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Phospholipids enhance nucleation but not elongation of apolipoprotein C-II amyloid fibrils

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Abstract

Amyloid fibrils and their oligomeric intermediates accumulate in several age-related diseases where their presence is considered to play an active role in disease progression. A common characteristic of amyloid fibril formation is an initial lag phase indicative of a nucleation-elongation mechanism for fibril assembly. We have investigated fibril formation by human apolipoprotein (apo) C-II. ApoC-II readily forms amyloid fibrils in a lipid-dependent manner via an initial nucleation step followed by fibril elongation, breaking and joining. We used fluorescence techniques and stopped-flow analysis to identify the individual kinetic steps involved in the activation of apoC-II fibril formation by the short-chain phospholipid dihexanoyl phosphatidylcholine (DHPC). Sub-micellar DHPC activates fibril formation by promoting the rapid formation of a tetrameric species followed by a slow isomerisation that precedes monomer addition and fibril growth. Global fitting of the concentration dependence of apoC-II fibril formation showed that DHPC increased the overall tetramerization constant from 7.5×10^{-13} to $1.2 \times 10^{-6} \mu\text{M}^{-3}$ without significantly affecting the rate of fibril elongation, breaking or joining. Studies on the effect of DHPC on the free pool of apoC-II monomer and on fibril formation by cross-linked apoC-II dimers further demonstrate DHPC affects nucleation but not elongation. These studies demonstrate the capacity of small lipid compounds to selectively target individual steps in the amyloid fibril forming pathway.

Keywords

Sedimentation velocity; protein self-assembly; kinetic mechanism; amyloid kinetics; Amyloid fibrils; phospholipid; nucleation

INTRODUCTION

Amyloid fibrils are formed by the self-assembly of proteins into linear aggregates that share a number of common characteristics including a cross- β morphology revealed by X-ray diffraction analysis of aligned fibrils and the ability to interact with the dyes Congo Red and Thioflavin T (ThT). The discovery that amyloid deposits are associated with several

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deilitating and age-related diseases including Alzheimer's and Parkinson's disease, type II diabetes and atherosclerosis has lead to considerable research effort aimed at defining and controlling the pathways for fibril formation. A general characteristic of amyloid fibril forming pathways is a distinct lag phase, indicative of a nucleation step that precedes fibril elongation. Evidence for this initial nucleation step is provided in "seeding" experiments where pre-formed fibrils or fibril fragments added to the solution, abolish the initial lag phase. The processes of nucleation and elongation, while kinetically distinct, are commonly thought to be related. Mutational studies of A β and β 2-microglobulin show that nucleation (as measured by the length of the lag phase) and elongation are correlated, suggesting that regions which are important for nucleation are also important for elongation^{1; 2}. External factors, such as the presence of anions, or macromolecular crowding agents have also been shown to affect nucleation and elongation to a similar extent^{3; 4}.

Human apolipoprotein (apo) C-II is a physiological activator of lipoprotein lipase and a typical member of the plasma apolipoprotein family. ApoC-II is one of a number of plasma apolipoproteins which accumulate in atherosclerotic plaques and co-localize with serum amyloid P, an *in vivo* marker of amyloid fibrils⁵. Lipid-binding proteins are prominent among the proteins known to form amyloid deposits *in vivo*⁶. In the absence of lipids, apolipoproteins show very little conformational stability^{7; 8}, perhaps explaining the high propensity of these proteins to form amyloid fibrils. Amyloid fibrils composed of either A β or apoC-II activate macrophages in a CD36 receptor-dependent process that has been proposed as an early step in foam cell formation and the development of atherosclerosis^{9; 10}.

ApoC-II forms fibrils *via* a reversible nucleation-elongation process coupled with fibril breaking and rejoining¹¹. This pathway is activated by the addition of sub-micellar phospholipids^{12; 13; 14}. Our previous work defined an on-pathway tetramer formed in the presence of sub-micellar dihexanoylphosphatidylcholine (DHPC) that correlates with the activating effect of this phospholipid on apoC-II fibril formation¹⁴. We have applied fluorescence techniques to monitor the rate of formation of this tetramer and to define the kinetic parameters associated with the activation of apoC-II fibril formation by phospholipids. The results of our study support the distinct nature of nucleation and elongation, clearly demonstrating that sub-micellar DHPC promotes the nucleation of apoC-II fibrils without significantly affecting the rate of fibril elongation or the rate of fibril breaking and joining.

