Conformational Switching and Fibrillogenesis in the Amyloidogenic Fragment of Apolipoprotein A-I*

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The N-terminal portion of apolipoprotein A-I corresponding to the first 93 residues has been identified as the main component of apolipoprotein A-I fibrils in a form of systemic amyloidosis. We have been able to characterize the process of conformational switching and fibrillogenesis in this fragment of apolipoprotein A-I purified directly from ex vivo amyloid material. The peptide exists in an unstructured form in aqueous solution at neutral pH. The acidification of the solution provokes a collapse into a more compact, intermediate state and the transient appearance of a helical conformation that rapidly converts to a stable, mainly β-structure in the fibrils. The transition from helical to sheet structure occurs concomitantly with peptide self-aggregation, and fibrils are detected after 72 h. The α-helical conformation is induced by the addition of trifluoroethanol and phospholipids. Interaction of the amyloidogenic polypeptide with phospholipids prevents the switching from helical to β-sheet form and inhibits fibril formation. The secondary structure propensity of the apolipoprotein A-I fragment appears poised between helix and the β-sheet. These findings reinforce the idea of a delicate balance between natively stabilizing interactions and fatally stabilizing interactions and stress the importance of cellular localization and environment in the maintenance of protein conformation.

Amyloid fibrils derived from apolipoprotein A-I (apoA-I) have been found in patients with autosomal dominant systemic amyloidosis who display particular mutations in the gene encoding apoA-I (Table I) as well as in elderly humans (10) and aged dogs (11) expressing the wild type protein. In all cases of apoA-I-associated amyloidosis, the main constituent of the fibrils is an N-terminal fragment of the protein, 80–93 residues long. A growing number of proteins and peptides have been shown to form amyloid fibrils in vivo and in vitro (12). The native structures of these polypeptides represent the spectrum of polypeptide conformation, from natively unstructured peptides through all β, mixed helix and β, to mainly helical proteins. Several apolipoproteins including apoA-I (1), apolipoprotein A-IV (13), murine and human apolipoprotein A-II (14), apo E (15), and serum amyloid A (16) have been shown to form amyloid or to be implicated in amyloidogenic diseases, and as such, they form a unique group of proteins, sharing structural and sequence similarities and an apparent propensity to form amyloid fibrils.

The plasma apolipoproteins play a critical role in lipid metabolism, and for this reason the sequences and structures of lipoproteins have been extensively studied. Apolipoprotein A-I is the major protein component of high density lipoprotein particles and plays a key role in the solubilization of lipids in these particles, the activation of lecithin cholesterol acyltransferase, the binding of high density lipoprotein to cell surfaces, and the promotion of cholesterol efflux from cells (17). Sequence analysis of apoA-I and other lipoproteins has indicated that these proteins contain repeated amphipathic helices, and this was confirmed in the crystal structure of truncated human apolipoprotein A-I (residues 44–243, i.e. Δ43) (18). These helices, 11 or 2 × 11 residues in length, make up the lipid binding regions of the proteins. The pattern of hydrophobic residues in the lipoprotein helical repeats is similar to the canonical heptad repeat of α-helical coiled coil proteins (19). It has been known for some time that apoA-I undergoes major structural changes when going from the free to the lipid-bound forms (20), and it has been suggested that the N-terminal 98 residues are responsible for maintaining a stable, lipid-free structure of apoA-I and that a conformational switch in residues 1–43 reveals a latent lipid binding domain (21). The shared ancestry of the lipoproteins and the conformational plasticity required for activity may underlie the observed common amyloidogenic nature of the lipoproteins.

In predictions of the secondary structure of apoA-I, the N-terminus has proved the most difficult to assign, and this may reflect the conformational switching that occurs upon lipid binding. A detailed analysis by Nolte and Atkinson (22) assigned random coil to residues 1–8, helix to 9–13, β-structure from 14 to 22, coil or turn from 23 to 33, possible helix to 34–40, and well defined helix between residues 55 and 83. The crystal structure of the Δ43 truncated form of the protein, which has been shown by biochemical and biophysical studies to have an overall structure similar to lipid-bound apoA-I (18), has coil structure between residues 44 and 49 and well defined helical structure in the rest of the molecule.

