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ORIGINAL PAPER

Effects of a lipid environment on the fibrillogenic pathway of the N-terminal polypeptide of human apolipoprotein A-I, responsible for in vivo amyloid fibril formation

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Abstract In amyloidosis associated with apolipoprotein A-I (ApoA-I), heart amyloid deposits are mainly constituted by the 93-residue ApoA-I N-terminal region. A recombinant form of the amyloidogenic polypeptide, named [1-93] ApoA-I, shares conformational properties and aggregation propensity with its natural counterpart. The polypeptide, predominantly in a random coil state at pH 8.0, following acidification to pH 4.0 adopts a helical/molten globule transient state, which leads to formation of aggregates. Here we provide evidence that fibrillogenesis occurs also in physiologic-like conditions. At pH 6.4, [1-93]ApoA-I was found to assume predominantly an α-helical state, which undergoes aggregation at 37°C over time at a lower rate than at pH 4.0. After 7 days at pH 6.4, protofibrils were observed by atomic force microscopy (AFM). Using a multidisciplinary approach, including circular dichroism (CD), fluorescence, electrophoretic, and AFM analyses, we investigated the effects of a lipid environment on the conformational state and aggregation propensity of [1-93] ApoA-I. Following addition of the lipid-mimicking detergent Triton X-100, the polypeptide was found to be in a helical state at both pH 8.0 and 6.4, with no conformational transition occurring upon acidification. These helical conformers are stable and do not generate aggregated species, as observed by AFM after 21 days. Similarly, analyses of the effects of cholesterol demonstrated that this natural ApoA-I ligand induces formation of α-helix at physiological concentrations at both pH 8.0 and 6.4. Zwitterionic, positively charged, and negatively charged liposomes were found to affect [1-93]ApoA-I conformation, inducing helical species. Our data support the idea that lipids play a key role in [1-93]ApoA-I aggregation in vivo.

Keywords Fibrillogenesis · Protein aggregation · Conformational analysis · Apolipoprotein A-I · Amyloidosis · Liposomes

Abbreviations

AFM	Atomic force microscopy
ANS	8-Anilino-1-naphthalenesulfonate
ApoA-I	Apolipoprotein A-I
[1-93]ApoA-I	Recombinant 93-residue N-terminal fragment of ApoA-I
CD	Circular dichroism

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DOTAP	1,2,-Dioleoyl-3-trimethylammonium-propane
GST	Glutathione-*S*-transferase
HDL	High-density lipoproteins
POPC	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPS	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine
ThT	Thioflavin T

Introduction

Amyloid depositions and plaques represent highly ordered aggregated biomaterial formed by amyloid fibrils. Fibrils are generated by protein aggregation based on repetitive, long-range interactions such as intermolecular hydrogen bonds. The amyloid motif consists of the cross-beta super-secondary structure with specific tinctorial and morphological properties (Dobson 2004). Amyloid aggregation of at least 20 different proteins is related to severe diseases, while an increasing number of nonpathogenic proteins have been found to be able to form amyloid fibrils in vitro, endowed with the same structure and properties (Merlini and Bellotti 2003). Protein aggregation occurs in vivo in eukaryotic cells (Ma et al. 2003) as well as in prokaryotic cells (Carrió and Villaverde 2002). Cells under stress conditions prevent protein aggregation by means of molecular (Muchowski 2002) or chemical (Papp and Csermely 2006) chaperones.

Isolation of amyloidogenic proteins or peptides is often difficult as they amorphously aggregate, fibrillate or precipitate depending on solution conditions such as pH, ionic strength, temperature, molecular crowding, and presence of cosolutes or cosolvents (Schmittschmittand and Scholtz 2003). Several amyloidogenic proteins produced in *E. coli* were localized in inclusion bodies, making their purification difficult (McParland et al. 2002; Legname et al. 2004; Lopes et al. 2004). Interestingly, generic bacterial inclusion bodies share amyloid-like properties (Carrió et al. 2005). To increase protein solubility, amyloidogenic proteins have been purified as fusion proteins with a highly soluble partner (Masino et al. 2002; Baxa et al. 2002; Serio et al. 1999; Masino et al. 2004). Alternatively, ionic and nonionic detergents may be used to increase protein solubility. Nevertheless, detergents may alter protein secondary and tertiary structures (Naeem et al. 2006; Ghosh 2008). For some proteins, discrete, high-affinity binding sites for detergents have been described (Singh and Kishore 2006; Goddard and Ananthapadmanabhan 1993). Apolipoproteins A-I and A-II bind sodium dodecyl sulfate (SDS) with relatively high affinity in their compact, globular state (Reynolds and Simon 1974). Several proteins naturally occurring in association with lipids are able to bind Triton X-100 in relatively large amount (le Maire et al. 2000).

Detergents, lipid molecules, and lipid mimics play a role in conformational change of many amyloid precursor proteins and in amyloid fibril formation in vitro (Andreola et al. 2003; Morillas et al. 1999; Ji et al. 2002). SDS induces some proteins or peptides to form aggregates or amyloid-like fibrils in vitro (Hagihara et al. 2002; Pertinhez et al. 2002). Stimulation and inhibition of fibril formation have been reported, depending on protein and cosolute nature and concentration, pH value, and incubation temperature. Yamamoto and coworkers demonstrated that low concentrations of SDS induce extension of β2-microglobulin-related amyloid fibrils, while the same concentrations of Triton X-100, lauryl sulfobetain or dodecyl trimethylammonium chloride have no effect (Yamamoto et al. 2004). Interactions of natively unfolded proteins with membrane lipids have been shown to induce large effects on protein conformation (Abedini and Raleigh 2009b).

Among the amyloidogenic proteins, apolipoprotein A-I (ApoA-I) is known to be responsible for hereditary, systemic amyloidoses, when specific mutations occur in the ApoA-I gene. The 13 amyloidogenic variants of ApoA-I described so far are directly associated with in vivo formation of amyloid deposits (Obici et al. 2006) that accumulate in tissues and organs, such as heart, kidney or liver, with severe consequences (Joya et al. 2003). Fibrils are mainly constituted by N-terminal fragments of ApoA-I, about 90–100 residues long. The 93-residue polypeptide was found to be the most abundant in heart amyloid deposits (Obici et al. 1999). Conformational analyses of the polypeptide isolated ex vivo revealed that a pH shift from 7.0 to 4.0 induces a transition from a random coil state to a transient helical conformation that rapidly converts to β-structure in fibrils (Andreola et al. 2003).

