrifuged and the protein concentration in the supernatant determined spectrophotometrically (\( c_{280 \text{nm}} = 12090 \) M\(^{-1}\) cm\(^{-1}\)), from which the amount of protein in the pellet was determined. The amount of protein in the pellet is plotted as a function of the final measured value of solution turbidity in Fig. 1B. The relationship between the two quantities is well described by the empirical relation,

\[
c_{\text{pellet}} = 5.957 A_{400} - 12.46 A_{400}^2 \quad \text{(Eq. 11)}
\]

which is plotted together with the data in Fig. 1B. Equation 1 was then fitted to each set of turbidity versus time data. The dependence of \( A_{400} \) on \( t \) for each protein concentration, calculated via Equation 1 using the best-fit values of \( t_{50} \) and \( A_{400}(\infty) \) given in Table I, is plotted together with the data in Fig. 1A. The limiting value of \( c_{\text{pellet}} \) in the limit of long time, denoted \( c_{\text{pellet}}(\infty) \), was calculated from \( A_{400}(\infty) \) using Equation 11 and is plotted as a function of total protein in Fig. 1C. It may be seen that this value is equal to the total protein to within experimental error, indicating that the equilibrium solubility of the aggregate is smaller than what can be measured by our present techniques. Equivalently, the dissociation rate constant for fiber growth may be regarded as negligibly small relative to the corresponding association rate constant.

**Effect of Dextran on the Kinetics of Aggregation**

The effect of added dextran T-10 on the rate of apoC-II amyloid formation was monitored via measurement of the time-dependent turbidity (Fig. 2) and the time-dependent amount of protein in the supernatant as measured by fluorescamine reactivity, after pelleting of high molecular weight aggregate (Fig. 3). Both measurements indicate that addition of dextran significantly accelerates the reaction. Equation 1 was fitted by the method of nonlinear least squares to the time-dependent turbidity data, and the best fit to each data set, calculated using the parameter values given in Table I, was plotted together with the data in Fig. 2. Equation 2 was fitted by the
method of nonlinear least squares to the time-dependent supernatant fluorescence data, and the best fit to each data set, calculated using the parameter values given in Table II, was plotted together with the data in Fig. 3. The dependence of the best-fit values of $\log t_{50}$ on dextran concentration, obtained from both the turbidity and the precipitation measurements, is plotted in Fig. 4. The difference between the values of $t_{50}$ (the value of $t_{50}$ for $c_{\text{dex}} = 0 \, \text{g/liter}$ for the precipitation assay and $0.4 \, \text{g/liter}$ for the turbidity assay) but may also be influenced by the difference between the two techniques of measurement. The essential feature to be noted is the fractional change in $t_{50}$ with increasing dextran concentration, which appears to be approximately the same for both methods of measurement.

Characterization of ApoC-II Aggregates Formed in the Presence of Dextran

Thioflavin T Binding—Thioflavin T is a diagnostic amyloid dye and undergoes a large spectral change in its fluorescence properties when bound to amyloid structures (37, 38). Previous studies have shown that the fluorescence of thioflavin T at fixed total concentration increases with the amount of apoC-II amyloid present (11, 22). We used thioflavin T reactivity to determine whether the acceleration of apoC-II aggregation by dextran was due to increased amyloid formation. The results in Fig. 5 show a time-dependent increase in thioflavin T reactivity of apoC-II that is accelerated by high concentrations of dextran (up to 150 g/liter). The systematic increases observed in the development of thioflavin T reactivity induced by dextran complement the results shown in Figs. 2 and 3 and support the conclusion that the dextran-induced alterations in the time development of turbidity and pelletable aggregate are due to

![Graph](https://i.imgur.com/3QZQZQ.png)

**FIG. 2.** Development of turbidity in solutions of apoC-II (0.4 g/liter) following dilution of GdnHCl, and addition of various concentrations of dextran T10. Data sets (from bottom to top): $c_{\text{dex}} = 0 \, \text{( ), 25 ( ), 50 ( ), 80 ( ), 100 ( ), 125 ( ), and 150 ( ) g/liter}$. Plotted curves are calculated according to Equation 1 with the best-fit parameter values given in Table I.