RESULTS

The equilibrium binding of DHPC to apoC-II

The interaction of phospholipids with apoC-II was initially explored by determining the change in Alexa-488 apoC-II fluorescence intensity as a function of DHPC concentration (Figure 2). These experiments were performed at a fixed apoC-II concentration of 5 μ M, where fibril formation is negligible. The Alexa-488 fluorescence shows a sigmoidal dependence on phospholipid concentration up to DHPC concentrations of 8 mM. The abrupt decrease in intensity above 8–10 mM correlates with the critical micelle concentration of DHPC, previously determined to be approximately 10mM¹⁴. The concentration of DHPC required to reach the maximum change in fluorescence intensity is consistent with DHPC-protein dissociation constants in the low millimolar concentration range. Our previous studies show that the change in fluorescence intensity induced by sub-micellar DHPC is accompanied by the formation of a discrete apoC-II tetramer¹⁴.

Stopped flow analysis of apoC-II tetramerisation

The rate of apoC-II tetramerisation induced by sub-micellar DHPC was investigated using stopped-flow kinetics and monitoring FRET between Alexa-488 apoC-II and Alexa-594 apoC-

II as described previously¹⁴. Alexa-594 apoC-II and Alexa-488 apoC-II at a 4:1 ratio (total apoC-II concentration 1.25, 2.5 μ M and 5 μ M) were mixed with buffer containing 1, 3, or 5 mM DHPC. Figures 3A and 3B show that DHPC induced a rapid increase in FRET, consistent with rapid tetramerisation. Since apoC-II fibrils do not form at these concentrations the data were fitted globally using Equations 1–2 with all parameters relating to kinetic steps subsequent to the initial tetramerisation constrained to zero. The analysis assumed that at the end point of the timecourse for 5 μ M apoC-II in the presence of 5 mM DHPC an equilibrium was reached corresponding to 50% tetramer by weight. This assumption is based on previous sedimentation analysis¹⁴. The solid lines in Figure 3 represent the best fit values for k_{on} and k_{off} (Table 1).

Kinetics of the slow step associated with tetramerisation

The fluorescence intensity timecourse for apoC-II fibril formation in the presence of DHPC displays a slow fluorescence change which follows the initial tetramerisation event¹⁴. This slow change has been attributed to either rearrangement of bound lipid or a slow conformational change¹⁴. The possibility of a conformational change was tested further using circular dichroism (CD) spectroscopy (Figure 4). The CD spectrum of apoC-II displayed a large transition upon addition of DHPC similar to that previously observed¹⁴ (Figure 4A). This fast spectral alteration indicated the rapid acquisition of α -helical structure and was followed by a further slow change in the spectrum. The CD spectrum of the sample after 2 hours incubation is consistent with a loss of α -helical structure. The time dependence of the slow CD change was monitored by measuring the change in mean residue ellipticity at 208 nm over time (Figure 4B). The 208 nm mean residue ellipticity decreased over 1–2 hours and was protein concentration dependent. While unimolecular isomerization reactions are independent of concentration we attribute the observed concentration dependence to the linked concentration-dependent tetramerization depicted in Figure 1. The curves for apoC-II at 5 and 2.5 μ M in the presence of 5 mM DHPC provided times to half maximum change ($t_{1/2}$) of 17.1 and 31.3 minutes, respectively. This change in secondary structure is comparable to the change in Alexa-488 fluorescence intensity over time for labeled apoC-II at 5 and 2.5 μ M in the presence of 5 mM DHPC (Figure 4C), which yield $t_{1/2}$ values of 16.4 and 35.4 minutes, respectively. The data in Figure 4C and similar data acquired for apoC-II in the presence of 1 and 3 mM DHPC were globally analyzed assuming a rapid monomer-tetramer equilibrium followed by a slow isomerisation using equations 1–3 with the rate constants for fibril elongation constrained to zero and the initial monomer-tetramer rate constants fixed to those presented in Table 1. The lines of best-fit shown in Figure 4C, yielded the kinetic parameters for rapid tetramerisation and slow isomerisation listed in Table 1.

