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Sub-micellar phospholipid accelerates amyloid formation by apolipoprotein C-II

Danny M. Hatters[a], Lynne J. Lawrence[b], Geoffrey J. Howlett[a,*]

[a] *Department of Biochemistry and Molecular Biology, The University of Melbourne, Parkville, Vic. 3010, Australia*
[b] *CSIRO, 343 Royal Parade, Parkville, Vic. 3052, Australia*

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Abstract Lipid-free human apolipoprotein C-II (apoC-II) forms amyloid fibrils with characteristic β-structure. This conformation is distinct from the α-helical fold of lipid-bound apoC-II. We have investigated the effect of the short-chain phospholipid, dihexanoylphosphatidylcholine (DHPC) on amyloid formation by apoC-II. The α-helical content of apoC-II increases in the presence of micellar DHPC (16 mM) and amyloid formation is inhibited. However, at sub-micellar DHPC concentrations (below 8 mM) amyloid formation is accelerated 6 fold. These results suggest that individual phospholipid molecules in vivo may exert significant effects on amyloid folding pathways. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Dihexanoylphosphatidylcholine; Amyloid nucleation; Folding pathway; Atherosclerosis; Analytical ultracentrifugation

1. Introduction

The primary sequence of mature human apolipoprotein (apo)C-II, composed of 79 amino acids, contains amphipathic α-helical regions that account for the increase in α-helical content upon binding to phospholipid [1,2]. In contrast, lipid-free apoC-II aggregates to form β-structure with all of the characteristics of amyloid fibrils [3]. In this regard apoC-II is similar to apoA-I and the C-terminal domain of apoE which also form amyloid in vitro [4,5]. Apolipoproteins are prevalent in amyloid deposits in vivo [6,7]. For instance, immunohistochemical studies demonstrate colocalization of apoA-I, apoE and apoB with amyloid in atherosclerotic plaques [8] while specific cases exist where apoA-I is the primary component of atherosclerotic amyloid [9]. These studies suggest a general propensity of apolipoproteins to form amyloid fibrils, an event that may relate to the structural requirements for lipid binding and the common ancestry of the apolipoprotein family [10].

To investigate the role of phospholipid on amyloid formation by apoC-II we used the short-chain phospholipid dihexanoylphosphatidylcholine (DHPC). DHPC has 6-carbon acyl chains that cause it to behave as a detergent micelle in aqueous solution, rather than forming the lipid bilayers characteristic of longer-chained phospholipids. This allowed an analysis of the effects of micellar and sub-micellar lipid on amyloid formation.

2. Materials and methods

2.1. Materials

DHPC was obtained from Avanti Polar Lipids (Alabaster, AL, USA). ApoC-II was expressed [11] and purified as described previously [3] and stored as a stock in 5 M guanidine hydrochloride (GuHCl) at a concentration of approximately 30 mg/ml. Refolded apoC-II was prepared by adding sequential aliquots of 50 μg to 1 ml of 100 mM sodium phosphate, pH 7.4, at 2 min intervals, to a final concentration of 0.3–0.45 mg/ml.

2.2. Spectroscopic analysis

Samples of apoC-II (0.3 mg/ml) containing a range of DHPC concentrations from 0 to 16.8 mM were prepared. Emission spectra were obtained using a SPEX τ-2 fluorolog fluorescence spectrometer over the wavelengths 330–375 nm at 5 nm intervals with excitation at 295 nm using a 0.5 cm quartz cuvette. The excitation and emission slit widths were 1.89 nm and 5 nm respectively. Circular dichroism (CD) spectra were recorded at 0.5 nm intervals with a 1 s integration time and the bandwidth was set at 1.5 nm using an Aviv 62DS CD spectrometer with 1 mm pathlength quartz cuvettes. The fluorescence and CD spectra were corrected by subtracting the spectra of samples containing no apoC-II at the respective DHPC concentrations. Amyloid formation by apoC-II (0.3 mg/ml) with 0.1% (w/v) sodium azide was monitored by turbidity measurements at 4 min intervals (Cary-5 spectrophotometer, Varian, Australia). Data were corrected for baselines determined for buffer alone. Controls containing no apoC-II indicated negligible changes in absorbance due to DHPC.