**FIG. 3.** Fluorescamine reactivity of apoC-II in the supernatant (initial concentration 0.2 g/liter) following dilution of GdnHCl, the addition of various dextran concentrations, and pelleting of aggregate. Data represent fluorescamine fluorescence intensity ($F$) as a function of incubation time prior to centrifugation ($t$). Data sets (from bottom to top): $c_{\text{dex}} = 100 \, \text{( ), 80 ( ), 60 ( ), 40 ( ), 20 ( ), 0 ( ) g/liter}$. Plotted curves are calculated according to Equation 2 with $F_0 = 290$ and the best-fit values of $t_{50}$ given in Table II.

**FIG. 4.** $\log t_{50}$ determined by modeling results of time-dependent turbidity experiments (○) and precipitation experiments (●) as described in the text, plotted as a function of the concentration of added dextran. Plotted curves represent the global best fit of Equation 10 to both data sets as described in the text.

**TABLE I**

| $c_{\text{apo}}$ (g/liter) | $c_{\text{dex}}$ (g/liter) | $A_{400}(\%)$ | $t_{50}$ (min) | $n$ |
|---------------------------|---------------------------|----------------|-----------------|-----|
| 0.4                       | 0                         | 0.0713         | 1667            | 1.44|
| 0.4                       | 25                        | 0.0894         | 1384            | 1.34|
| 0.4                       | 50                        | 0.1151         | 1127            | 1.18|
| 0.4                       | 75                        | 0.130          | 794             | 1.14|
| 0.4                       | 100                       | 0.166          | 614             | 1.12|
| 0.4                       | 125                       | 0.240          | 449             | 1.16|
| 0.4                       | 150                       | 0.332          | 245             | 1.25|
| 0.5                       | 0                         | 0.110          | 1193            | 1.27|
| 0.6                       | 0                         | 0.152          | 934             | 1.20|
| 0.7                       | 0                         | 0.193          | 714             | 1.13|

**TABLE II**

| $c_{\text{dex}}$ (g/liter) | $t_{50}$ (min) |
|---------------------------|----------------|
| 0                         | 9268           |
| 20                        | 9585           |
| 40                        | 4615           |
| 60                        | 3391           |
| 80                        | 2566           |
| 100                       | 1907           |
the effect of dextran on amyloid formation.

Congo Red Binding—Congo Red is another diagnostic amyloid dye (39) and was used to characterize the amyloid nature of apoC-II aggregates formed in the presence of dextran T10. Congo Red alone has an absorbance maximum at 490 nm (Fig. 6). The addition of apoC-II (0.4 g/liter), freshly prepared in the absence or presence of 150 g/liter dextran, produced overlapping spectra with a small red shift and rise (13%) in the peak centered at 490 nm, consistent with the presence of predominantly monomeric apoC-II (Fig. 6). ApoC-II, incubated in the absence and presence of 150 g/liter dextran for 24 h at 20°C, produced dramatic changes in the absorbance spectrum of Congo Red. The spectra in the presence and absence of dextran T10 overlap and show a 20-nm further red shift and 45% increase in the absorbance at the maximum compared with the freshly prepared samples, shown in Fig. 6. These changes are characteristic of the presence of substantial amounts of amyloid (11).

Circular Dichroism Spectroscopy—The secondary structure of apoC-II (0.3 g/liter) was monitored using CD spectroscopy as a function of time in the absence and presence of 100 g/liter dextran. The spectra of freshly prepared apoC-II in the absence and presence of dextran (Fig. 7, A and B) are similar, reflecting a largely disordered conformation. The minor differences observed suggest the onset of a small degree of aggregation in the solution containing dextran during the 20-min period of CD data acquisition (cf. Fig. 1). Similarly, the spectra of the two solutions after 2 days of incubation, which reflect extensive β-structure characteristic of apoC-II amyloid (11, 22), are nearly identical. The only significant difference appears to be a more rapid shift toward the final spectrum in the solution containing dextran.