Global analysis of the kinetics of apoC-II fibril formation in the presence of phospholipid

Measuring the rate of the initial tetramerisation step provided insight into the effect of sub-micellar phospholipids on the early stages of fibril formation. A global approach was employed to investigate the effect of these lipids on fibril elongation. Previous studies on the kinetics of lipid-free apoC-II fibril formation took advantage of the solubility and relative homogeneity of the fibrillar end products, which allowed the use of sedimentation velocity analysis to determine the size distribution of fibrils¹¹. The data acquired from these experiments was fitted using a mathematical description of the evolution of nuclei and fibrillar species over time¹¹. This approach was adopted for the analysis of the kinetics of apoC-II fibril formation in the presence of sub-micellar DHPC. Fibril formation was monitored by collecting radial absorbance scans at 8,000 rpm where fibrillar, but not monomeric apoC-II, sediments. The non-sedimenting absorbance provided a measure of the proportion of non-fibrillar and fibrillar apoC-II. The evolution of this parameter over time yields the timecourse of fibril formation. Example data sets are provided in Figure 5, showing sedimentation profiles of apoC-II alone (Figure 5A) and in the presence of 5 mM DHPC (Figure 5B) after 5 hours incubation. The non-sedimenting optical density in the absence of phospholipid is 30 % greater when compared to

the data acquired in the presence of DHPC, consistent with the slower rate of fibril formation in the absence of phospholipids. The sedimentation data were fitted to a $c(s)$ model where the relationship between the molecular mass and the sedimentation coefficient was calculated assuming a worm-like chain model¹⁵. This model provided sedimentation coefficient distributions of the sedimenting, fibrillar material. Integration of the distributions provided a measure of the weight average sedimentation coefficient (S_w), which shows a time dependent increase in the modal sedimentation coefficient from approximately 40 S to 104 S (Figure 5C). The final value of approximately 100 S is similar to the values obtained for apoC-II fibrils previously¹⁶ suggesting that DHPC has little effect on the size of the final fibrillar product. The small peak at approximately 40 S which is observable in the 72 hour data is attributable to the formation of a small population of closed loop fibrillar structures^{17; 18}.

Figure 6 shows the evolution of the proportion of fibrillar material and the weight-average sedimentation coefficient data over time. This kinetic data was analyzed globally in terms of a reversible kinetic model (Equations 1–4) in which monomeric apoC-II initially tetramerises, followed by isomerisation of this tetramer to form an elongation-competent nucleus. This nucleus then elongates by rapid addition of monomeric apoC-II to form fibrils which can break and rejoin (Figure 1). Initially the kinetic parameters for the rapid formation of tetramer and tetramer isomerisation were fixed to the values in Tables 1. Upon obtaining reasonable fits to this restricted model, these values were allowed to vary to obtain the best fit parameters (Table 2). These results show that 5 mM DHPC increases the overall equilibrium constant for nucleation of apoC-II approximately 1.6×10^6 fold. However, the lipid exerts no effect on the rate constants for fibril elongation or breaking and joining.

Size of the free pool of apoC-II in equilibrium with fibrils

A key finding from this analysis is that the rate constants describing fibril elongation are unaffected by the addition of sub-micellar phospholipid. We have previously observed that the nucleation rate constants do not have a significant effect on the equilibrium between the free apoC-II pool and fibrillar apoC-II¹¹ and that the size of the free pool is determined primarily by the equilibrium constant for fibril elongation. The free pool of apoC-II was determined by a centrifugal pelleting assay. In the absence of phospholipid, apoC-II fibrils were in equilibrium with a free apoC-II pool of $3.0 \pm 1.8\%$, consistent with previous results¹¹. Addition of sub-micellar DHPC (5mM) did not significantly alter this value, yielding a free pool of apoC-II of $3.0 \pm 1.5\%$. These observations indicate that sub-micellar DHPC has no effect on the equilibrium constant for fibril elongation. In contrast, addition of micellar DHPC (25mM) increased in the free pool to $12 \pm 3.2\%$ indicating that DHPC micelles disaggregate apoC-II fibrils. Lipid micelles have previously been shown to stabilise the α -helical fold of apoC-II^{19; 20}, thereby reducing the free pool of partially unfolded apoC-II available for fibril formation.

Effect of DHPC on cross-linked dimeric apoC-II fibril formation

The results of the stopped-flow analysis of the early stages of apoC-II self-association and the global modeling of apoC-II fibril formation indicated that lipids activate fibril formation by enhancing the initial self-association of apoC-II into a tetramer. This raises the question of what happens to the rate of fibril formation if this initial oligomerisation is induced prior to the addition of phospholipid. Since tetramer formation is likely to proceed via initial dimer formation followed by a rapid dimer-dimer interaction to form tetramer we sought to determine whether lipids exerted any effect on fibril formation when the dimers were preformed. Preformed apoC-II dimers were formed using a cysteine to serine substitution mutant which was allowed to air oxidize. This produced a stable population of cross-linked dimeric apoC-II, as measured by SDS-PAGE (data not shown), consistent with previous studies using cysteine cross-linked apoC-II²¹. Figure 7A shows the ThT fluorescence time course for cross-linked dimeric apoC-II in the presence and absence of 5 mM DHPC. Cross-linked dimeric

apoC-II formed fibrils rapidly with a t_{50} of 31 min, comparable to previous studies²¹. The addition of DHPC to the preformed apoC-II dimers also yielded a t_{50} of 31 min, indicating that phospholipids do not exert a significant effect on the rate of fibril formation by cross-linked apoC-II dimers. This finding adds further support to the conclusion that lipids act by promoting the initial nucleation event rather than affected subsequent fibril elongation.

