Structure-guided Protein Engineering Modulates Helix Bundle Exchangeable Apolipoprotein Properties*

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Robert S. Kiss‡‡, Paul M. M. Weers**, Vasanthy Narayanaswami, Jenny Cohen, Cyril M. Kay‡, and Robert O. Ryan‡‡

From the Department of Biochemistry and Protein Engineering Network of Centers of Excellence, University of Alberta, Edmonton, Alberta T6G 2S2, Canada and Lipid Biology in Health and Disease Research Group, Children’s Hospital Oakland Research Institute, Oakland, California 94609

Apolipoprotein (apo) E plays a major role in lipid metabolism by mediating cellular uptake of lipoprotein particles through interaction with members of the low density lipoprotein (LDL) receptor family. The primary region of apoE responsible for receptor binding has been limited to a cluster of basic amino acids between residues 134 and 150, located in the fourth helix of the N-terminal domain globular helix bundle structure. To investigate structural and functional requirements of this “receptor binding region” we engineered an apolipoprotein chimera wherein residues 131–151 of human apoE were substituted for residues 146–166 (helix 5) of Manduca sexta apolipopophorin III (apoLp-III). Recombinant hybrid apolipoprotein was expressed in Escherichia coli, isolated, and characterized. Hybrid apolipoprotein and apoE3-N-terminal, but not apoLp-III, bound to heparin-Sepharose. Far UV circular dichroism spectroscopy revealed the presence of predominantly α-helix secondary structure, and stability studies revealed a urea denaturation midpoint of 1.05 M, similar to apoLp-III. Exchangeable Apolipoprotein Properties*

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The physiological relevance of apoE is demonstrated by the progression of severe atherosclerosis in knockout mouse models and naturally occurring mutations in human apoE (2–4). apoE serves as a ligand for the low-density lipoprotein (LDL) receptor and the LDL receptor-related protein, thereby mediating removal of lipoproteins from the plasma compartment. The ability of apoE to redistribute among lipoproteins in response to fat metabolism and bind multiple lipoprotein receptors is crucial to its role in lipoprotein metabolism. apoE also interacts with cell surface heparan sulfate proteoglycans (5), and this interaction appears to work in concert with the LDL receptor-related protein receptor as a means to internalize lipoprotein particles (6).

apoE is composed of two independently folded domains (7). Residues 1–191, which forms the N-terminal domain, has been shown to be responsible for the receptor binding function (8) whereas residues 210–299 comprise the C-terminal domain that has a high lipid binding affinity (9). X-ray crystallography of the N-terminal domain showed that it exists as a globular bundle of four elongated amphipathic α-helices (10, 11). The helix bundle conformation is quite stable in solution (12) yet is proposed to undergo a dramatic structural alteration upon lipid interaction wherein it adopts a receptor competent conformation (4, 13, 14).

Numerous studies have led to the conclusion that the major receptor binding region of apoE is localized to residues 130–150 within the NT domain (4). This region is rich in basic amino acids and is located entirely within the boundaries of helix 4. Helix 4 is amphipathic and orients its hydrophobic face toward the interior of the bundle. Thus, in the absence of lipid, helical segments are stabilized by hydrophobic helix-helix contacts. In isolation, peptides encompassing apoE residues 141–155 (15), 130–152 (16), or 126–183 (17) are largely unstructured in aqueous medium. These results suggest tertiary contacts in the bundle conformation are necessary for maintenance of the α-helix conformation of peptides derived from this region of apoE. From other studies it is known that helical structure of the basic residue cluster within residues 140 and 150 is critical for normal binding to the LDL receptor (18). Upon interaction with an appropriate lipid surface, substitution of helix-helix interactions for helix-lipid interactions preserves the helicity of apoE.

The abbreviations used are: apo, apolipoprotein; DMPC, dimyristoylphosphatidylcholine; LDL, low density lipoprotein; PL-C, phospholipase C; DMEM, Dulbecco’s modified Eagle’s medium; FAFA, fatty acid-free albumin; ANS, 1-anilino-8-naphthalenesulfonate; WT, wild-type; apoLp-III, apolipopophorin III; mAb, monoclonal antibody; NT, N-terminal; TFE, trifluoroethanol.
this region and promotes adoption of a receptor-active state, possibly through greater exposure of basic residue side chains (19).

Using an alternative strategy to study apoE peptides, Mims et al. (20) covalently attached a lipophilic anchor to apoE residues 129–169. Although this peptide was able to bind the LDL receptor when bound to an LDL substrate lipoprotein, the same peptide lacking the lipid modification was not. In other work Datta et al. (21, 22) attached apoE residues 141–150 to an idealized, synthetic lipid binding peptide, producing a fused peptide that was capable of binding to LDL and very low density lipoprotein, thereby enhancing cellular uptake and degradation of these particles via a heparan sulfate proteoglycan-facilitated pathway. The peptide also elicited a decrease in plasma cholesterol levels in apoE knockout mice, although the effect was relatively short-lived.