Recently, we reported production of a recombinant version of the N-terminal 93-residue polypeptide (henceforth denoted as [1-93]ApoA-I) as a fusion protein with glutathione-*S*-transferase (GST), from which the polypeptide was then released by site-directed proteolysis (Di Gaetano et al. 2006). We demonstrated that in aqueous solutions at pH 7.0 the recombinant polypeptide is natively unfolded, and upon acidification to pH 4.0 it undergoes a conformational transition towards a helical state, similarly to that described for the natural polypeptide. We hypothesized that the helical species is a key intermediate in the fibrillogenic pathway leading to formation of a β-sheet-based polymeric structure that evolves to typical amyloid fibrils (Di Gaetano et al. 2006).

As the functional role of ApoA-I is strictly related to its interactions with lipids, we analyzed the effects of lipids on the propensity of [1-93]ApoA-I to undergo fibrillogenesis under different experimental conditions. Our analyses

include the lipid-mimicking detergent Triton X-100, the ApoA-I natural ligand cholesterol, and liposomes. From our data a picture emerges indicating that a lipid environment affects the [1-93]ApoA-I aggregation pathway by inducing and stabilizing helical intermediates.

Materials and methods

Materials

Anti-human ApoA-I polyclonal antibodies were from DAKO, Denmark. For Western blot analyses, immunopositive species were detected by a chemiluminescence detection system (SuperSignal® West Pico, Pierce, Rockford, IL) using a Phosphoimager (Biorad). POPC, POPS, and DOTAP were purchased from Avanti Polar Lipids (Alabaster, AL). Reagents were from Sigma–Aldrich (St. Louis, MO).

Expression and isolation of [1-93]ApoA-I

Recombinant [1-93]ApoA-I, cloned in the expression vector pGEX-4T-3, was expressed in BL21DE3 *E. coli* cells following the procedure described by Di Gaetano et al. (2006) with some modifications. Briefly, bacteria were resuspended in phosphate-buffered saline (PBS) containing 20% sucrose and protease inhibitors (Roche, Germany). Lysates were sonicated, incubated for 30 min at 4°C, and centrifuged. GST-containing species, selected by affinity chromatography, were digested with thrombin to release [1-93]ApoA-I. The recombinant product was isolated by high-performance liquid chromatography (HPLC) reverse phase chromatography on a Ultrapure C8 column (Vydac, Grace, IL, USA) with a gradient of buffer B [90% acetonitrile in 0.1% trifluoroacetic acid (TFA)] in buffer A (0.1% TFA) using a PerkinElmer chromatographic system (Series 200). The eluted fractions containing the recombinant product were immediately neutralized by addition of ammonium hydroxide (0.12% final concentration). The final yield of the procedure was estimated to be 2.5 mg/l of bacterial culture. Pure [1–93]ApoA-I was lyophilized and stored at −70°C until use. For experimental purposes, the polypeptide was dissolved in the appropriate buffer and centrifuged before use.

Mass-spectrometric analyses

Aliquots of 20 μg lyophilized recombinant [1–93]ApoA-I were dissolved in phosphate buffer (12 mM, pH 6.6) and then desalted by reversed-phase HPLC on a Phenomenex Jupiter C4 column (250 × 2.1 mm, 300 Å pore size) with a linear gradient from 20% to 95% acetonitrile in 0.1% TFA over 10 min, at flow rate of 200 μl/min onto an Agilent Technologies 1100 HPLC. Peaks were monitored at 220 nm, and the fractions were manually collected and analyzed by electrospray MS using a Quattro Micro triple quadrupole mass spectrometer (Waters, Micromass) by sample direct injections in the ion source. Mass data were acquired and processed using Mass-Lynx software. Horse heart myoglobin (average molecular mass 16,951.5 Da) was employed as calibrant; all masses are reported as average mass.

CD spectra

[1-93]ApoA-I far-ultraviolet (UV) circular dichroism spectra were obtained using a Jasco J815 spectropolarimeter (Jasko, Essex, UK), equipped with a temperature control system, using a 1-mm quartz cell in the far-UV range 194–260 nm (50 nm/min scan speed). Each spectrum was the average of three scans with the background of the buffer solution subtracted. Measurements were performed at 25°C at protein concentration of 0.3 mg/ml in 3 mM glycine, 3 mM sodium acetate, and 3 mM sodium phosphate at pH 6.4 or pH 8.0. The acidification of the protein solution was carried out by progressive addition of 0.1 N HCl. For CD spectra, 26 mM cholesterol stock solution was prepared in ethanol. Stock solutions of liposomes were 26 mM in water. CD spectra of lipids alone, i.e., in the absence of the polypeptide, were subtracted from the data. CD data were expressed as the CD absorption coefficient on a mean residue weight basis. Spectra deconvolution was performed following Continll, Selcon3, and CDSSTR algorithms of the CDPro program pack (http://lamar.colostate.edu/~sreeram/CDPro/) (Sreerama and Woody 2000).

Liposome preparation

To prepare negatively charged, positively charged, and zwitterionic liposomes, POPC/POPS 85:15 (M/M), POPC/DOTAP 85:15 (M/M), and pure POPC were dissolved in chloroform. After mixing, the solvent was evaporated under a stream of nitrogen and the last traces of solvent were removed under vacuum overnight. The dry lipid film was then hydrated to form multilamellar liposomes. The lipid concentration was 20 mg/ml. A bath-type sonicator was employed to obtain a homogeneous liposomal dispersion. Unilamellar vesicles were then obtained using a probe-type sonicator (Ultrasonics Ltd., UK).