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The amyloidogenic N-terminal fragment of apoA-I forms long, straight amyloid fibrils with a dominant cross-β-structure similar to that seen in all other forms of amyloid (12). However, we have recently shown that elements of ordered, oriented helical structure are present in some preparations of apoA-I amyloid material, notably in material purified from two different patients with amyloid composed of apoA-I(1–93) and expressing the L174S mutation (23). The superstructure of apoA-I amyloid can apparently accommodate some residual helical structure, whereas the dominant stabilizing structure is β-sheet. The successful purification of fragment 1–93 from ex vivo tissue has made possible the study of the process of fibrillogenesis in this polypeptide. It has not been possible to produce this fragment in recombinant expression systems to date as it is very unstable and sensitive to bacterial proteases. Here we have been able to use material purified from patients to study the pathway of fibril formation and to probe the environmental triggers that may initiate the conformational switching. These results may shed light on the process of amyloid formation in the whole family of lipoproteins that appears to be particularly susceptible to the formation of these stable aggregates.

**EXPERIMENTAL PROCEDURES**

**Purification of ApoA-I(1–93) from Tissue—**Amyloid fibrils were extracted from patient PER by the water extraction procedure of Pras et al. (24) as described in Mangione et al. (23). The protein was then purified by gel filtration in the presence of 5 M guanidine hydrochloride. After the removal of denaturant by dialysis against water, the protein was lyophilized and then resolubilized in water in a concentrated stock solution. The polypeptide extracted from the fibrils was submitted to N-terminal sequencing and mass spectrometry as previously described (23).

**Fluorescence Spectra—**Fluorescence spectra were measured with a PerkinElmer LS50 spectrofluorimeter at 20 °C with excitation at 295 nm. ApoA-I(1–93) was dissolved at a concentration of 0.06 mg/ml in 3 mM sodium acetate, 3 mM sodium phosphate adjusted to a pH range from 3 to 8.

**FT-IR Spectroscopy—**FT-IR spectra were recorded on a Nicolet Magna 560 spectrometer (Madison, WI) purged with Nz, and equipped with a MCT/A detector cooled with liquid nitrogen. The samples, typically 250 μl, were loaded into an ATR cell prepared for liquid samples and sealed with Teflon stoppers. The internal reflection element assembly was a trapezoidal (50 × 2 × 20 mm) 25-reflection germanium plate from Grasy Specac (Kent, UK). A cavity for the sample was created using a 0.5-mm-thick Teflon spacer between the internal reflection element and the metal plate. Spectra were collected at room temperature and without a polarizer.

ApoA-I(1–93) was dissolved at a concentration of 1 mg/ml in 10 mM d5-acetate pH 7.4 (corrected for deuterium effects) and incubated overnight to allow HD exchange. The pH 7 was adjusted to 4, and 300 interferograms were collected at a resolution of 1 cm−1 (collection lasted approximately 1 min), which were averaged and processed with zero filling Happ-Genzel apodization. Data were collected at regular times over a period of 16 h.

**Results**

Effect of pH on Compactness and Secondary Structure Content—The apoA-I(1–93) polypeptide was purified from ex vivo material and displayed the N-terminal sequence of mature apoA-I and a molecular mass of 10720 Da. As previously shown, it adopts a random coil conformation at neutral pH in aqueous buffer (23). Knowing that the polypeptide assumes a cross-β conformation in the amyloid fibrils, we initially probed the acquisition of more compact structure by monitoring the total tryptophan fluorescence signal arising from the three tryptophan residues at positions 8, 50, and 72. It is clear from Fig. 1a that the pH of the solution has an effect on the protein conformation such that the environment of the tryptophans is altered. The tryptophan emission maximum of a protein usually shifts from shorter wavelengths to around 350 nm, the fluorescence maximum of tryptophan in aqueous solution, when proteins are unfolded. At neutral pH the fluorescence emission maximum from apoA-I(1–93) is at 347 nm, but this shifts to close to 341 nm at pH 3, consistent with transfer of the tryptophan side chains into a hydrophobic environment and the development of a more compact structure. Studies of full-length apoA-I in n-propyl alcohol buffered with Tris at pH 8.2 indicate that in this helix-inducing solvent, mimicking the effect of lipids, the wavelength of maximum fluorescence occurs at 342 nm (only one other Trp occurs outside of the N-terminal amyloidogenic fragment, at position 108). Leroy and Jonas postulate (20) that the similarities in the spectroscopic proper-