2.3. Analytical ultracentrifugation

Solutions of apoC-II (0.3 mg/ml) with the addition of 0.1% (w/v) sodium azide were analyzed using the XL-A analytical ultracentrifuge (Beckman/Coulter). The sedimentation boundaries at different time points were analyzed to obtain molecular masses and sedimentation coefficients using a model for a single sedimenting species [12]. The partial specific volume (0.732 ml/g) and degree of hydration (0.4 g/g) for apoC-II were calculated from the amino acid composition [13].

2.4. Electron microscopy

Samples of apoC-II (0.3 mg/ml) in the presence or absence of DHPC were negatively stained with 2% potassium phosphotungstate as described [3]. The samples were imaged using a JEOL 2000FX transmission electron microscope operating at 120 kV. Images were photographed at a nominal magnification of 60 000× or 80 000×.

3. Results

3.1. Spectroscopic analysis of the interaction of apoC-II with DHPC

The effect of DHPC on the secondary structure of apoC-II

*Corresponding author. Fax: (61)-3-9347 7730.
E-mail: ghowlett@unimelb.edu.au

Abbreviations: apoC-II, apolipoprotein C-II; DHPC, dihexanoylphosphatidylcholine; CD, circular dichroism; GuHCl, guanidine hydrochloride; CMC, critical micelle concentration

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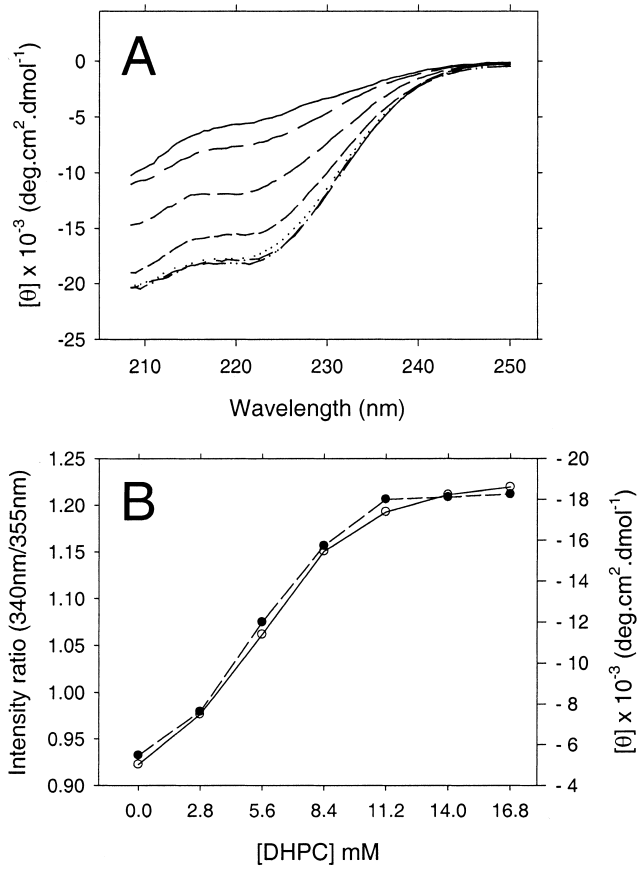

Fig. 1. A: DHPC-induced changes in the CD spectrum of apoC-II. The spectrum of apoC-II (0.3 mg/ml) was measured in the absence of DHPC (solid line) and in the presence of increasing concentrations of DHPC (2.8 mM – long dashed, 5.6 mM – medium dashed, 8.4 mM – short dashed, 11.2 mM – dotted, 14 mM – dashed-dotted, 16.8 mM – dashed-dotted-dotted). B: DHPC titration of apoC-II (0.3 mg/ml) monitored by CD and fluorescence emission spectroscopy. The data are presented as the average molar ellipticity at 220–222 nm (closed circles, dashed line), and the ratio of emission intensities at 340 and 355 nm upon excitation at 295 nm (open circles, solid line).