Electron Microscopy—The effect of high concentrations of dextran T10 on amyloid morphology was investigated using electron microscopy. Micrographs of apoC-II incubated for 3 days in the absence of dextran (Fig. 8A) reveal the presence of helical twisted amyloid ribbons with no evidence of amorphous aggregates, similar to those previously observed (11). Aggregates formed in the presence of 100 g/liter dextran (Fig. 8B) appeared indistinguishable from those formed in the absence of dextran. No evidence of mixed aggregates of dextran and apoC-II was found. These results indicate that high concentrations of dextran do not promote amorphous aggregation of apoC-II or the formation of apoC-II/dextran heterocomplexes.

Sedimentation Velocity—The size of the amyloid fibrils formed by apoC-II in the presence of dextran was investigated by sedimentation velocity analysis. Fig. 9 shows sedimentation...
by the gray lines in Fig. 9 (A and B). The sedimentation coefficient distribution for apoC-II in the absence of dextran (Fig. 9C, solid line) reveals a broad population of species between 30 and 300 S with a maximum at 100 S, consistent with previous estimates of 50–100 S for apoC-II amyloid (9, 11, 22). The sedimentation of apoC-II was slowed by the higher viscosity of 100 g/liter dextran (Fig. 9B). The sedimentation coefficient distribution, when standardized to the viscosity of 100 mM sodium phosphate buffer, generates a distribution of species in the range 30–400 S, with a maximum at 150 S (Fig. 9C, dashed line). Within the uncertainties of the method, the results indicate similar size distributions of the amyloid formed in the presence and absence of dextran, consistent with the electron micrographs indicating no significant alteration in the overall morphology of the fibrils.

Elimination of Possible Effects Due to Contamination or Specific Interaction

In principle, addition of large quantities of a cosolute such as dextran could affect the behavior of apoC-II by a variety of mechanisms as follows: 1) The cosolute could alter the pH and/or the ionic strength of the solution, two variables that are known to influence the kinetics of amyloid formation by apoC-II.\(^4\) We therefore investigated the effect of high dextran concentration on these parameters. The conductivity of 100 mM sodium phosphate solutions did not vary significantly upon addition of dextran at concentrations between 50 and 150 g/liter. Moreover, the addition of 150 g/liter dextran to 100 mM sodium phosphate, pH 7.4, had negligible effect on the pH. 2) The cosolute could strongly bind to and coaggregate with apoC-II. Sedimentation velocity experiments were therefore carried out on apoC-II in the absence and presence of 2 g/liter dextran T10. No difference in sedimentation coefficient of the apoC-II was observed, ruling out the presence of a significant abundance of high affinity heterocomplexes between dextran and apoC-II.

Estimate of Excluded Volume Effect

Our various experimental measurements (CD, Congo Red, and thioflavin T binding assays, electron microscopy, sedimentation velocity analysis, pH, and conductivity measurements) collectively demonstrate that the effect of dextran on the rate of apoC-II amyloid formation can be attributed neither to the presence of a contaminant nor to the formation of apoC-II-dextran complexes. It follows that the effect must be due to a repulsive interaction between the added polymer and apoC-II that destabilizes the monomeric form of the apolipoprotein relative to the aggregated form, in effect stabilizing the aggregate relative to the soluble form. Although electrostatic repulsion could play a part, the reduction of volume available to apoC-II because of the added polymer (“macromolecular crowding”) is the most likely source of such interaction, because the charge density of dextran is quite low, and, moreover, macromolecular crowding is known to promote a variety of macromolecular associations (25, 26). This conclusion is supported by the observation that apoC-II amyloid formation is accelerated in a qualitatively similar manner by the addition of another watersoluble polymer, heparin, which possesses a substantially higher net charge per unit mass than that of dextran (data not shown).