Binding of [1-93]ApoA-I to ANS

The 8-anilino-1-naphthalenesulfonate (ANS, 350 μM) emission fluorescence spectra were acquired in the presence of [1-93]ApoA-I (3 μM) in 3 mM glycine, 3 mM

sodium acetate, and 3 mM sodium phosphate at pH 8.0 at 25°C, using a LS55 luminescence spectrometer (PerkinElmer). Spectra were recorded in the range 400–600 nm at excitation wavelength of 380 nm (5 nm slit width). The acidification of the protein solution was carried out as described above. To return to the initial pH value, 0.1 N NaOH was added to the protein solution.

ThT assays

[1-93]ApoA-I (20 μM) was incubated at 25°C in 12 mM sodium phosphate at pH 6.4 in the presence of 15 μM thioflavin T (ThT). ThT fluorescence emission spectra were acquired in the range 455–600 nm at 1 h intervals with scan speed of 300 nm/min, upon excitation at 450 nm. Excitation and emission slits were set at 5 and 10 nm, respectively. Fluorescence intensity values at 482 nm were plotted as a function of time.

Atomic force microscopy

[1-93]ApoA-I was incubated at concentration of 0.4 mg/ml in sodium phosphate 12 mM, pH 6.4, 37°C. For AFM inspection, an aliquot of the sample was extracted at fixed time and diluted 100 times with Milli-Q water; then a 10 μl aliquot of the diluted sample was deposited on freshly cleaved mica and dried under a gentle nitrogen stream. Sample dilution before deposition on mica was required to avoid salt crystallization. AFM measurements were performed with a Dimension 3100 scanning probe microscope (Digital Instruments, Santa Barbara, CA) equipped with a G scanning head (maximum scan size 100 μm) and controlled by a Nanoscope IIIa controller. AFM images were acquired in tapping mode in air; single-beam uncoated silicon cantilevers (type Olympus AC160TS, Santa Barbara, CA) were used. The drive frequency was around 300 kHz; the scan rate was between 0.5 and 2.0 Hz. Images were collected with 512 data points per line. Aggregate size was measured from the height in cross-section in topographic AFM images.

Results

Effects of lipid-mimicking detergent Triton X-100 on [1-93]ApoA-I conformation

The effects of the detergent Triton X-100 on the aggregation propensity of [1–93]ApoA-I were first investigated by conformational analyses. Following the procedure described in the "Materials and methods" section, pure recombinant [1–93]ApoA-I was obtained, as confirmed by mass-spectrometric analyses. The mass spectrum of the desalted protein sample showed molecular mass of 10,864.6 ± 0.3 Da, in perfect agreement with the theoretical mass value of recombinant [1–93]ApoA-I carrying a Gly-Ser dipeptide at the N-terminus (10,863.95 Da) (Di Gaetano et al. 2006).

The recombinant polypeptide was dissolved at pH 8.0 and analyzed by far-UV CD spectroscopy. A minimum at 204 nm in the far-UV CD spectrum of [1–93]ApoA-I was detected (Fig. 1a), indicating that the polypeptide is highly unstructured at pH 8.0, accordingly to previous results at pH 7.0 (Di Gaetano et al. 2006). We then added increasing amounts of Triton X-100 up to 0.01% (v/v). In the presence of the detergent, the polypeptide conformation switched towards an α-helical state, as shown by the shift of the major minimum from 204 to 208 nm and the presence of a second minimum at 222 nm. The helical content was found to increase with increasing detergent concentration (Fig. 1a). In the experiments reported below, aimed at further investigation of the effects of the detergent on [1–93]ApoA-I aggregation propensity, Triton X-100 was used at 0.004% (v/v) final concentration, a value four times lower than the critical micellar concentration.

In Fig. 1b the CD spectrum obtained upon acidification from pH 8.0 to pH 4.0 is shown. In the absence of the detergent, the shift of the major minimum from 204 to 208 nm and the change in CD signal at 222 and 194 nm indicated that a transition from a predominant random coil structure to a predominant helical structure was induced by acidification. Using CDPro software, a twofold increase of the α-helix content (from 20% to about 40%) was estimated to occur. These observations are in line with those previously obtained upon acidification from pH 7.0 (Di Gaetano et al. 2006). When the same experiment was performed in the presence of Triton X-100, i.e., under conditions in which helical conformers were already present at pH 8.0, no significant spectral changes were found to occur upon acidification to pH 4.0 (Fig. 1b). These data indicated that, in the presence of the detergent, the polypeptide gains a stable helical conformation that remains unchanged following pH decrease.

The effect of Triton X-100 on the propensity of [1–93] ApoA-I to aggregate upon exposure to acidic conditions (pH 4.0) was investigated by incubating the polypeptide for 72 h at 37°C. In the absence of Triton X-100, a decrease of the CD signal, suggestive of protein aggregation, was detected (Fig. 1c), whereas in the presence of Triton X-100 a switch towards a random coil structure was observed. This conformation was found to be stable, as after 30 days incubation the spectrum was found to be unchanged (data not shown). These results suggested that the fate of the helical conformers is strongly influenced by the detergent.

The aggregation propensity of [1-93]ApoA-I was also investigated in quasi-physiologic conditions; the polypeptide