was measured by CD spectroscopy (Fig. 1A). Increasing concentrations of DHPC caused a systematic decrease in ellipticity in the wavelength range 210–240 nm. The decrease in the molar ellipticity at 222 nm (Fig. 1B) and the change in the shape of the spectra in the presence of 16.8 mM DHPC (Fig. 1A) indicated a change from largely unordered structure to a more ordered, α-helical structure. Addition of increasing concentrations of DHPC resulted in a systematic blue shift in the emission wavelength maxima of the single tryptophan in apoC-II from 350 to 335 nm. This change was also used to monitor the effect of DHPC binding (Fig. 1B). The overlay of the CD and fluorescence data in Fig. 1B suggests that each method monitors the same conformational event. The sigmoidal titration curve defines a cooperative transition with a midpoint at approximately 6 mM DHPC. This concentration is significantly lower than the values of 10–16 mM reported for the critical micelle concentration (CMC) of DHPC [14]. Sedimentation equilibrium experiments with varying concentrations of DHPC under the conditions used in Fig. 1 yielded values for the CMC of approximately 8–10 mM (C. MacRaild and G. Howlett, personal communication). The data in Fig. 1 therefore demonstrate a significant interaction between apoC-II and sub-micellar concentrations of DHPC consistent with either the binding of individual molecules of DHPC to apoC-II or the induction of DHPC micelle formation by apoC-II.

3.2. Characterization of apoC-II aggregation

Sedimentation velocity profiles for apoC-II alone or in the presence of sub-micellar DHPC (2.8 mM) are presented in Fig. 2. Initial scans reveal a clearly resolved fast moving boundary in the presence of 2.8 mM DHPC (Fig. 2B) that is not evident in the data for apoC-II alone (Fig. 2A). The rate of movement of the midpoint of this fast moving boundary indicates sedimentation coefficients in excess of 30 S and in the range reported previously [3]. This rapidly migrating material accounts for approximately 10% of the optical density at this wavelength and is depleted from the solution plateau after 40 min of centrifugation. Analysis of the slower moving boundary over the time interval 50–250 min of sedimentation, gave good fits to a single species of molecular mass close to monomer (Table 1). Sedimentation velocity data for apoC-II alone (Fig. 2A) over the same time interval also gave good fits to a single species and a molecular mass close to monomer (Table 1). These molecular mass values suggest the predominance of apoC-II monomer (MW 8900) and the lack of any significant induction of micelle formation by apoC-II. For comparison, sedimentation velocity experiments were also performed using micellar concentrations of DHPC (16.8 mM). The molecular mass obtained under these conditions (Table 1) is significantly higher than for apoC-II alone, consistent with the binding of apoC-II to DHPC micelles.

The fast moving boundary in Fig. 2B suggests that sub-micellar DHPC promotes amyloid formation by apoC-II. Sedimentation velocity experiments were used to characterize the time course for this process. ApoC-II samples were incubated at room temperature for 24 h in the presence and absence of DHPC (2.8 mM). Sedimentation velocity profiles for apoC-II alone (Fig. 2C) show a rapidly moving boundary corresponding to approximately 50% of the total absorbing material and a slower boundary corresponding to predominantly monomeric apoC-II as observed in Fig. 2A. The data shown in Fig. 2D for apoC-II after 24 h in the presence of DHPC (2.8 mM) indicate more extensive formation of the fast sedimenting species with only minor amounts of slow moving material.

Electron microscopy was used to further characterize the aggregates formed by apoC-II in the presence of sub-micellar DHPC (2.8 mM DHPC). The fibrils formed in the presence of 2.8 mM DHPC have a fibrous morphology indistinguishable to fibrils formed in the absence of DHPC [3]. There was no

Table 1
Effect of DHPC on the molecular mass and sedimentation coefficient of apoC-II[a]

Addition	MW	*s*
None	8 700 (+300, −300)	0.96 (+0.017, −0.016)
DHPC (2.8 mM)	9 300 (+300, −300)	1.00 (+0.009, −0.009)
DHPC (16.8 mM)	12 700 (+200, −200)	1.16 (+0.006, −0.004)

[a]Molecular masses (MW) and sedimentation coefficients (*s*) were determined by non-linear least squares analysis of multiple scans at different time points using the program SEDFIT [13]. Values in brackets represent errors calculated using F-statistics with a 68% confidence interval.