The contribution of excluded volume to the effect of dextran on the rate of apoC-II amyloid formation may be estimated using the excluded volume model presented above, together with rough estimates of the molecular dimensions of the vari-

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\(^4\) D. M. Hatters and G. J. Howlett, unpublished data.
ous macromolecular species. The effective radius of monomeric apoC-II, \( r_{1} \), is estimated to be equal to 2.1 nm, the Stokes' radius of the equivalent hydrodynamic particle, calculated from the molar mass, partial specific volume, and experimentally measured sedimentation coefficient (40). The radius of the effective spherocylinder representing the apoC-II fiber, \( r_{c}\), is taken to be 2.4 nm, corresponding to a cross-sectional area approximately equal to that obtained from analysis of electron and atomic force micrographic images of the actual fiber. The increment of fiber length corresponding to the addition of a single molecule of apoC-II to the growing fiber, \( \Delta \), is taken to be equal to 0.5 nm. The radius of the effective cylindrical rod representing the dextran polymer, \( r_{\text{dex}}\), is taken to be 0.7 nm, in accordance with previous estimates in the literature (41, 42).

The specific exclusion volume of dextran, \( v_{\text{dex}} \), is expected to be in the vicinity of 0.8 cm\(^3\)/g, in accordance with previous evaluations (42), but was evaluated by least-squares modeling as described below.

The value of log \( t_{50}^{1/2} \) was allowed to be different for each of two measurements due to differences in apoC-II concentration and in the technique of measurement, but the value of the second term on the right hand side of Equation 10, describing the dependence upon \( c_{\text{dex}} \), was assumed to be common to both measurements. The dimensions of the effective hard particles were allowed to vary to achieve a best fit of Equation 10 to the combined data on the dependence of log \( t_{50} \) on dextran concentration, as measured by both techniques. The best-fit calculated dependence of log \( t_{50} \) on \( c_{\text{dex}} \) is plotted together with the data in Fig. 4. The best-fit value of \( v_{\text{dex}} \), 0.75 cm\(^3\)/g, is in good agreement with previous estimate of the specific excluded volume (42). It is therefore concluded that the effect of dextran on the overall rate of apoC-II amyloid formation may be accounted for semiquantitatively by the hypothesis that the transition state for addition of a monomer of apoC-II to a growing oligomer excludes less volume to inert macromolecules than to the fully separated reactive species.