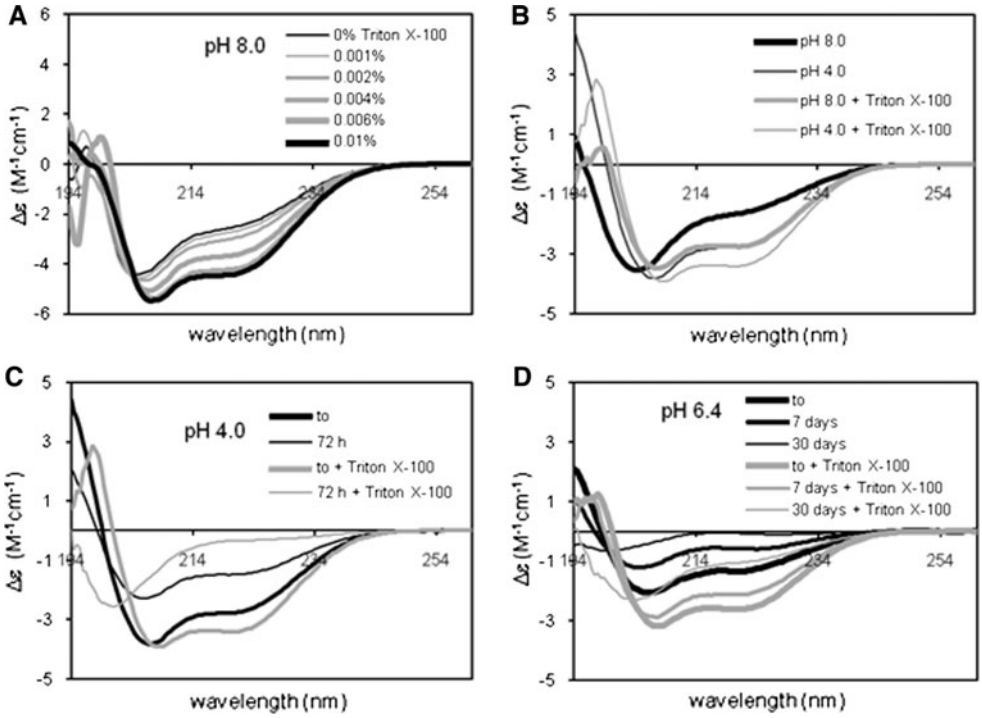

Fig. 1 Spectroscopic analyses of [1-93]ApoA-I conformation in presence and absence of Triton X-100. **a** Far-UV CD spectra of [1-93]ApoA-I recorded at pH 8.0 in absence or presence of increasing concentrations of Triton X-100. **b** CD spectra of [1-93]ApoA-I after a pH jump from 8.0 to 4.0. **c** CD spectra of [1-93]ApoA-I at pH 4.0 recorded at time 0 (t0) and after 72 h incubation at 37°C. **d** Time dependence of [1-93]ApoA-I aggregation upon incubation at pH 6.4 at 37°C. In **b**, **c**, and **d**, Triton X-100 concentration was 0.004% (v/v)

was dissolved at pH 6.4 and analyzed by CD spectroscopy (Fig. 1d). Interestingly, helical conformers were found to be present, at content estimated to be higher than that determined at pH 8.0 but lower than that occurring at pH 4.0 (Fig. 1b). Following acidification to pH 4.0, a slight increase in helical content was detected (not shown), reaching the expected level at this pH value. When [1-93] ApoA-I was incubated at 37°C at pH 6.4 up to 30 days, a progressive decrease of CD signal was observed (Fig. 1d), indicative of protein aggregation. Thus, [1-93]ApoA-I is able to aggregate even at pH 6.4, although with lower propensity than at pH 4.0.

When the protein was tested at pH 6.4 in the presence of the detergent, the content of helical species was found to be higher than that in the absence of the detergent (Fig. 1d) and comparable to that observed at pH 8.0 and pH 4.0 in the presence of the detergent (Fig. 1b). This confirmed that the detergent is able to induce formation of stable helical species. When the protein was incubated at pH 6.4 in the presence of Triton X-100, over time only a slight decrease of the CD signal was observed, associated with an increase in random coil content.

To further investigate the transient helical conformers in the fibrillogenic pathway, assays with the apolar fluorescent dye ANS were performed. ANS is frequently used to detect partially folded, molten-globule-like intermediate states of globular proteins (Uversky et al. 1996), as it binds to solvent-exposed hydrophobic surfaces of polypeptide chains. The polypeptide was dissolved in buffer at pH 8.0 and analyzed in the presence of ANS before and after acidification at pH 4.0. A conformational switch towards a molten globule state was clearly evident, which reversed when the pH was returned to 8.0 (Fig. 2a), in line with our previous results determined at pH 7.0 (Di Gaetano et al. 2006). The reversibility of the pH-induced conformational switch was also demonstrated by intrinsic fluorescence measurements. The decrease in tryptophan fluorescence emission, induced by low pH and due to the acquisition of a more compact conformation, was reversed when the pH returned to 8.0 (Fig. 2b).

By contrast, in the presence of Triton X-100 no increment in ANS fluorescence was observed upon pH switch from 8.0 to 4.0 (Fig. 2c), confirming the inhibiting effect of the detergent on the fibrillogenic process.

When the same experiments were repeated at pH 6.4, either in the presence or in the absence of the detergent, no conformational switch was observed upon acidification to pH 4.0 (data not shown). According to the CD data, these results indicated that, as the protein was already in an α-helical state at pH 6.4, no conformational switch is expected to occur upon acidification.

Effects of lipids on [1-93]ApoA-I conformation

As ApoA-I is known to play a key role in reverse cholesterol transport, this lipid was tested to shed light on the impact that natural lipid ligands may have on [1-93]ApoA-I conformation. First, the effects of cholesterol were analyzed at pH 8.0, i.e., in conditions in which the polypeptide assumes a random coil structure. Far-UV CD spectra of

