Electron Paramagnetic Resonance Spectroscopy of Site-directed Spin Labels Reveals the Structural Heterogeneity in the N-terminal Domain of ApoA-I in Solution*

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Apolipoprotein A-I (apoA-I) is the major protein constituent of high density lipoprotein (HDL) and plays a central role in phospholipid and cholesterol metabolism. This 243-residue long protein is remarkably flexible and assumes numerous lipid-dependent conformations. Consequently, definitive structural determination of lipid-free apoA-I in solution has been difficult. Using electron paramagnetic spectroscopy of site-directed spin labels in the N-terminal domain of apoA-I (residues 1–98) we have mapped a mixture of secondary structural elements, the composition of which is consistent with findings from other insoluble methods. Based on side chain mobility and their accessibility to polar and non-polar spin relaxers, the precise location of secondary elements for amino acids 14–98 was determined for both lipid-free and lipid-bound apoA-I. Based on intermolecular dipolar coupling at positions 26, 44, and 64, these secondary structural elements were arranged into a tertiary fold to generate a structural model for lipid-free apoA-I in solution.

High density lipoprotein (HDL)3 plays a central role in lipid metabolism as a principal facilitator of reverse cholesterol transport, a process wherein cholesterol is mobilized from peripheral tissues and delivered to the liver and steroidogenic organs. Low levels of HDL represent a major risk factor for cardiovascular disease, and there is growing interest in therapeutic interventions that can enhance HDL plasma levels or its anti-atherogenic activity (1).

Apolipoprotein A-I (apoA-I) comprises ~70% of the protein content of HDL and is a primary determinant of HDL structure, composition, and stability. ApoA-I is a prominent member of the exchangeable apolipoprotein class of proteins, and HDL derives a large portion of its functionality from the ability of apoA-I to sequester phospholipid and cholesterol and functionally interact with plasma enzymes and cellular receptors (for a recent review see Ref. 2).

In a previous study, we applied site-directed spin label electron paramagnetic resonance (SDSL-EPR) spectroscopy to determine the conformation of the C-terminal domain of apoA-I (3). This approach allowed us to both describe the structure of the lipid-free apoA-I C terminus (residues 163–241) and also report on the structural changes that arise because of lipidation. Using a similar approach, we have targeted the N-terminal one-third of apoA-I to identify regions of conformational flexibility and sites of inter- and intramolecular interaction. The unique genetic (4, 5), pathophysiological (6, 7), and structural (8, 9) features associated with the N terminus highlight the need to ascertain the structure and dynamics of this region of apoA-I in the presence and absence of lipid.

The present SDSL-EPR analysis of residues 14–98 of full-length apoA-I reveals a mixed secondary structure composition for this region. We also identify sites of more stable tertiary interactions in an otherwise conformational dynamic segment of the protein. To observe the effect of lipid binding on the structure of the N terminus, the spin-labeled samples were analyzed in reconstituted HDL discs, providing insight into the adaptive response that the secondary structure of apoA-I undergoes during lipidation.

EXPERIMENTAL PROCEDURES

Materials—Thio-specific nitrooxide spin label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate (MTS-SL) was received as a kind gift from Dr. K. Hideg (University of Pecs, Hungary). Standard molecular biology procedures were used to create the different plasmid constructs. All mutagenic primers were purchased from Sigma-Genosys (The Woodlands, TX), and Davis Sequencing (Davis, CA) performed DNA sequence determination.

Construction, Expression, Purification, and MTS Labeling of ApoA-I Mutants—PCR mutagenesis was applied on an apoA-I template enhanced for bacterial expression (10). Dideoxy DNA sequencing confirmed the substitutions and the absence of unwanted changes. The resulting constructs expressed apoA-I mutant proteins with single substitutions of

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3 The abbreviations used are: HDL, high density lipoprotein; apoA-I, apolipoprotein A-I; CD, circular dichroism; CroX, chromium oxalate; EPR, electron paramagnetic resonance; MTS-SL, methanethiosulfonate spin label; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-sn-glycerophosphocholine; SDSL, site-directed spin label; TBS, Tris-buffered saline.
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A