**Table I**

List of the apoA-I amyloidogenic mutations so far reported and the sequence limits of the corresponding polypeptides isolated from natural fibrils

| ApoA-I variant | Ethnic origin | Length of the apoA-I polypeptide in the fibrils | Ref. |
|----------------|---------------|-----------------------------------------------|-----|
| G26R           | British, Irish, Scandinavian | 1–93                                         | 1   |
| L60R           | English       | 1–88, 1–92, 1–93                              | 2   |
| T50R           | Ashkenazi     | 1–86, 1–92, 1–93                              | 3   |
| Glu70–Phe71–Trp72 Deletion | Not specified | Not characterized                             | 4   |
| L90P           | Not specified | 1–88, 1–94                                    | 5   |
| R173P          | Caucasian     | 1–90 to 1–100                                 | 6   |
| 60–71 Deletion/Val-Thr insertion | Spanish   | 1–83, 1–92                                   | 7   |
| L174S          | Italian       | 1–93                                         | 8   |
| L178H          | French        | Full-length apoA-I and uncharacterized fragments | 9   |

- D. Booth, personal communication.
ties observed in apoA-I in 30% n-propyl alcohol and in reconstituted high density lipoprotein were such that it was likely that most of the Trp residues are in or near a helix but are accessible to solvent. In the recombinant high density lipoprotein complexes the phospholipids make contact with the tryptophans and shield them from solvent. Strikingly, our CD data from polypeptide 1–93 suggested that the structural collapse from a predominantly unstructured form at neutral pH (Fig. 1b), presenting a minimum at 203 nm, was accompanied by acquisition of helical structure at lower pH (Fig. 1c) in which the minima are at 208 and 222 nm. The helical conformation induced at pH 4 is not stable, however (Fig. 2), and the formation of insoluble material is observed after ~5 min. This is accompanied by loss of CD signal. The polypeptide in a helical conformation at pH 4 appears to undergo intermolecular association and precipitation.

**The Process of Fibril Formation**—We have used FT-IR spectroscopy to follow the formation of aggregates at low pH because this technique can be used for monitoring secondary structure changes in a mixture of soluble and insoluble material. The loss of helical signal and the increase in β-structure that accompanies the formation of aggregates are illustrated in Fig. 3a. After the pH of the solution was adjusted to 4, acquisition of spectra was initiated rapidly and continued over a period of 16 h. After 5 min at pH 4, an increase in the signal at 1615 and 1684 cm⁻¹ was observed. The signal centered at 1615 cm⁻¹ corresponds to the contribution of β-structure, and the signal at 1684 cm⁻¹ is specific for the presence of anti-parallel β-structure. After 12 h the process of conversion into β-sheet is complete. This can be compared with the FT-IR spectra at pH 3 and 7 (Fig. 3b) and with the spectrum of amyloid fibrils purified from patient PER (Fig. 3c). These are particularly interesting because they contain a substantial residual helical element (1655 cm⁻¹), and this information is consistent with the α-helical component we have detected by x-ray diffraction in natural fibrils (23). The search for fibrils in the aggregate was performed by electron microscopy every 24 h, and apparently only after 72 h at 4 °C, under these acidic conditions, very long, straight fibrils were detected by negative stain electron microscopy (Fig. 4). The material analyzed by electron microscopy does not display fibrillar structure in the first 72 h. The lag phase between the α- to β-structure conversion and the fibril formation is not surprising, in general, for protein fibrillogenesis. In the case of apoA-I in particular, the coexistence of non-fibrillar aggregate and typical amyloid fibrils is noted in the natural fibrils analyzed by atomic force microscopy in which the non fibrillar aggregate has a globule like structure presenting a mean height around 4.5 nm. Therefore, it is possible that the process of fibrillogenesis in vitro requires a slow conversion of oligomeric aggregates into long fibrils, and it is likely that an equilibrium of the two states of association exists in vivo. Unfortunately the relatively small amounts of material available have so far made the alignment of fibrils for x-ray fiber diffraction and comparison of in vitro and ex vivo fibrils impossible.