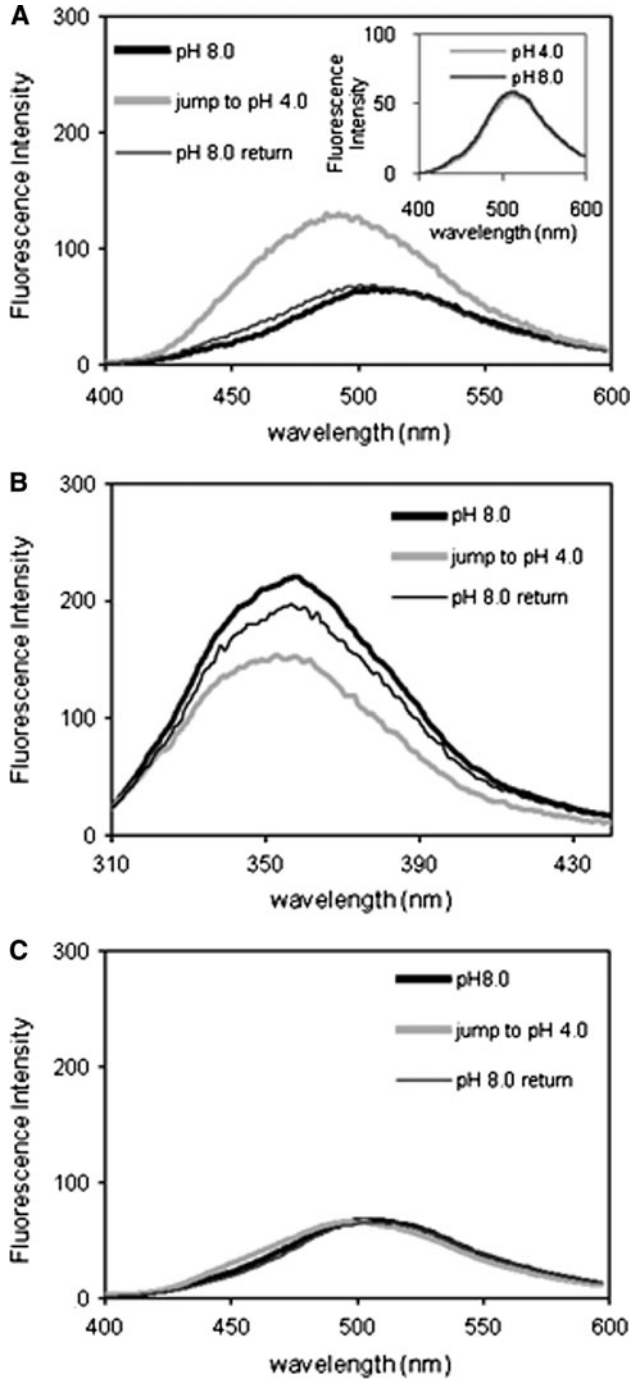

Fig. 2 Spectrofluorimetric analyses of [1-93]ApoA-I. **a** Binding of ANS to [1-93]ApoA-I at pH 8.0, after a pH jump to pH 4.0 and then a return to pH 8.0; spectra of the free dye at pH 8.0 and 4.0 are shown in the inset. **b** Intrinsic fluorescence of [1-93]ApoA-I. Tryptophan emission was monitored at pH 8.0, after a pH jump to pH 4.0 and then a return to pH 8.0. **c** As in **a**, but in the presence of Triton X-100 (0.004%, v/v)

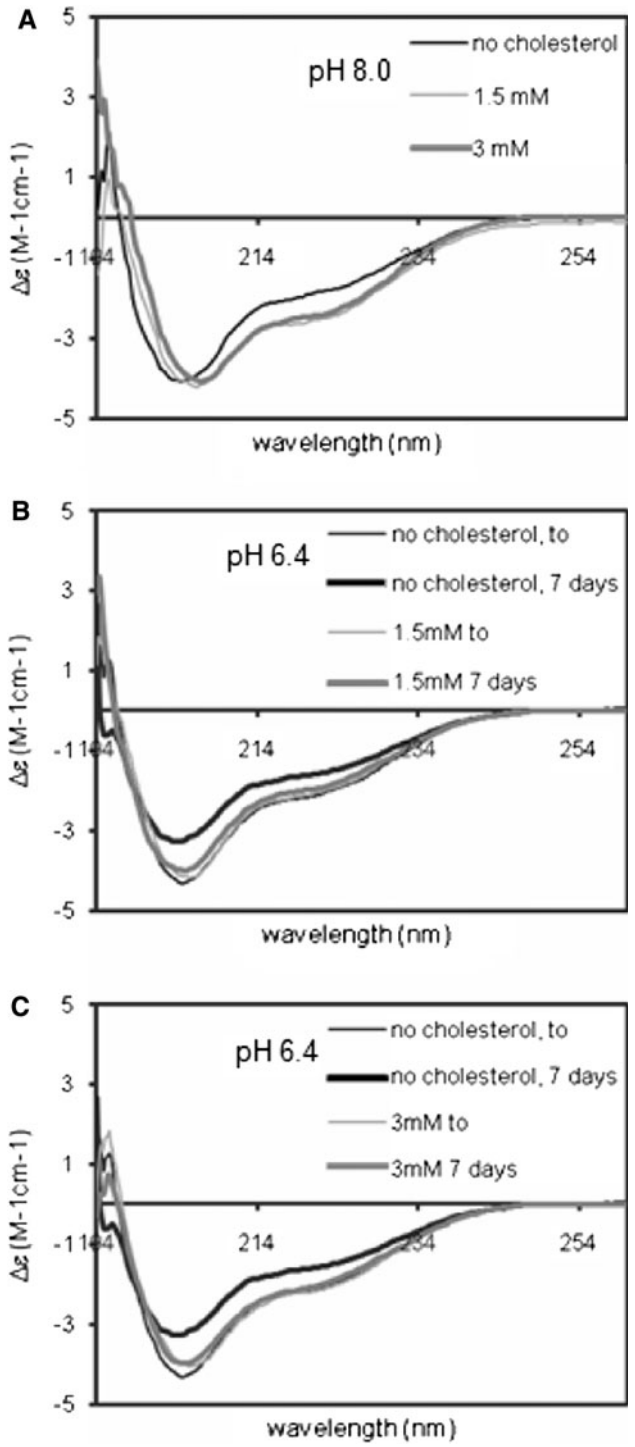

Fig. 3 Conformational analyses of [1-93]ApoA-I in the presence of cholesterol. Far-UV CD spectra of [1-93]ApoA-I were recorded at pH 8.0 (**a**), or at pH 6.4 in the presence of 1.5 mM (**b**) or 3 mM (**c**) cholesterol. In **b** and **c**, spectra were recorded at time 0 (t0) and after 7 days incubation at 37°C in absence or presence of cholesterol

[1-93]ApoA-I in the presence of 1.5 mM cholesterol, which represents the physiological level of HDL cholesterol, revealed a shift of the polypeptide conformation towards a helical state (Fig. 3a). By increasing the cholesterol concentration (3 mM), the helical content was found to increase to 32%.

The effect of cholesterol was then tested at pH 6.4 (Fig. 3b, c), i.e., in conditions that per se promote a helical

state of the polypeptide. Spectra deconvolution indicated that addition of 3 mM cholesterol increased the helical content. Interestingly, in the presence of cholesterol the helical state was found to be stable over time, as after incubation at 37°C for 7 days the CD spectrum was found to be unchanged (Fig. 3b, c), whereas in the absence of cholesterol a loss of signal, indicative of protein aggregation, occurred. These results indicated that cholesterol induces α-helix and inhibits aggregation, similarly to Triton X-100 (Fig. 1d).