Effect of DHPC on apoC-II fibril elongation

A further test of the effect of DHPC on apoC-II fibril elongation was conducted by adding DHPC to apoC-II at different stages of fibril formation. The results in Figure 7B show that addition of 5 mM DHPC at the start of fibril formation caused a rapid increase in the rate of fibril formation as described previously¹⁴. In contrast, the addition of 5 mM DHPC 24 hours after the start of fibril formation and after the lag phase is complete had no significant effect on the time course for fibril formation. These results confirm that DHPC enhances nucleation but not elongation of apoC-II fibrils

DISCUSSION

We have presented a systematic investigation of the effect of lipid binding on the kinetics of apoC-II fibril formation. The significance of the work lies in the observation that lipids are common components of amyloid deposits and a high proportion of proteins that accumulate in amyloid deposits are lipid binding proteins. Lipids and lipid-like molecules therefore have considerable potential to modulate the rate of formation and stability of disease-related amyloid fibrils. Examples include the affect of phospholipids on fibril formation by A β peptide²², α -synuclein²³ and islet amyloid polypeptide²⁴. Lipid bilayers composed of negatively charged phosphatidylserine or other anionic phospholipids accelerate amyloid fibril formation by several proteins relative to control neutral lipids²⁵. More recent studies show that anionic phospholipids also accelerate the conversion of recombinant prion protein (PrP^C) to the aberrant isoform PrP^{Sc} leading to *de novo* prion formation²⁶. In the case of apoC-II submicellar phospholipids and oxidized cholesterol derivatives accelerate amyloid fibril formation²⁷ while high concentrations of micellar lipids stabilize α -helical structures and prevent fibril formation¹². In contrast, low concentrations of micellar phospholipids and lipid bilayers, while initially inhibiting fibril formation, ultimately promote the formation of apoC-II fibrils with distinct rod-like morphologies¹². These studies underlie the potentially complex effects of lipids on amyloid fibril metabolism.

Analysis of the equilibrium binding of DHPC with Alexa-488 labeled apoC-II indicates a cooperative interaction at sub-micellar concentrations of DHPC (Figure 2). One explanation for the sigmoidal shape of the titration is that apoC-II contains multiple lipid binding sites, with various affinities and fluorescence yields. Experimental evidence for multiple phospholipid binding sites on apoC-II is provided by mass spectrometry analysis²⁸. Another possibility for this cooperativity is that DHPC binds preferentially to the tetrameric form of the apoC-II leading to a lipid-induced shift in the monomer-tetramer equilibrium. The data in Figures 3 and Table 1 support this explanation with the equilibrium constant for the rapid tetramerisation increasing from 7.8×10^{-6} , 453×10^{-6} and $1796 \times 10^{-6} \mu\text{M}^{-3}$ in the presence of 1, 3 and 5 mM DHPC, respectively. Alternatively, the cooperativity could arise from self-association of the lipid component. Evidence that DHPC self associates is provided by sedimentation equilibrium and NMR analysis of DHPC micelle formation which is consistent with a monomer-dimer-micelle equilibrium^{14; 29}. According to this scheme the phospholipid dimerises, then binds to the monomeric apoC-II, thereby inducing the formation of the tetrameric form of apoC-II. The observation that DHPC does not accelerate fibril formation by cross-linked apoC-II (Figure 7) is consistent with a mechanism where DHPC initially promotes a dimeric apoC-II intermediate which then rapidly associates to form a discrete

tetramer. It is also possible that a combination of the above mechanisms gives rise to the observed cooperativity.

Analysis of the kinetic parameters associated with apoC-II tetramer formation indicates that phospholipids exert significant effects on the rate of tetramer association and dissociation (Table 1). The rate constants associated with the initial rapid tetramerisation are dependent on the lipid concentration, while those associated with the subsequent isomerisation are independent of lipid concentration. Thus rapid tetramerisation is the primary driving force for the large increase in the overall nucleation constant K_{nuc} which increases by a factor of 1.6×10^6 in the presence of 5 mM DHPC (Table 2). The slower conformational change in the tetramer detected by both CD and fluorescence spectroscopy (Figure 4) is largely independent of the presence of DHPC. As suggested previously, this conformational change may involve proline isomerisation or domain swapping¹⁴. Further high resolution structural analysis is required to determine the exact nature of this isomerisation event.