L14C A15C T16C V17C Y18C V19C D20C V21C
L22C K23C D24C S25C G26C R27C D28C Y29C
V30C S31C G32C F33C E34C G35C S36C A37C
L38C G39C K40C Q41C L42C N43C L44C K45C
L46C L47C D48C N49C W50C D51C S52C V53C
T54C S55C T56C F57C S58C L59C L60C R61C
E62C Q63C L64C G65C V67C T68C Q69C E70C
F71C W72C D73C N74C L75C E76C K77C E78C
E80C L82C R83C Q84C E85C M86C S87C K88C
D89C L90C E91C E82C V83C K94C A95C K96C
V97C Q98C

B

[Graph showing residue numbers and correlation analysis]

C

[Graph showing residue numbers and correlation analysis]

D

[Graph showing residue numbers and correlation analysis]
the native amino acids for cysteines, and the mutants were purified and labeled with MTS-SL as described previously (3). Briefly, the apoA-I protein was sequentially incubated with 100 μM Tris-(2-carboxyethyl)phosphine and 300 μM MTS spin label on a Ni2+-chelated HiTrap column (GE Healthcare) under denaturing conditions (3 M guanidine-HCl). The protein purity was estimated higher than 95% by SDS-polyacrylamide gel electrophoresis analysis and Coomassie Brilliant Blue staining. The protein concentration was determined by use of the MicroBCA assay kit from Pierce (Rockford, IL). Bovine serum albumin was used as a standard.

ApoA-I Lipidation—Reconstituted nascent HDL were prepared by a modified method originally described by Nichols et al. (11, 12). To 0.5 ml of 16.3 mM 1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine (POPC) in TBS, pH 8 was added an equal volume of TBS, pH 8, 22 mM sodium deoxycholate. The mixture was vortexed and incubated at 37 °C until completely clear. 3 mg of spin-labeled apoA-I was added to the cleared dispersion (1:2.04 w/w apoA-I:POPC) and incubated for 1 h at 37 °C. Deoxycholate was removed by extensive dialysis against TBS, pH 8. Reconstituted nascent HDL was recovered by KBr density gradient ultracentrifugation at 50,000 × g for 3 h in a Beckman Optima TLA 100.4 rotor. Fractions containing both protein and lipid were pooled and dialyzed against TBS, pH 7.4. The size of lipiddated discoidal complexes was confirmed by gradient gel electrophoresis (11).

EPR Analysis—EPR measurements were carried out in a JEOL X-band spectrometer fitted with a loop-gap resonator (13, 14). For room temperature spectra, an aliquot (5 μl) of purified, spin-labeled protein (100 μM) was placed in a sealed quartz capillary contained in the resonator. Spectra were acquired at room temperature (20–22 °C) from a single 60-s scan over a field of 100 G at a microwave power of 2 milliwatt and a modulation amplitude optimized to the natural line width of the individual spectrum (0.5–1.5 G). The spectra were normalized by the intensity of their integrals. For frozen spectra (−100 °C), samples were first flash-frozen in sealed capillaries by rapid immersion into a butane bath cooled in liquid nitrogen. Samples were then placed in a TE cavity and scanned over 100 G using a microwave power of 0.1 milliwatt and modulation width of 1 G.

Molecular accessibility of spin-labeled side chains to chromic oxalate (CrOx) and O2 was determined using successive power saturation scans as described (15). II1/2 values (which also were used to calculate the contrast function (Φ)) were calculated using software provided by C. Altenbach.

Molecular Modeling—Insight II software (version 2005) on the Octane work station by Silicon Graphics was used for structural modeling. The protein data bank coordinates of crystalline lipid-free apoA-I (code 2A01) (16) were used as templates.

RESULTS

SDSL-EPR Analysis of Lipid-free ApoA-I—The chemical and structural properties of the N-terminal one-third of apoA-I were examined by EPR spectroscopy. Here, 82 individual cysteine substitution mutants of apoA-I were created between residues 14 and 98.4 These 82 cysteine substitution variants of apoA-I were expressed in bacteria, purified, and labeled at the cysteine sulfhydryl with nitroxide label. The EPR spectra obtained from these protein samples (Fig. 1A) reported on the immediate milieu of the labeled side chains. Strong immobilization of the side chain is observed as spectral broadening (for example, see spectra for V30C and L75C in Fig. 1A), whereas a high degree of side chain motional freedom is characterized by a sharpness of the spectra (Fig. 1A, G35C and V53C spectra). The inverse central line width (δ−1, Gauss−1), a parameter that directly reflects the side chain mobility (17), was used to determine the secondary structure (Fig. 1B, bottom). In addition, the collision frequency between the nitroxide on the labeled side chain and the polar or non-polar relaxation agents (chromium oxalate (CrOx) and oxygen (O2), respectively) was used to determine the accessibility (II; Fig. 1B, upper graph) of the respective compound and thus also the chemical environment at the side chain. Based on the periodicity (generated from asymmetrical solvent or tertiary contacts) of these values, several regions were identified as α-helical with a helical pitch of 3.6 residues/turn.