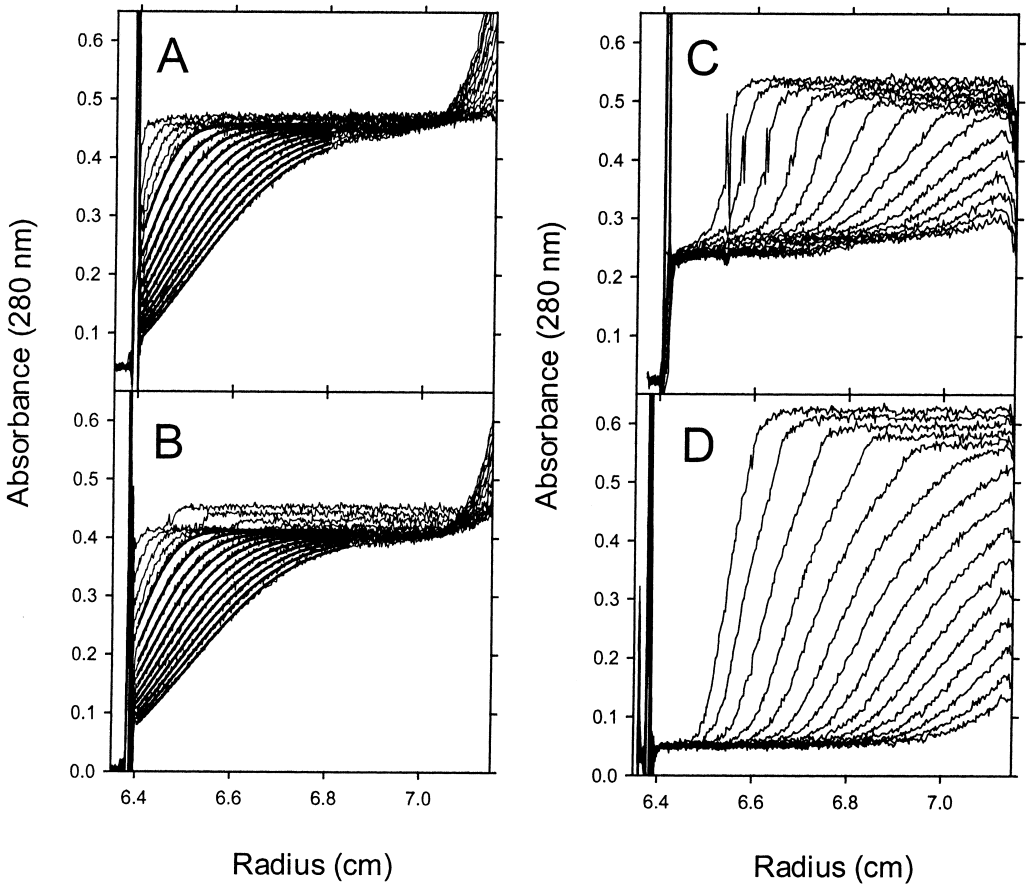

Fig. 2. Sedimentation velocity analysis of apoC-II (0.3 mg/ml) in the presence and absence of sub-micellar DHPC. For freshly prepared apoC-II samples (A: apoC-II alone; B: apoC-II in the presence of 2.8 mM DHPC), the first five sequential scans were taken at 10 min intervals at a rotor speed of 40 000 rpm. The 10 subsequent scans were taken at 20 min intervals. Lines through the data represent numerical fits to the data assuming a single sedimenting species (thick lines). For aliquots of the same samples incubated for 1 day (C: apoC-II alone; D: apoC-II in the presence of 2.8 mM DHPC), scans were taken at 5 min intervals at a rotor speed of 15 000 rpm.

indication of amorphous material. Parallel samples of apoC-II were also prepared in the presence of 16.8 mM DHPC. For these samples, no fibrillar or amorphous material was observed in repeated grids/samples or of material incubated for 1 week at room temperature, indicating an inhibitory effect of micellar DHPC on amyloid formation by apoC-II.