In contrast to the large effect of DHPC on the initial tetramerization and overall nucleation process, DHPC had little effect on the rate of fibril elongation. Evidence for this conclusion is provided by several observations. First the global analysis of the sedimentation velocity data indicated that the rates of elongation and fibril breakage and joining were not significantly different in the presence of sub-micellar lipid (Table 2). Second, the free pools of apoC-II in equilibrium were unchanged by the addition of sub-micellar lipid. This is significant as the rate of elongation (k_e) and the rate of fibril dissociation (k_{off}) govern the proportion of free apoC-II, and these constants are not significantly affected by the addition of lipid. Third, the rate of fibril formation by the cross-linked dimeric apoC-II is also unaffected by the addition of lipid (Figure 7A). Finally, the addition of lipid after the lag phase of fibril formation is complete has no effect on the rate of fibril formation (Figure 7B). This lack of an effect on elongation by lipids suggests that the structural factors affecting the nucleation and elongation steps of fibril formation by apoC-II are separate from each other. The ability to separate the effects of DHPC on fibril nucleation and elongation is important in terms of the design of amyloid fibril inhibitors and activators. The observation that sub-micellar DHPC activates *de novo* apoC-II fibril formation but has no effect on elongation demonstrates the ability of lipids to selectively modulate discrete steps in the amyloid fibril forming pathway. In the development of inhibitors and activators of amyloid fibril formation it is important to distinguish effects on nucleation compared to effects on elongation since it is unlikely that small compounds that solely modulate nucleation will affect fibril growth from pre-existing seeds or the reversal of fibril formation. The model and experiments described in this study provide methods to investigate the independent processes of elongation and nucleation. The ability to identify compounds which impact on these two dominant processes of fibril formation may provide potential compounds to reverse or slow the progression of amyloid related diseases.

MATERIALS AND METHODS

Alexa 488 C₅ maleimide and Alexa 594 C₅ maleimide were obtained from Invitrogen Molecular Probes (Eugene, Oregon). DHPC was obtained from Avanti Polar Lipids, Inc. (Alabaster, Alabama). ApoC-II was expressed and purified as described previously (12). Purified apoC-II stocks were stored in 5M guanidine hydrochloride, 10 mM Tris.HCl, pH 8.0 at a concentration of ~ 45 mg/ml. ApoC-II_{S61C} was provided by Dr. Chi Pham (University of Melbourne) and was conjugated with Alexa dyes as described previously¹⁴.

Fibril formation by apoC-II

ApoC-II was refolded by dilution to 0.3 mg/ml from a stock solution into refolding buffer (100mM sodium phosphate, 0.1% sodium azide, pH 7.4). Thioflavin T (ThT; 10 μ M) fluorescence intensity was measured, in triplicate, using an fmax fluorescence plate reader

(Molecular Devices, Sunnyvale, California) equipped with 444 nm excitation and 485 nm emission filters.

Fluorescence titrations of the interaction of apoC-II with phospholipids

The emission spectrum of Alexa-488 labeled apoC-II (5 μ M) in a total volume of 120 μ L of refolding buffer was recorded on a Cary Eclipse spectrophotometer (Varian, Palo Alto, California) using an excitation wavelength of 495 nm and collecting the emission over the range 495–700 nm using a 495 nm long-pass filter. 2 μ L of DHPC (150 mM) was added to the cuvette. The solution was allowed to equilibrate for 5 minutes and the Alexa-488 spectrum was recorded again as described above.

Stopped flow measurements

Stopped flow measurements were conducted using an RX-6200 portable stopped flow device (Applied Photophysics, Leatherhead, Surrey, UK) equipped with a pneumatic drive and two 2 mL syringes for the ligand and acceptor solutions. In all cases stopped flow experiments were conducted using a stopping volume of 150 μ L. The time courses presented are the average values of 5 separate mixing experiments. ApoC-II tetramerisation was monitored by measuring the rate of change in FRET between Alexa-594 labelled apoC-II and Alexa-488 labelled apoC-II populations using excitation at 480 nm, and monitoring emission at 620 nm with a Cary Eclipse spectrophotometer (Varian Inc, Palo Alto, California).