For example, residues 26–34 display an α-helical pattern with a periodicity between 3 and 4 for both the mobility and accessibility parameters. This can be seen in the EPR spectra of residues 26, 30, 33, and 34, the line shapes of which display immobilized, broadened features. The intervening residues exhibit more mobile spectra, and their II-values indicate they are in a more hydrophilic environment. Moreover, the residues with immobilized spectra are concentrated within a hydrophobic region in the helical wheel plot (Fig. 1D). The same approach was used to identify other α-helical regions. These are comprised of residues 36–42, 44–51, 55–85, and residues 92–98 (Fig. 1B–D). Of these segments, the mobility parameter for residues 36–42 do not exhibit this periodicity, suggesting a more complex modulation of side chain dynamics in this region. However, the accessibility of the relaxation agents for these residues bears a helical periodicity, allowing for an assignment of helical secondary structure for residues 36–42.

In addition, residues 20–25 bore an alternating periodicity of mobility consistent with β-strand structure. This con-

4 Pro-66 was not substituted in order to preserve its unique backbone conformation. Thr-79 was omitted due to poor expression.
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cluision is also supported by the non-polar accessibility parameter for this stretch of amino acids (Fig. 1B, IIO$_2$). The mobility parameter for residues 87–92 also demonstrates such a periodicity. However, because the accessibility data for this region do not support such a pattern and also because the overall mobility is high, we propose this region to be a loop; although the presence of β-strand structure cannot be completely excluded.

In contrast to the clearly defined structural regions of the N-terminal third of apoA-I, there are segments of this region that appear unstructured (Fig. 1B, white). These loop regions typically exhibit spectra with high mobility and are also readily accessible by EPR relaxation agents. For example, the line shapes for spectra 51–55 are sharp with high δ$^{-1}$ values (>$0.4$ Gauss$^{-1}$). In particular, position 53 exhibits a spectrum that is similar in appearance to the spectra of unbound nitroxide. These residues thus clearly define a loop region. Similarly, the spectra of nitroxides at residues 35 and 43 also display high mobility, which does not fit with the helical pattern of the neighboring residues. Thus, residues 35 and 43 may also represent interruptions in the regular helical structure. By the same criteria, residues 86 to 91 have been assigned coil/turn secondary structure. The final assignment of secondary structure is summarized in Fig. 1C.

Several of the EPR spectra of lipid-free apoA-I display a broadening (Fig. 2) indicative of dipolar coupling between two nitroxides. For proteins labeled with only one disulfide-linked nitroxide, the dipolar interaction must be intermolecular. At the concentrations of apoA-I used in this study, the quaternary state of apoA-I exists as a dynamic equilibrium between monomers, dimers, and higher order oligomers (18). Because of the limited range in which dipolar broadening is observable (<20 Å) by continuous wave EPR and the small subset of residues, which exhibit dipolar spin coupling, we assign residues 26, 44, and 64 within the central plane of an antiparallel dimer. Further evaluation of the broadening as a function of the distance-dependent dipolar interaction was performed by collecting the EPR spectra of frozen samples. In the absence of motion (i.e. frozen in liquid N$_2$), a semiquantitative analysis of the extent of broadening as a function of the distance-dipolar interaction was performed by collecting the EPR spectra of frozen samples. In the absence of motion (i.e. frozen in liquid N$_2$), a semiquantitative analysis of the extent of broadening as a function of the distance-dipolar interaction can be obtained from the $d_1/d$ spectral ratio (19, 20) (Fig. 2B). All four examined positions reveal a $d_1/d$ ratio between 0.38 and 0.41, confirming a weak dipolar coupling and therefore the close proximity of spin labels in the self-associated population. This alignment of amino acids allows for the synthesis of a tertiary model in which segments of secondary structure containing sites of self-interaction can be aligned relative to the central plane of the protein (3).