We also analyzed the effects of membrane-mimicking structures, such as liposomes, on [1-93]ApoA-I conformation. For this purpose, zwitterionic, negatively charged, and positively charged liposomes were prepared, made of POPC, POPC/POPS, and POPC/DOTAP, respectively. First, far-UV CD analyses of [1-93]ApoA-I were performed at pH 8.0 in the presence of increasing concentrations of each liposome type, over a wide range of lipid-to-polypeptide ratios from 1:1 to 50:1 (M/M). Significant spectral changes were detectable when the lipid-to-protein ratio was higher than 10:1 (data not shown). In Fig. 4a, the results obtained in the presence of zwitterionic, negative or positive liposomes (50:1 ratio) are shown. While a slight increase of helical content was evidenced in the presence of zwitterionic liposomes, a significant increase was induced by charged liposomes. In the case of negatively charged liposomes, the α-helical species became predominant.

Analyses were also performed at pH 6.4 in the presence of zwitterionic (Fig. 4b), positively charged (Fig. 4c), or negatively charged (Fig. 4d) liposomes (10:1 lipid-to-protein ratio). [1-93]ApoA-I CD spectra were recorded immediately after liposome addition (t0), as well as after incubation at 37°C for 48 h or 7 days in the presence of liposomes. Spectral changes, consistent with a progressive transition towards a random coil state, were observed. This behavior was particularly evident in the case of negatively charged liposomes.

Effects of Triton X-100 on [1-93]ApoA-I aggregation

Further studies on the effects of Triton X-100 on the aggregation propensity of [1-93]ApoA-I were performed. First, the kinetics of aggregation of [1-93]ApoA-I was recorded by ThT assays measuring the typical shift of fluorescence emission maximum from 440 to 482 nm usually associated with ThT binding to fibrillar structures. [1-93]ApoA-I was incubated at pH 6.4 in the presence of ThT, and the increase of ThT emission fluorescence at 482 nm was measured over time. A typical S-shaped curve was obtained with a midpoint at about 40 h, in line with previously published results (Guglielmi et al. 2009), whereas in the presence of the detergent, no fluorescence increase was detected up to 220 h (not shown).

Then, the aggregation state of [1-93]ApoA-I was inspected by electrophoretic analyses. The polypeptide, dissolved at pH 6.4 (2 mg/ml), was incubated at 37°C up to 7 days and then analyzed by gel electrophoresis in native conditions, followed by Western blotting with anti-ApoA-I antibodies. As shown in Fig. 5, at time 0 the immunopositive signal was found to be associated with a unique protein

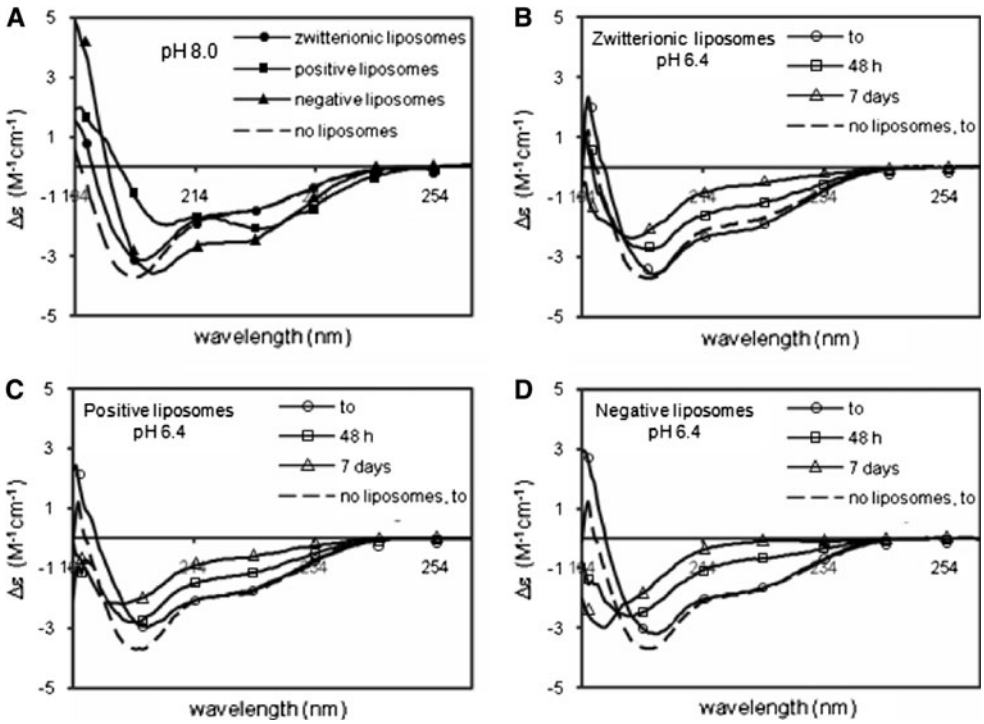

Fig. 4 Conformational analyses of [1-93]ApoA-I in the presence of liposomes. Far-UV CD spectra of [1-93]ApoA-I were recorded at pH 8.0 (**a**) or at pH 6.4 (**b**–**d**) in the absence or in the presence of POPC zwitterionic liposomes (**b**), POPC/DOTAP positively charged liposomes (**c**), or POPC/POPS negatively charged liposomes (**d**). The lipid-to-protein concentration ratio was 50:1 (M/M) in **a** and 10:1 in **b**–**d**. Spectra were recorded immediately after liposomes addition (time 0, t0) and after incubation of the polypeptide at 37°C for 48 h and 7 days in the presence of liposomes. The spectrum of [1-93]ApoA-I in the absence of liposomes is shown for comparison