Some fibrillar material was also detected after incubation at pH 7, although the fibrils formed under these conditions were

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3 A. Relini et al., manuscript in preparation.
not as long or as straight as those produced at pH 4. Full-length apoA-I has also been shown to form fibrils in vitro at pH 7, and at least some fragments of apoA-I appear to be colocalized with Aβ in senile plaques (25).

Effect of Phospholipids on Secondary Structure—The apoA-I-(1–93) peptide was incubated with a range of concentrations of DHPC at pH 4 and pH 7 to study the effect of a lipid environment on this lipid-associated protein (Fig. 5). This is a short (6-carbon) chain phospholipid that exhibits a critical micelle concentration of 16 mM (26) and behaves as a detergent micelle in aqueous solution rather than forming lipid bilayers. The low dielectric constant medium provided by the lipid acyl chains promotes the formation of a helical structure, possibly by increasing intramolecular hydrogen bonding and electrostatic and dipolar interactions and decreasing competition with water. At pH 4, the effect of low concentrations of DHPC (up to 7.5 mM) is to induce some helical structure into the polypeptide, but this form is not stable and, with time, signal is lost. With increasing DHPC concentration (from 10 mM and above), the helical conformation is stabilized to a point where no signal is lost over 3 h (Fig. 6). Increasing the DHPC concentration beyond this point increases the strength of the helical signal slightly. At pH 7, concentrations of DHPC up to 10 mM have only a limited effect on the conformation of the peptide. Above 10 mM DHPC, a decrease in the ellipticity at 222 nm indicates that some helix is present, but the spectra suggest that there is a substantial coil element present. This altered conformation appears to be stable at pH 7. DHPC appears to be able to induce some helical structure under favorable pH conditions and to mask intermolecular interactions that can occur between molecules in the helical form. Therefore, high concentrations of this short chain lipid prevent fibril formation at low pH but do not induce helical conformation at pH 7.

Effect of TFE on Secondary Structure—To study the role of the helical conformation in apoA-I-(1–93) fibrillogenesis, the polypeptide was incubated with a range of TFE concentrations at pH 4 and at pH 7 (Fig. 7). TFE can promote α-helical structure in proteins and polypeptides (27), but high concentrations of TFE have been used to destabilize the native states of proteins and to populate amyloidogenic forms of proteins such as acylphosphatase (28) as well as to induce fibril formation in an amphipathic peptide derivative of apo C-II (29). The presence of TFE induces a strong helical signal from apoA-I-(1–93) at both pH 4 and 7, and this helical form is stable in TFE, presumably because of increased intramolecular hydrogen bonding and a reduced hydrophobic effect. More TFE is required to induce or to stabilize equivalent amounts of helix at pH 4 than at pH 7. Therefore there is an apparent dichotomy between a high intrinsic tendency to populate a helical structure at low pH and the apparent requirement for higher concentrations of TFE to produce equivalent amounts of helical structure at pH 4 and at pH 7. This may be explained by the formation of intermolecular coiled-coil structure at low pH. As discussed previously, the 1–93 peptide is predicted to form a coiled-coil structure in the full-length protein. An analysis of the relative ellipticity exhibited at 222 and 208 nm can distinguish between the coiled-coil structure and the α-helical structure (30). A 222:208 ellipticity ratio of −1 indicates interhelical contacts, whereas values around 0.9 suggest helix without interhelical contacts. We have measured the ellipticity ratio for apoA-I-(1–93) at pH 4, and we obtain a ratio of 1.07 in the absence of TFE and 0.87 in the presence of 40% TFE, suggesting interacting helices in the absence of TFE but loss of these contacts in the presence of the solvent. At pH 7, the ratio falls to 0.78 at 20% TFE. We propose that there may be a competing effect of the TFE at pH 4 to destabilize the coiled-coil interactions on the one hand and to stabilize the α-helical structure on the other, and higher concentrations are therefore required to stabilize equivalent amounts of helical structure compared with pH 7. At pH 7, the helix induced by 20% TFE is stable. However, the same proportion of TFE at pH 4 appears to accelerate the loss of signal and, by inference, precipitation.