Time course of Alexa-488 labeled apoC-II fluorescence

Refolded Alexa-488 labelled apoC-II in the presence or absence of DHPC was transferred to a 96 well fluorescence plate and 30 μ L of mineral oil was layered on top of each sample to reduce evaporation. The fluorescence emission of the samples was measured using a Paradigm fluorescence plate reader (Beckman Coulter Instruments, Inc., Fullerton, California) equipped with 485 nm excitation and 538 nm emission filters. Control experiments showed that oil overlay did not affect the lipid-induced fluorescence changes of the Alexa 488-labelled apoC-II.

Circular dichroism (CD) measurements of apoC-II

CD measurements of apoC-II (0.05 and 0.025 mg/mL) in the presence and absence of 5 mM DHPC were acquired with an Aviv 62DS CD spectrophotometer using 1 mm pathlength quartz cuvettes. CD spectra were acquired with an averaging time of 2 s and a step size of 0.5 nm from 250 to 190 nm. The spectra are an average of two separate measurements and were corrected for the ellipticity of the phospholipid. The ellipticity at 208 nm was measured over a 4 hour time period, at 2 minute intervals, using an averaging time of 15 s.

Sedimentation velocity experiments

Sedimentation velocity experiments were conducted using an AnTi50 rotor and analytical ultracentrifuge cells equipped with quartz windows and double sector charcoal epon centerpieces (Beckman Coulter, Inc, CA). Sedimentation velocity data for fibrillar apoC-II (0.1–0.6 mg/mL) were collected using absorbance optics over radial positions from 6–7.25 cm at a rotor speed of 8,000 rpm and a wavelength of 280 nm at 7 minute intervals. The data from these sedimentation velocity experiments were analyzed to obtain the weight-average sedimentation coefficient (S_w) and associated error bars using the $c(s)$ model^{30; 31} in combination with a worm-like chain model which allows the effects of fibril diffusion to be accounted for in the analysis^{15; 31}

A kinetic model of fibril formation via a tetrameric intermediate

Fibril formation by apoC-II, as measured by sedimentation velocity experiments of apoC-II incubated in the presence of sub-micellar lipid for varying times, was modeled as a rapid tetramerisation, followed by a slower conformational change that precedes fibril elongation, breakage and joining^{11; 14} (Figure 1). This model of fibril formation was used to derive the kinetic equations 1–4 which describe the evolution of the concentration of monomer, tetramer, tetrameric isomer and fibrillar products over time:

$$\frac{dm}{dt} = -4k_{on}m^4 + k_{off}4n - 2k_e m(f + n') + 2k_{eoff}f \quad (1)$$

$$\frac{dn}{dt} = k_{on}m^4 - k_{off}n - k_{iso}n + k_{isob}n' \quad (2)$$

$$\frac{dn'}{dt} = k_{iso}n - k_{isob}n' - 2k_e mn' \quad (3)$$

$$\frac{df}{dt} = 2k_e mn' + k_b(co - m - 4n - 4n' - f) - k_j f^2 \quad (4)$$

Where m represents the molar monomer concentration, n represents the molar concentration of the fibril forming incompetent prenucleus, n' represents the fibril forming competent nucleus, k_{on} and k_{off} are the rate constants for the initial formation and dissociation of tetrameric apoC-II and k_{iso} and k_{isob} are the rate constants for the slow change in the apoC-II tetramer, co is the total molar concentration of subunits in the experiment, f represents the molar concentration of fibrillar material, k_e and k_{eoff} are the rate constants for the formation and dissociation of fibrillar material and k_b and k_j are the rate constants for fibril breaking and joining. These rate equations are a simplification of the actual process as the heterogeneous population of fibrillar end products is represented as a single average fibrillar species, f . Further simplifications result in this model not taking into account the rate of nucleus formation by dissociation of monomeric apoC-II from fibrillar material or breakage of fibrillar material into nuclei. This model can calculate the average size of apoC-II fibrils using $(co - m - 4n - 4n')/f$ which provides a further constraint on the fit of the data.

Equations 1–4 were used to analyze the global sedimentation data simultaneously. The stopped flow data were analyzed simultaneously using the equations 1–2 with all rate constants other than k_{on} and k_{off} constrained to zero. The slow isomerisation step, monitored by the increase in Alexa-488 labeled apoC-II fluorescence intensity, was analyzed globally with equations 1–3, with the values of k_{on} and k_{off} constrained to the values obtained from stopped flow analysis, and k_e and k_{eoff} constrained to zero. The fitting of these equations to the data was conducted using the differential equation solver functionality of MATLAB.