**REFERENCES**

1. Kelly, J. W. (1996) Curr. Opin. Struct. Biol. 6, 11–17
2. Faccini, P. (1999) Methods Enzymol. 309, 256–274
3. Dobson, C. M. (1999) Trends. Biochem. Sci. 24, 329–332
4. Booth, D. R., Sunde, M., Bellotti, V., Robinson, C. V., Hutchinson, W. L., Fraser, P. E., Hawkins, P. N., Dobson, C. M., Radford, S. E., Blake, C. F. C., and Pops, M. B. (1997) Nature 385, 787–793
5. Fandrich, M., Fletcher, M. A., and Dobson, C. M. (2001) Nature 410, 165–166
6. Havel, R. J., Fielding, C. J., Olivera, T., Shore, V. G., Fielding, P. E., and Fielding, P. A. (1973) Biochemistry 12, 1829–1833
7. Jackson, R. L., and Holdsworth, G. (1986) Methods Enzymol. 128, 288–297
8. Tajima, S., Yokoyama, S., Kawai, Y., and Yamamoto, A. (1982) J. Biochem. 91, 1279–1279
9. Hatters, D. M., Lawrence, L. J., and Howlett, G. J. (2001) FEBS Lett. 494, 220–224
10. MacRaid, C. A., Hatters, D. M., Howlett, G. J., and Gooley, P. R. (2001) Biochemistry 40, 5414–5421
11. Hatters, D. M., MacPhee, C. E., Lawrence, L. J., Sawyer, W. H., and Howlett, G. J. (2000) Biochemistry 39, 8276–8283
12. Westerman, P., Mariani, L., Maghini, B., Johnson, K. H., and Sletten, K. (1995) Am. J. Pathol. 147, 1186–1192
13. Wisniewski, T., Gelabak, A. A., Kida, E., Wisniewski, K. E., and Frangione, B. (1995) Am. J. Pathol. 147, 238–244
14. Higuchi, K., Rigatow, K., Naiki, H., Hanada, K., Hosokawa, M., and Takeda, T. (1991) Biochem. J. 279, 427–433
15. Benson, M. D., Liepniets, J. J., Yazaki, M., Yasahita, T., Hamidi Asl, K., Kishimoto, T., and Frangione, B. (1995) J. Biol. Chem. 270, 272–277
16. Bergstrom, J., Murphy, C., Eulitz, M., Weiss, D. T., Westerman, G. T., Solomon, A., and Westerman, P. (2001) Biochem. Biophys. Res. Commun. 285, 903–908
17. Wisniewski, T., Lalowski, M., Gelabak, A., Vogel, T., and Frangione, B. (1995) Lancet 345, 956–958
18. El-Agnaf, O. M., and Irvine, G. B. (2000) J. Struct. Biol. 130, 360–369
19. Husebekk, A., Skogen, B., Husby, G., and Marhaug, G. (1985) Scand. J. Immunol. 21, 283–287
20. Hatters, D. M., and Howlett, G. J. (2002) Eur. Biophys. J., in press
21. Nettleton, E. J., Sunde, M., Lai, Z., Kelly, J. W., Dobson, C. M., and Robinson, C. V. (1998) J. Mol. Biol. 281, 553–564
22. Hatters, D. M., Lindner, R. A., Carver, J. A., and Howlett, G. J. (2001) J. Biol. Chem. 276, 33755–33761
23. Zimmerman, S. B. (1993) Biophys. J. 64, 114–119
24. Lamsbury, P. T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3342–3344
25. Minton, A. P. (2000) Curr. Opin. Struct. Biol. 10, 34–39
26. Wang, C. S., Downs, D., Dashit, A., and Jackson, K. W. (1996) Biochim. Biophys. Acta 1302, 224–230
27. Bohlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Arch. Biochem. Biophys. 153, 213–220
28. Schuck, P., and Rosemanllit, P. (2000) Biopolymers 54, 328–341
29. Schuck, P., MacPhee, C. E., and Howlett, G. J. (1996) Biophys. J. 74, 466–474
30. Minton, A. P. (2001) Biophys. J. 80, 1841–1846
31. Minton, A. P. (1998) Method. Enzymol. 295, 127–149
32. Ogston, A. G. (1956) Trans. Faraday Soc. 54, 1754–1757
33. Giddings, J. C., Kucera, E., Russell, S. P., and Myers, M. N. (1968) J. Mol. Biol. 47, 3487–4408
34. LeVine, H. (1999) Protein Sci. 8, 404–410
35. LeVine, H. (1999) Methods Enzymol. 309, 274–284
36. Klunk, W. E., Jacob, R. F., and Mason, R. P. (1999) Methods Enzymol. 309, 285–305
37. Tanford, C. (1963) Physical Chemistry of Macromolecules, pp. 317–456, John Wiley & Sons, Inc. New York
38. Laurent, T. C., and Killander, J. (1964) J. Chromatogr. 14, 317–330
39. Rivas, G., Fernández, J. A., and Minton, A. P. (1999) Biochemistry 38, 9379–9388
40. Zimmerman, S. B., and Minton, A. P. (1993) Annu. Rev. Biophys. Biomol. Struct. 22, 27–65
41. Naher, D., Korte, U., and Krack, K. (1979) Exp. Geront. 14, 59–63
42. Nagy, I., Nagy, K., Nagy, V., Kalmar, A., and Nagy, E. (1981) Exp. Gerontol. 16, 229–240
43. Nagy, I. Z., Nagy, K., and Lustigk, G. (1982) Exp. Brain Res. Suppl. 5, 118–122
44. Barber, B. J., Babbitt, R. A., Parneswaran, S., and Dutta, S. (1995) J. Gerontolol. A: Biol. Sci. Med. Sci. 50, B282–B287