These results appear to support the idea that the formation of these amyloid aggregates requires the peptide to pass from its random conformation through a helical conformation to the stable β-sheet structure. If all of the polypeptide material is stabilized in this helical form, with masking of intermolecular interactions, then aggregation and fibril formation are prevented. No development of soluble β-structure was observed during fibril formation by this apoA-I polypeptide, and this is different to what has been reported in numerous amyloidogenic peptide systems and also in other lipoproteins that form amyloid (31). Instead, the aggregating conformer that is transiently populated below the pl (4.35) is mainly helical, and the conformational switching to form fibrils with β-structure occurs after precipitation.

Hydrophobicity, Charge Distribution, and the Effect of Mutations—The mean hydrophobicity and the net charge of apoA-I...
full-length and truncated 1–93 were calculated as suggested by Uversky et al. (32) and are reported in Fig. 8. Through these parameters it was also possible to calculate the “boundary” mean hydrophobicity value, \((H)_{\text{boundary}}\), by means of the equation (33) \((H)_{\text{boundary}} = (R + 1.151)/2.785\).

This equation, in which \(R\) corresponds to the mean net charge of the polypeptide, predicts a natively unfolded state for proteins that have a mean hydrophobicity below the \((H)_{\text{boundary}}\) value. Both full-length apoA-I and the polypeptide 1–93 fulfill the requirements for natively unfolded proteins.

There is clearly an effect of pH on this amyloidogenic polypeptide; the helical conformation is populated at pH values below the pI, and the polypeptide has a random coil structure at neutral pH. The sequence of apoA-I is very rich in charged residues. Of the 93 residues, 20 are negatively charged (10 Asp and 10 Glu), and 11 are positively charged (4 Arg and 7 Lys) (Fig. 8). Protonation of the acidic residues appears to be the trigger for the transient helix formation and subsequent intermolecular association and conversion to \(\beta\)-structure. At neutral pH the clusters of acidic residues may result in charge-charge repulsion and a more random conformation, whereas neutralization of the negative charges removes the local repulsion effects and allows helical structure. The \(pK_a\) values of glutamate and aspartate suggest that lowering of the pH will result in protonation of the glutamate residues first. These acidic residues are mainly situated in the C-terminal part of the polypeptide (Fig. 8), so neutralization of these side chains may allow the helical conformation to be adopted. Several of the amyloidosis-associated mutations identified to date have had the effect of adding an extra positive charge to the sequence of the N-terminal fragment. However, because of the large number of charges in the sequence, the additional charge actually has a relatively small effect on the pI of the polypeptide (increasing from 4.35 to 4.45). The mutations at 26, 50, and 60 will result in the positioning of opposite charges separated by one residue. This may favor extended \(\beta\)-sheet structure.

Many other amyloidogenic proteins have been shown to be affected by acidification, notably transthyretin and lysozyme. Dobson (12) and Kelly (34) propose that the lysosomes may play a key role in initiation of fibrillogenesis, although the pH of the lysosome may not be as low as the pH used to initiate amyloidosis \textit{in vitro}. An acidic environment may destabilize some protein structures and allow conformational switching or it may allow the population, even to a small extent, of a misfolded species that is aggregation prone. In the case of the apoA-I

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**Fig. 3. Development of \(\beta\)-structure.** a, time course showing the amide I region of the IR spectrum of apoA-I-(1–93) after the pH* (corrected for deuterium effects) was adjusted to 4. Spectra are as follows: no symbol, 5 min; □, 32 min; ○, 68 min; ▲, 127 min; ●, 183 min; ▼, 240 min; ▲, 322 min; ▲, 340 min; dashed line, overnight. b, amide region of the IR spectra of apoA-I-(1–93) at pH 3 (○) and 7 (●). c, secondary structure in full-length native apoA-I and natural apoA-I amyloid fibrils. Amide I region of the IR spectra of native full-length apoA-I (solid line) and amyloid fibrils extracted from patient PER (broken line).
fragment, the very large number of charged residues in the N-terminal fragment that will be directly affected by pH changes are almost certainly influential in the initiation of conformational change. Our studies of the effect of acidification by fluorescence indicated that reducing the pH caused some conformational change, presumably collapse of the random coil structure. At the relatively low protein concentrations used for the fluorescence experiments, protein aggregation was not observed after the pH was reduced. However, monitoring of the acidification process by CD at higher protein concentrations indicated that aggregation succeeded conformational change.