Measurement of the free pool of apoC-II

Fibrils formed from Alexa-488 apoC-II (100 μ l) in the absence and presence of sub-micellar or micellar DHPC and DHPS were centrifuged for 30 min at 100,000 rpm (350000 g) in an OptimaMax centrifuge using a TL-100.1 rotor (Beckman Coulter Instruments, Inc., Fullerton,

California). The pellet was washed with refolding buffer and dissolved in 100 μ L of 5 M guanidine hydrochloride. The Alexa-488 emission spectra of the pre-centrifuged material and the supernatant and pellet fractions were recorded on a Cary Eclipse (Varian, Palo Alto, California) using an excitation wavelength of 495 nm and collecting the emission over the range 495–700 nm. The degree of fluorescence that was retained in the supernatant was taken as a measure of the free pool of apoC-II.

Fibril formation by cysteine cross-linked apoC-II dimers

ApoC-II_{S61C} was induced to form disulfide cross-linked apoC-II dimers by air oxidation as described ²¹. The rate of fibril formation by these dimers (0.3mg/mL) in the absence and presence of sub-micellar DHPC (5mM) was assayed using the ThT assay described above.

Effect of DHPC on apoC-II fibril formation

The ThT fluorescence of apoC-II samples (0.3 mg/mL, 100 μ L in sodium phosphate buffer, 100mM, pH 7.4) containing 10 μ M ThT was monitored continuously using a Paradigm Plate reader equipped with a ThT cartridge (Beckman Coulter, Inc. CA). At 0 and 24 hours, 5 μ L of a concentrated stock solution of DHPC was added to the test samples to give a final DHPC concentration of 5mM.

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Abbreviations

apo	apolipoprotein
CD	circular dichroism
DHPC	1,2-Dihexanoyl- <i>sn</i> -Glycero-3-Phosphocholine
FRET	fluorescence resonance energy transfer
ThT	thioflavin T

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Figure 1.

Kinetic model of fibril formation by apoC-II. The model assumes a rapidly formed tetrameric intermediate, which undergoes a slow conformational change prior to fibril elongation and fibril breaking and joining; k_{on} and k_{off} refer to the rate constants governing the formation and dissolution of the initial tetramer, k_{iso} and k_{isob} represent the rate constants for the slow conformational change and k_e and k_{eoff} govern the elongation step. The fibrils undergo breaking and rejoining, governed by the rate constant k_b and k_j .

Figure 2.

Steady state binding of DHPC to apoC-II. The binding of DHPC was monitored by the change in Alexa-488 labeled apoC-II upon addition of DHPC. Samples of Alexa-488 apoC-II (5 μ M) were titrated with DHPC. The fluorescence intensity in arbitrary fluorescence units (a.f.u.) at 520 nm after excitation at 495 nm was measured after each addition of lipid. The error bars represent the standard error for triplicate measurements.

Figure 3.

Stopped flow analysis of the change in FRET between Alexa-488 and Alexa-594 labelled apoC-II. Stocks of 4:1 Alexa-594: Alexa-488 apoC-II was rapidly mixed with buffer containing DHPC to final concentrations of 1.25 (triangles), 2.5 (squares) and 5 (circles) μM apoC-II and (A) 5 or (B) 3 mM DHPC. The changes in FRET, monitored by the change in 620 nm emission intensity after excitation at 480 nm, are the average values of 5 separate mixing experiments and the fits in panels A and B are the results of global fitting of the curves obtained at the three different apoC-II concentrations. While best-fit lines show some non-random distribution of residuals the maximum variation in the residuals observed at the early timepoints is less than

5% for the high concentration data, 1.4% for the middle concentration data and 1.1% for the low concentration data, and less than 0.03% for all curves at the middle and later timepoints.

Figure 4.

Characterization of the slow change that follows the rapid formation of tetramer. A) CD spectra for apoC-II alone (long dashed line), and in the presence of 5mM DHPC immediately after addition of DHPC (dotted line) and after 1 hr incubation (solid line). B) The change in mean residue ellipticity (MRE) at 208nm over time for apoC-II at 0.05 mg/mL (triangles) and 0.025mg/mL (squares) in the presence of 5mM DHPC. C) Alexa-488 fluorescence intensity timecourse for apoC-II at 2.5 (squares) and 5 (circles) μM apoC-II in the presence of 5mM DHPC. The fluorescence intensity is in arbitrary fluorescence units (a.f.u.), and was acquired using 495 nm excitation and monitoring the intensity of the 520 nm emission.

Figure 5.