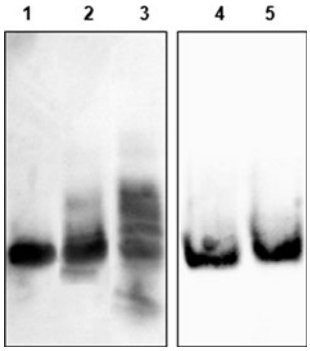

Fig. 5 Analysis of the effects of Triton X-100 on the aggregation state of [1-93]ApoA-I. [1-93]ApoA-I was analyzed by gel electrophoresis (15% acrylamide) in native conditions, followed by Western blotting with anti-ApoA-I antibodies. The polypeptide was analyzed upon incubation at 37°C at pH 6.4 in absence (*lanes* 1–3) or presence (*lanes* 4 and 5) of Triton X-100 (0.004%, v/v), at time 0 (*lanes* 1 and 4) and after 7 days (*lanes* 2 and 5). *Lane* 3, the polypeptide analyzed after 90 days incubation in the absence of Triton X-100

species. After 1 week, this signal was found to be less pronounced and additional immunopositive species were detected. After 90 days, a significant decrease of the signal intensity was accompanied by an increased number of molecular species. By contrast, when [1-93]ApoA-I was incubated in the presence of Triton X-100, no additional species were generated during 1 week incubation (Fig. 5), supporting the evidence that the detergent does affect the aggregation process.

Finally, tapping-mode atomic force microscopy was employed to analyze the morphology of [1-93]ApoA-I incubated at pH 6.4. At time 0 globular structures were observed (Fig. 6a), with mean height of 3.3 ± 0.1 nm. After 7 days incubation, in addition to globular material, short protofibrils were found (representative examples are indicated by arrows in Fig. 6b). The mean aggregate height was 3.7 ± 0.1 nm. On the other hand, in the presence of Triton X-100, after 21 days incubation the sample still displayed globular morphology (Fig. 6c), with globule height of 2.3 ± 0.1 nm; elongated protofibrils such as those observed after 7 days in the absence of detergent were absent. These results demonstrated that the detergent inhibits the aggregation process of [1-93]ApoA-I.

Discussion

Our previous results demonstrated that a pH shift from 7.0 to 4.0 is able to induce a reversible conformational transition from a random coil structure to a helical/molten globule state in the recombinant fibrillogenic polypeptide of ApoA-I (Di Gaetano et al. 2006), similarly to the natural polypeptide isolated ex vivo (Andreola et al. 2003). As the helical conformer plays a key role in the fibrillogenic pathway, it is of interest to study those factors that may stabilize or destabilize the helical/molten globule state. Using a multidisciplinary approach, based on intrinsic and extrinsic fluorescence, CD, electrophoresis, and AFM methodologies, we analyzed the effects of natural and artificial lipids on the propensity of [1-93]ApoA-I to undergo fibrillogenesis.

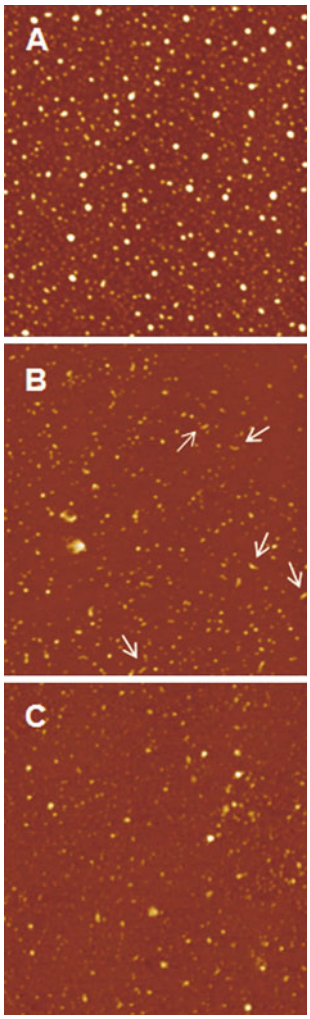

Fig. 6 Tapping-mode atomic force microscopy images (height data) of [1-93]ApoA-I before (**a**) and after (**b**) 7 days incubation at 37°C and pH 6.4 in absence of Triton X-100. Elongated prefibrillar structures formed after 7 days are indicated by arrows in (**b**). **c** After 21 days incubation at 37°C and pH 6.4 in the presence of Triton X-100 (0.004%, v/v). Scan size (**a**, **b**) 2.0 μm; **c** 1.7 μm Z range (**a**, **c**) 5 nm, (**b**) 8 nm

In the absence of lipids we found that: (1) [1-93]ApoA-I is predominantly in a random coil state at pH 8.0; (2) a conformational switch to a helical state is induced by acidification to pH 4.0; (3) upon acidification, a molten-globule-like state, able to bind the apolar dye ANS, is detected as a reversible intermediate species; and (4) upon incubation at pH 4.0 at 37°C aggregation occurs. The presence of a lipid-mimicking detergent, namely Triton X-100, greatly affected

conformational changes of [1-93]ApoA-I following pH variation. The polypeptide adopts a stable helical conformation at pH 8.0 that remains unchanged upon acidification to pH 4.0. Moreover, when incubated under acidic conditions, [1-93]ApoA-I is unable to aggregate and a shift towards a random coil conformation could be observed over time. Therefore, Triton X-100 induces and stabilizes helical conformers of [1-93]ApoA-I, thus hampering aggregation.

The data reported above are in line with the general knowledge that the destabilization of protein and peptide conformation, induced by a drastic variation of physicochemical parameters such as pH, temperature or ionic strength, may trigger the fibrillogenic pathway. In order to understand whether the fibrillogenic pathway of [1-93]ApoA-I can be activated even under quasi-physiological conditions, we carried out the experiments at pH 6.4. We found that, at pH 6.4, in the absence of lipids, [1-93]ApoA-I is predominantly in an α-helical state. As demonstrated by ThT assays, and electrophoretic and AFM analyses, upon incubation at pH 6.4 at 37°C protein aggregation in amyloid-like structures did occur. A shift from pH 6.4 to 4.0 is thus not required to induce fibrillogenesis, although it accelerates the process. AFM analysis of the sample after 7 days incubation showed a change from an entirely globular morphology to coexistence of globular structures and short elongated protofibrils. Collectively, these results suggest that fibrillar species can be generated in physiologic-like conditions, with formation of transient helical structures being an essential prerequisite. In the presence of Triton X-100, the kinetics of aggregation of [1-93]ApoA-I at pH 6.4 was found to be strongly impaired. AFM inspection of the sample revealed the presence of globular structures even after 21 days incubation; no elongated protofibrils whatsoever were observed in these conditions.