DISCUSSION

In this study we have identified environmental conditions that drive the conversion of the polypeptide 1–93 of human apolipoprotein A-I from random coil to a predominantly antiparallel β-sheet fibrillar aggregate, passing through a helical conformation. We have shown that this polypeptide has chameleon properties (35). Under certain conditions, random, helical, or β-sheet conformations can be stabilized, with a barrier to reciprocal conversion. Aqueous solutions at neutral pH favor the random coil structure, trifluoroethanol and phospholipids stabilize the helical conformation, and low pH conditions prime the self-aggregation into β-sheet structure. The persistence of a predominantly random coil structure in aqueous solution at neutral pH is unusual for a polypeptide as long as the apoA-I-(1–93) peptide but is not unique among fibrillogenic proteins. Synuclein, one of the human proteins able to create pathologic fibrils in vivo, has been demonstrated to behave in a similar manner. α-Synuclein is unfolded in its native state (36), but the presence of an organic solvent like hexafluoroisopropanol induces the acquisition of helical structure, and in this conformation the protein is very sensitive to the heat-induced aggregation (37). Phospholipids have a similar effect on synuclein and apoA-I-(1–93); in fact both proteins undergo large conformational changes and an increase in and stabilization of helical structure in the presence of phospholipids (38). It is worth noting that the most fibrillogenic portion of α-synuclein is the N-terminal polypeptide 1–87 (39); however, despite the similar

**FIG. 5.** Effect of DHPC on conformation at pH 4 and at pH 7. Comparison of the far-UV CD spectra of apoA-I-(1–93) incubated with 0 (no symbol), 2.5 (●), and 7.5 mM □ DHPC at pH 4 and 7.

**FIG. 6.** Effect of DHPC and time on the conformation of apoA-I-(1–93). The far-UV CD spectra of the polypeptide incubated at pH 4 with 0 mM DHPC (○), 5 mM DHPC ( ), 10 mM DHPC (●), and 15 mM DHPC (■).
behavior, the level of sequence homology between α-synuclein and the apoA-I N terminus is not significant. A comparison that may be more significant though is the fact that both apoA-I-(1–93) and α-synuclein belong to the category of natively unfolded proteins (32). The experimental data presented here and previously performed NMR studies (23) are fully consistent with the prediction of apoA-I-(1–93) being a natively unfolded protein on the basis of the algorithm that considers the mean hydrophobicity and the mean net charge in aqueous solution at neutral pH (32, 33). On the basis of these parameters full-length apoA-I in the lipid-free state also fulfills the criteria for the inclusion in this protein category. Conditions permissive for in vitro fibrillogenesis of lipid-free full-length apoA-I were discovered by Wisniewski et al. (25), but it is uncertain if this process is relevant to the deposition in vivo. Biomedical data so far available suggest that the cleavage of full-length apoA-I and the release of the N-terminal polypeptide could represent the regulatory step in the fibrillogenic pathway. The accuracy of the site of the proteolytic cleavage is apparently crucial for any further metabolic processing of apoA-I polypeptide. In fact, enhanced proteolytic susceptibility is described for apoA-I variants associated with metabolic abnormalities, but without amyloid disease. Of interest is the metabolic pathway of apoA-I Finland associated with hypolipoproteinemia (40). In this case the mutation (L159R) affects the protein secretion, reduces the half-life of circulating apoA-I, and has a dominant negative effect on wild type apoA-I, a behavior quite similar to that hypothesized for the apoA-I (L174S) variant (23). In the case of the apoA-I Finland mutation, a 18-kDa N-terminal polypeptide (40) is released in plasma and appears to prime a degradative, rather than a fibrillogenic, pathway.

The tissue compartment where the putative primary proteolytic cleavages occur for both the wild type and amyloidogenic species awaits definition; at present, the chemical environment and the nature of the proteolytic enzyme(s) are largely unknown. We believe that new data in this area will complement the data we have presented on the folding dynamics of this apoA-I polypeptide and will allow the molecular mechanism underlying this disease to be elucidated.
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Fibril Formation by the Human ApoA-I-(1–93) Polypeptide 2451
Conformational Switching and Fibrillogenesis in the Amyloidogenic Fragment of Apolipoprotein A-I

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