Sedimentation velocity analysis of fibril formation. Sedimentation velocity profiles acquired at 8,000 rpm for apoC-II (0.4 mg/mL) alone (A) and in the presence of 5 mM DHPC (B) after 5 hours of incubation at 20°C. C. Size distribution analysis of apoC-II fibrillar material in the presence of 5 mM DHPC. Data similar to that in panel A) and B) was analyzed in terms of a $c(S)$ model for samples of apoC-II incubated for 5, 12 and 72 hours providing distributions 1, 2 and 3, respectively.

Figure 6.

Global analysis of the kinetics of apoC-II fibril formation in the presence of 5 mM DHPC. The proportion of fibrillar material (A) and the weight average sedimentation coefficient, S_w (B) for apoC-II at concentrations of 0.6 (open circles), 0.5 (closed circles), 0.4 (diamonds), 0.3 (triangles) and 0.2 squares) mg/mL in the presence of 5 mM DHPC were fitted to the Eq. 1–4 to obtain best fit values for the rate constants for each step in the pathway (Table 2).

Figure 7.

A. Fibril formation by cross-linked dimers of apoC-II_{S61C}. Fibril formation of apoC-II_{S61C} cross-linked dimers in the absence (open squares) and presence (closed squares) of 5 mM DHPC was measured using the ThT assay. The rate of fibril formation was estimated by measuring the time to the half maximal fluorescence change (t_{50}) obtained by fitting the data to a Hill Plot (solid lines). B) The time-dependent response of apoC-II fibril formation to the addition of DHPC. DHPC (5mM) was added to apoC-II (0.3mg/mL) solutions which had been incubated for 0 (dotted circles) and 24 (open squares) hours (addition time indicated by arrow). In addition, a sample of apoC-II in the absence of DHPC (filled circles) was also measured. The ThT timecourse of these solutions was monitored continuously for 96 hours.

Table 1

Rate constants for apoC-II tetramer formation and isomerisation in the presence of DHPC.

[DHPC] (mM)	k_{on} ($\mu\text{M}^{-3}\text{hr}^{-1}$) ^a	k_{off} (hr^{-1}) ^a	k_{iso} (hr^{-1}) ^b	k_{isob} (hr^{-1}) ^b
1	0.0045	580	1.194	2310
3	0.0204	45	1.209	2320
5	0.0413	23	1.206	2290

^aValues for k_{on} and k_{off} were obtained by global analysis of stopped flow data for apoC-II (Figure 3) using equations 1–2 with all other rate constants constrained to zero

^bValues for k_{iso} and k_{isob} were obtained by global analysis of fluorescence intensity timecourse data for apoC-II (Figure 4C) using equations 1–3 with k_e and k_{eoff} constrained to zero and k_{on} and k_{off} constrained to the values obtained from stopped flow data for apoC-II

Table 2

Rate constants for apoC-II fibril formation in the presence and absence of DHPC

Parameter ^a	+ 5 mM DHPC ^b (confidence interval) ^d	-DHPC ^c (confidence interval) ^d
k_{on} ($\mu\text{M}^{-3}\text{hr}^{-1}$)	0.089 (0.043 – 0.15)	-
k_{off} (hr^{-1})	63.01 (48.2 – 70.4)	-
k_{iso} (hr^{-1})	2.21 (1.91 – 2.41)	-
k_{isob} (hr^{-1})	2521 (2381 – 2743)	-
K_{nuc} (μM^{-3}) ^e	1.2×10^{-6} ($[0.72 - 1.88] \times 10^{-6}$)	7.5×10^{-13} ($[4.6 - 13.9] \times 10^{-13}$)
k_{eoff} (hr^{-1})	27.42 (10.1 – 37.8)	26.6 (1.5 – 87.9)
k_e/k_{eoff} (μM^{-1})	0.19 (0.12 – 0.22)	0.16 (0.13 – 0.20)
Fibril breaking (k_b) (hr^{-1})	4.55×10^{-5} ($[2.5 - 5.32] \times 10^{-5}$)	9.06×10^{-6} ($[4.6 - 17.9] \times 10^{-6}$)
Joining/breaking	2.2×10^5 ($[1.8 - 2.9] \times 10^5$)	2.9×10^5 ($[2.4 - 3.3] \times 10^5$)

^aThe parameters are described in Figure 1.^bBest fit values obtained from global analysis of the data in Figure 6 using equations 1–4^cResults for previous work ¹¹ describing fibril formation by apoC-II in the absence of lipid.^dConfidence limits were determined using Monte-Carlo analysis of the fits of the data in Figure 6 to equations 1–4. This analysis provided an RMSD for the kinetic and S_W data of 5.5 and 6.4, respectively.^e K_{nuc} describes the overall equilibrium constant for nucleation and is defined as the product of k_{on}/k_{off} times k_{iso}/k_{isob}