**SDSL-EPR Analysis of Lipid-bound ApoA-I**—ApoA-I undergoes major structural changes as it binds lipids. During this process the helical structure content increases from 45–58% (21) to ~80% for lipid-bound apoA-I (22). To examine the structural consequences of lipid binding, the spin-labeled apoA-I protein preparations described above were utilized in the formation of recombinant HDL. This allowed for a direct comparison of the EPR spectra of the N-terminal domain in two environmentally distinct states. Clearly, a majority of nitroxide-labeled side chains experienced a higher steric hindrance in the lipid-bound state compared with the lipid-free state. This is typified by generally broader spectral shapes for lipid-bound forms of the protein (Fig. 3A). The exceptions include positions Ser-31, Thr-56, and Leu-64, which all display an increased mobility in the lipid-bound state, whereas some side chains, such as Leu-42 and Arg-83, appear to be structurally unaffected.

To define the helical faces in the apoA-I N terminus relative to lipid, the contrast function ($\Phi$), which reflects the ratio between the accessibility parameters for O$_2$ and CrOx (Fig. 3B). This value is especially useful for membrane-embedded proteins and lipoproteins because O$_2$ dissolves particularly well in the hydrophobic lipid phase, and CrOx is highly hydrophilic. A helical pitch of 3.67 was used to define helical regions (Fig. 3B). This periodicity corresponds to an 11/3 helix, which has been proposed to better conform to the curvature of the periphery of the reconstituted HDL disc and arrange the hydrophobic and charged residues around the helical axis in comparison to a 3.6 helical pitch (23). The SDSL-EPR analysis of lipid-bound apoA-I suggests that residues 13–38 and 49–98 form two individual α-helices, and that the intervening residues display α-helical character (residues 43–47). The helical wheel display (Fig. 3C) shows that the distribution of hydrophobic and charged residues around the helical axis is in agreement with the expected amphipathic character of helices in lipid-bound apoA-I.

**FIGURE 2. Dipolar coupling between spin-labeled residues in apoA-I dimers.** A, the EPR spectra from spin labels at residues 26, 44, 64 (this study), and 167 (3) indicate dipolar coupling between residues on two dimerized apoA-I molecules. B, frozen spectra were used to calculate the $d_1/d$ line height ratio.
DISCUSSION

The first 90 amino acids of apoA-I compose a unique feature of this molecule. The distinctiveness of this region is highlighted by the presence of proteolytically derived N-terminal fragments (amino acids 1 to ~83) of apoA-I within senile amyloid plaques. Interestingly, the first 43 amino acids of apoA-I are encoded by exon 3, which is the most highly conserved region of apoA-I, whereas the remainder of apoA-I is encoded by exon 4 (4). The structure of the apoA-I gene suggests that this region bears a critical role distinct from the remainder of apoA-I. Furthermore, lipid-free apoA-I lacking residues 1–43 adopts a conformation resembling lipid-bound apoA-I (8, 9). Thus it has been hypothesized that these residues serve to stabilize the compact lipid-free conformation by protecting a latent lipid-binding domain, available only after initial lipid interaction (8).

Although the majority of positions throughout apoA-I display dynamics and molecular accessibility that is consistent with the recently solved crystal structure (16), results from EPR analysis suggest the conformation of the N-terminal domain in solution diverge significantly from the crystalline state. The first 90 amino acids in the crystal structure compose of two long α-helices flanked by loop regions, composing half of a four-helix bundle. However, lipid-poor apolipoproteins are in general noted for their dynamic properties, especially at their termini, and thus the highly ordered structure of crystalline apoA-I may not be representative of a prevalent conformer in solution. Because the thermodynamics of lipid binding are frequently driven by reorganization of secondary and tertiary structural elements, mapping the dynamics of the apoA-I polypeptide at its N-terminal region is an important step in elucidating the mechanism of lipid recognition and lipid-driven reorganization.

As shown recently with the KvAP potassium channel (24) and the MsbA multi-drug resistance protein (25), EPR of site-directed spin labels is useful for identifying conformers in solution that may not be observable when the protein is in a crystalline state. Such discrepancies are not entirely unexpected for apolipoproteins, the global
conformational rearrangements of which require a dynamic lipid-free state for lipid sensing and structural conversion. In particular, the EPR data complement the crystal structure by identifying regions involved in hinge bending and misfolding (6, 7) but also in describing the secondary structure, dynamics, and arrangement of specific domains (3, 26, 27).