3.3. Effect of DHPC on amyloid formation by apoC-II

Turbidity measurements were used to monitor the effects of DHPC on amyloid formation by apoC-II (Fig. 3A). Sub-micellar concentrations of DHPC (2.8 and 5.6 mM) induce a more rapid and extensive change in turbidity compared to the data obtained for apoC-II alone. Conversely, DHPC at micellar concentrations (>11 mM) causes a systematic reduction in the turbidity increase over the same time scale. These changes in the rate of amyloid formation by apoC-II are summarized by the results in Fig. 3B where the rate of change, expressed as the change in optical density over the first 8 h is plotted as a function of DHPC concentration. The data illustrate the acceleration in rate induced by sub-micellar DHPC and the substantial inhibition observed at DHPC concentrations above the CMC.

4. Discussion

The effect of micellar DHPC on apoC-II aggregation can be compared with the effect of lipids on other systems known to form insoluble fibrils. The Alzheimer's amyloid peptide Aβ(1–40), binds to ganglioside containing membranes with a transition from random coil to β-sheet, accompanied by accelerated fibrillogenesis [15]. A similar transformation to β-structure aggregate is seen when Aβ(1–40) binds to negatively charged lipids at low lipid-to-peptide ratios [16]. The neuronal protein α-synuclein binds to acidic phospholipid vesicles and undergoes a random coil to α-helical transition [17]. A mutant form of α-synuclein (A53T), linked to early-onset familial Parkinson's disease, shows reduced membrane binding and more rapid oligomerization kinetics [18]. These results demonstrate that the binding of amyloidogenic peptides and proteins to lipid surfaces can induce secondary structure with the potential to either increase or decrease the relative rates of amyloid fibril formation.

An unexpected finding of the present work was the enhancement of aggregation of apoC-II at sub-micellar DHPC concentrations (Fig. 3). This was in contrast to the inhibition observed at micellar concentrations and leads us to propose the following model (Fig. 4). Under denaturing conditions apoC-II exists as a random coil (state 1) lacking ordered secondary structure. Removal of the denaturant generates lipid-free apoC-II (state 2) characterized by a limited degree of secondary structure. Lipid-free apolipoproteins in general, for example apoA-I and the C-terminal domain of apoE,

have a low energy of stabilization and a loosely defined tertiary structure [19,20]. Such partially folded conformations are a common characteristic of several proteins that form amyloid [21,22]. The sedimentation coefficient and molecular mass of lipid-free apoC-II (Table 1) indicate an extended conformation characterized by a high axial ratio (4.9) rather than a value close to unity as expected for a globular conformation. The model in Fig. 4 proposes that loose, flexible structures, represented by state 2, account for the time dependent aggregation of apoC-II to form amyloid (state 6). Upon binding to lipid, apoC-II gains significant α-helical content (state 3). This satisfies the requirements for compact folding with hydrogen-bound peptide bonds and hydrophobic groups sequestered from the aqueous environment and with the lipid providing core structure. According to the model, events that dramatically perturb lipid binding, such as mutations in the case of apoA-II [6] or truncations in the case of apoA-I [7,9], promote amyloidosis by preventing compact interaction of the protein or peptide with lipid. Lipid modification by enzymes such as lipoprotein lipase or by oxidation may also favor the amyloid state relative to lipid binding.

Comparison of Fig. 2A,B with Fig. 2C,D indicates sub-micellar concentrations of DHPC induce a cooperative and more extensive polymerization of apoC-II monomers to large aggregates. This increased aggregation coupled with the lack of a significant increase in the molecular mass of slow moving apoC-II in the presence of sub-micellar DHPC (Table 1) suggests a capacity of apoC-II to interact with individual molecules of DHPC (state 4). CD spectroscopy suggests this intermediate has increased secondary structure (Fig. 1). We propose that this state has a propensity to aggregate (state 5) and to nucleate amyloid formation (state 6). This would explain the significant increase in the rate of amyloid formation by apoC-II at sub-micellar concentrations of DHPC (Fig. 3). The ability of apoC-II to bind to sub-micellar DHPC raises the question of whether apoC-II might also bind to the individual molecules of the longer chain phospholipids present in vivo. Atherosclerotic plaques contain a necrotic core that includes free-radical damaged lipids [23] and micellar lipids such as lysolecithin and fatty acids which may also play a role in nucleating apolipoprotein aggregation. The common ancestry of the apolipoprotein family and structural conservation of the class A amphipathic helices suggests that the results presented here for apoC-II may be generally relevant to the high incidence of apolipoproteins in amyloid related diseases.