The effects of natural lipids on [1-93]ApoA-I conformational behavior were then investigated. To mimic a native-like environment, cholesterol, a natural ApoA-I ligand, was tested at 1.5 mM (its physiological concentration in HDL) and at 3 mM, i.e., above its critical micellar concentration (about 25 nM at 25°C). Interestingly, cholesterol was found to affect the polypeptide conformation in a way that parallels the effects of Triton X-100. Both at pH 8.0, i.e., in conditions in which [1-93]ApoA-I is in a random coil state, and at pH 6.4, i.e., where the polypeptide is mainly in a helical state, cholesterol acts as an inducer of helical conformers and an inhibitor of protein aggregation in a concentration- and time-dependent manner.

Furthermore, unilamellar vesicles were used to analyze the effects on [1-93]ApoA-I conformation of lipids mimicking the assembly of biological membranes. Similarly to cholesterol, zwitterionic, negatively charged, and positively charged liposomes were able to increase the helical content, with negatively charged liposomes showing the greatest effect in inducing helical species. It could be hypothesized that, as [1-93]ApoA-I is very rich in charged residues (Andreola et al. 2003), upon interaction with charged lipids, either positive or negative, intramolecular repulsion effects are masked and helical conformation is favored. The observation that, over time, a random coil structure is generated is suggestive of a negative effect of liposomes on polypeptide aggregation. Therefore, our observations suggest that the composition of membranes may influence the fate of ApoA-I fibrillogenic polypeptide. Further studies will be performed to investigate in detail the interaction of [1-93]ApoA-I with membranes and their effects on fibrillogenesis.

By integrating the data acquired, we propose a model for [1-93]ApoA-I aggregation, depicted in Fig. 7. The N-terminal polypeptide of ApoA-I at pH 7.0 or 8.0 is in a random coil state (RC), stable over time. A "long-range" switch from pH 8.0 to pH 4.0 induces a conformational rearrangement and the appearance of α-helical conformers able to undergo fibrillogenesis (fast process in Fig. 7). At pH 6.4 the polypeptide appears to be in a helical conformation able to slowly evolve into fibrillar species (slow process). At this stage, a "short-range" pH switch from 6.4 to 4.0 is not essential to activate fibrillogenesis, although it accelerates the process by increasing the population of helical conformers. In the tau protein, involved in Parkinson's and Alzheimer's diseases, when 40% α-helical content is reached, heterogeneous aggregates are generated, including amorphous, amyloid-like, and helical fibrillar aggregates (Kunjithapatham et al. 2005).

The effect of a lipid environment is that of trapping the polypeptide in a helical state, thus blocking, or slowing down, the fibrillogenic process. In this regard, lipids may be considered as stabilizing agents able to freeze the helical transient species of [1-93]ApoA-I. This is of interest, because for other aggregating proteins any attempt to analyze the intermediate species of the fibrillogenic pathway failed, due to the rapid formation of insoluble species (Ferreira et al. 2006). On the other hand, from a general

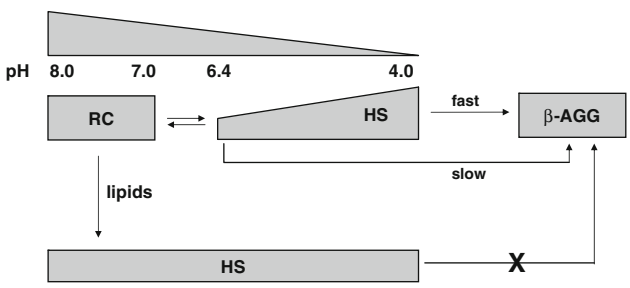

Fig. 7 Schematic representation of the effects of lipids on [1-93] ApoA-I conformational states and their impact on the aggregation process. RC, random coil; HS, helical state; *β*-AGG, beta-aggregation

point of view, our data strengthen the concept that the helical conformer plays a key role in the fibrillogenic process (Pagel et al. 2005; Abedini and Raleigh 2009a) and that stabilization of the aggregation-competent state inhibits fibril formation.

Our results are in line with data in the literature obtained for other fibrillogenic peptides or proteins. Lipid binding was found to inhibit α-synuclein fibril formation (Zhu and Fink, 2003). In the presence of membrane-mimicking detergents at concentrations close to the critical micellar concentration, Alzheimer's Abeta(1–40) peptide adopts a helical conformation (Wahlström et al. 2008). The results reported here are also consistent with the observation that a synthetic polypeptide, corresponding to region 1–44 of ApoA-I, which was found to be unfolded in aqueous solution, in the presence of the lipid-mimicking detergent *n*-octyl-*β*-D-glucopyranoside and of SDS was found to assume an extensive helical structure (Zhu and Atkinson 2004). Moreover, it has been reported that the phospholipid dihexanoylphosphatidylcholine, tested below its critical micellar concentration, is able to induce and stabilize a helical conformation in the 93-residue N-terminal fragment of ApoA-I extracted from ex vivo fibrils (Andreola et al. 2003). Our study expands this observation, as unilamellar vesicles with different lipid composition were used to mimic the effects of a membrane-like lipid assembly.

In this study we demonstrated that a correlation exists between α-helical content and polypeptide aggregation rate and that the fibrillogenic process is strongly affected by a hydrophobic environment that favors formation of α-helices. Moreover, our data confirm and support the general view that freezing the conformation of a key amyloidogenic intermediate along the fibrillogenic pathway impairs fibril formation. Hydrophobic interactions are known to be of key importance in modulating amyloidogenic protein conformation. This is of interest considering that the in vivo role of ApoA-I is mediated by its interactions with lipids, which certainly play an important role in the maintenance of the protein native structure. It has to be noted that the N-terminal region of ApoA-I is involved in lipid binding in the native protein (Frank and Marcel 2000). In this regard, a comprehensive study on factors that may influence the critical balance between folded and partially unfolded states of the fibrillogenic polypeptide, i.e., factors that may act as inhibitors or inducers of fibrillogenesis, is of interest for deep investigation of the fibrillogenic pathway and for developing targeted therapies against this amyloid disease.

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