The dynamic nature of the N-terminal domain of apoA-I is evident from the appearance of more than one component in the majority of EPR spectra from sites within this region. Thus we find regions within both the C- (3) and N-terminal domains display conformational heterogeneity, consistent with two states in equilibrium. Our model for secondary structure in the N terminus of lipid-free apoA-I is based on the accessibility of side chains to paramagnetic relaxers (II) and side chain mobility (\(\delta^{-1}\)). In the case of the former, the values will reflect the most predominant conformational state, whereas the simple \(\delta^{-1}\) evaluation of side chain mobility may over-represent the more dynamic species when considering comparable populations of restricted and mobile side chains. The general agreement of the parameters (II and \(\delta^{-1}\)) as a function of N-terminal sequence position, along with the identification of sites displaying dipolar interaction, is reflected in the model shown in Fig. 4. Here the structure of the ground-state crystal conformer was modified according to the EPR results to produce a time-averaged conformer of lipid-free apoA-I in solution (Fig. 4B).

The most distinguishing feature between the crystal structure (16) and the EPR-based solution model is the secondary structure content in the N-terminal domain. The assignment of \(\beta\)-strand secondary structure in lipid-free apoA-I is in agreement with circular dichroism (CD) measurements of the protein in solution (28, 29). Although the crystal structure of apoA-I provides the basis for an elegant model of nascent HDL formation, the fixed ground-state conformation of crystal structure does not reflect the substantial conformational dynamics inherent to apoA-I in solution. For example, the thermodynamics of folding suggest the protein experiences a loosely packed molten globule in solution (30).

Other features of lipid-free apoA-I in solution are not apparent from the crystal structure. These include sites of protease sensitivity in the N-terminal domain (31), the environment of tryptophan residues within the protein (32), and a \(\beta\)-strand secondary structure content on the order of 10% determined by CD spectroscopy (28, 29). In addition, the \(\alpha\)-helical content of lipid-free apoA-I is measured to be in the range of 45–58% (21), much lower than the 82% observed in the crystal structure (16). The secondary structure content of the crystal structure resembles more the lipid-bound structure (\(\sim 70–78\%\)) (33) and is in notable agreement with the secondary structure of lipid-bound apoA-I presented in this study. An additional detail not reported in the crystal structure is derived from the relatively high motional freedom of residues 33–44 in both lipid-free and lipid-bound states. This suggests these residues reside within a flexible linker region, consistent with NMR structure analysis of apoA-I in a lipid-mimetic solvent (34). It is noteworthy that in the short segment of \(\beta\)-strand structure we identify, the N terminus of apoA-I is similar in length to the \(\beta\)-strand we observe in its C terminus (3). Ongoing studies in our group and elsewhere (28) are exploring the potential of these regions to participate in inter- or intra-domain interaction.

The antiparallel association apoA-I in solution facilitates identification of positions that fall within a central plane of the molecule. In our previous study (3), we found position 167 resides near the center of a long helix that is followed by a flexible loop, an arrangement present in the crystal structure of apoA-I (16). EPR of site-directed spin labels has now been used to examine nearly all sequence positions within N-terminal (1–90) and C-terminal (160–240) regions of apoA-I. In each study we have identified three to four sites that display a weak dipolar coupling as determined from a broadening of the continuous wave EPR spectrum, with the spectra from all other positions in these regions free of dipolar broadening. A
similar occurrence of weakly interacting sites is observed in the central one-third of the protein\(^5\). The sporadic appearance of self-interacting sites throughout the sequence of apoA-I reflects an antiparallel arrangement of apoA-I dimers, whereas the absence of any strong coupling suggests a loosely associated oligomer, a finding consistent with earlier measurements showing apoA-I in solution exists in a dynamic equilibrium of monomers, dimers, and higher order oligomers (18).

In summary, EPR spectroscopy of site-directed spin labels reveals a dynamic N-terminal domain that is heterogeneous in its secondary structure. In this lipid-bound state, the N-terminal region of apoA-I adopts an elongated helix on the particle surface, where its hydrophobic face partitioned into an aliphatic environment. Dynamic regions in apolipoproteins play a key role in lipid sensing and structural conversion (3). Thus, these findings provide important insights into the thermodynamics of lipid binding and the associated structural rearrangements that occur in apoA-I and related apolipoproteins.

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