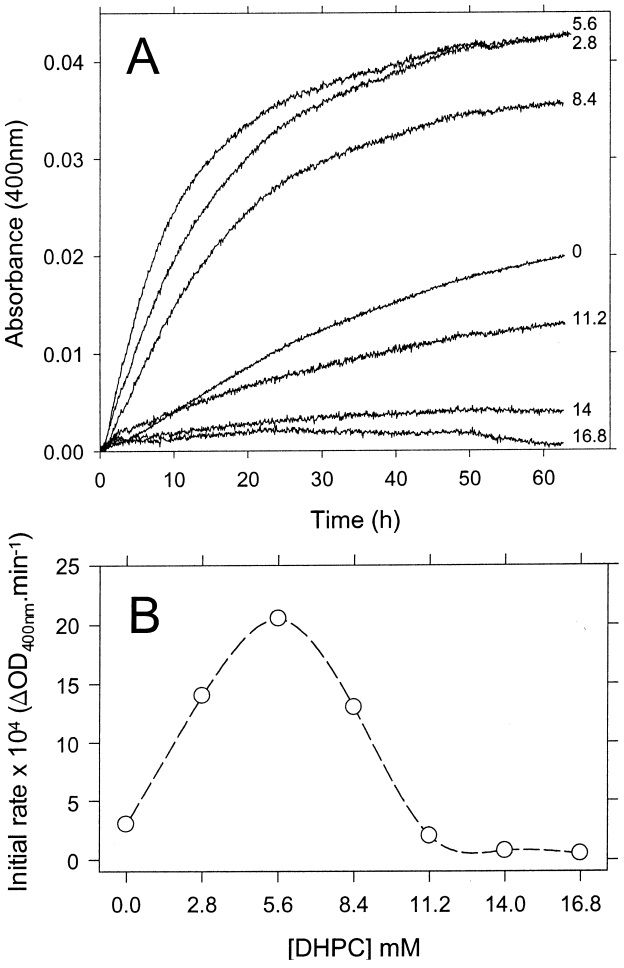

Fig. 3. The effect of DHPC on the time dependence of apoC-II turbidity. A: The absorbances of apoC-II samples (0.3 mg/ml) as a function of time were measured in the presence of DHPC at the concentrations (mM) indicated. B: Rate of turbidity change (first 8 h) as a function of DHPC concentration.

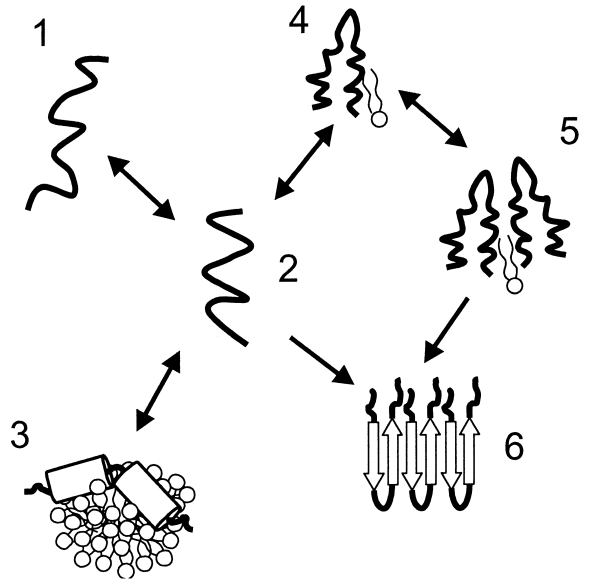

Fig. 4. Schematic of the proposed folding pathways for apoC-II in the presence and absence of lipid. State 1: Unfolded apoC-II. State 2: Loosely folded, mostly unordered lipid-free conformation. State 3: Lipid-bound α-helical conformation formed in the presence of phospholipid micelles or bilayers. State 4: ApoC-II bound to sub-micellar phospholipid monomers or dimers. State 5: Self-association of sub-micellar phospholipid/apoC-II complexes with increased β-sheet content. State 6: ApoC-II amyloid.

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