In the present study, residues 131–151 of apoE have been substituted for residues 146–166 of an unrelated model helix bundle apolipoprotein, Manduca sexta apolipophorin III (apoLp-III), creating an apolipoprotein chimera that provides a helix bundle structural scaffold to preserve the α-helix structure of apoE residues 131–151 in the absence of lipid (23, 24). The hybrid apolipoprotein retains physicochemical and functional characteristics of apoLp-III, yet manifests the heparin and LDL receptor binding characteristics of apoE.

**EXPERIMENTAL PROCEDURES**

*Materiales—* Bovine serum albumin, dimyristoylphosphatidylcholine (DMPC), trifluoroethanol (TFE), and *Bacillus cereus* phospholipase C (PL-C) were obtained from Sigma. Dubecco's modified Eagle's medium (DMEM) was obtained from Bio-Whittaker (Walkersville, MD), and fetal bovine serum and lipoprotein-deficient serum were obtained from Hyclone (Logan, UT). Human LDL was isolated from the blood of healthy male volunteers by sequential ultracentrifugation between the density limits of 1.019 and 1.063 g/ml, and LDL was radiiodinated according to Langer et al. (25). The specific activity of the 125I-LDL was at least 100 cpm/ng LDL protein. Recombinant apoE3-NT (N-terminal domain residues 1–183) was prepared as described previously (26) whereas *M. sexta* apoLp-III was prepared according to Ryan et al. (27).

**Plasmid Vector Construction**—The human apoE3-NT/pET plasmid (26) served as the template for amplification of the nucleotide sequence encoding residues 131–151 of apoE. Oligonucleotides containing non-annealing Stul and HindIII restriction endonuclease sites served as primers for polymerase chain reaction amplification of the apoE cDNA fragment. Subsequently, the coding sequence of *M. sexta* apoLp-III was amplified using the apoLp-III/pET plasmid as template (27), and the cDNAs were digested with Stul and HindIII. The apoE cDNA digestion product was subcloned into the digested apoLp-III/pET vector. The resulting plasmid construct included the coding sequence for residues 1–145 of apoLp-III followed directly by the nucleotide sequence encoding residues 131–151 of apoE. Thus, in the hybrid apolipoprotein construct, nucleotides encoding residues 146–166 of apoLp-III were replaced by nucleotides encoding residues 131–151 of apoE. The sequence of the hybrid vector construct was verified by DNA sequencing.

For bacterial expression, the hybrid apolipoprotein coding sequence was cloned into the pTYB2 vector (New England Biolabs). Oligonucleotides containing non-annealing NdeI and Smal restriction sites served as primers for polymerase chain reaction amplification of the hybrid apolipoprotein coding sequence in the pET vector. The amplified product was ligated into the pTYB2 vector at its unique NdeI and Smal restriction sites, downstream of the T7 promoter, in-frame with the translation start codon and the vector-encoded intein/chitin binding domain fusion protein sequence, producing the hybrid apolipoprotein/pTYB2 vector. The sequence of the hybrid apolipoprotein insert was confirmed by DNA sequencing.

**Recombinant Protein Synthesis and Purification**—Escherichia coli BL-21 (DE3) cells were transformed with the hybrid apolipoprotein/pTYB2 plasmid. Saturated overnight cultures (grown in 2× YT medium) with ampicillin (50 μg/ml culture medium) were grown with initial OD50 of 0.3–0.5 and grown at 37 °C until the optical density at 600 nm reached 0.6. Following induction with 2 μM isopropyl-β-D-thiogalactopyranoside, the cells were cultured for 4 h at 37 °C. Bacteria were pelleted by centrifugation, resuspended in buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1 mM EDTA), and sonicated in the presence of 0.1% Triton X-100. After sonication and centrifugation, the soluble fraction was loaded onto a chitin-agarose column. Recombinant protein production utilized the IMPACT (New England BioLabs) system, wherein a ternary hybrid apolipoprotein-intein-chitin binding domain fusion protein is generated. Passage of the soluble fraction obtained from the bacterial cell pellet over a chitin-agarose column and washing was followed by an in-column overnight incubation in which buffer containing 100 mM dithiothreitol at room temperature to induce intein-mediated self-cleavage and liberation of the hybrid apolipoprotein. Elution of the column yielded a single major protein of the expected size, as judged by SDS-PAGE. The hybrid apolipoprotein was further purified by reversed-phase high pressure liquid chromatography using an RCK-8 Zorbax 300SB column with a linear AB gradient of 0.5%/min, where Solvent A was 0.05% trifluoroacetic acid in water, and Solvent B was 0.05% trifluoroacetic acid in acetonitrile. Fractions were monitored at 210 nm, and fractions containing the hybrid apolipoprotein were pooled, lyophilized, and stored at −20 °C until use. The final protein product contained a vector-encoded N-terminal methionine and a C-terminal glycine residue.

**Immunology—*Immunoblotting was performed after transferring separated proteins from SDS-polyacrylamide gels onto a polyvinylidene difluoride membrane. The presence of apoLp-III epitopes was detected using a rabbit polyclonal antibody directed against *M. sexta* apoLp-III at a dilution of 1:5,000. Monoclonal antibodies (mAb) specific for the receptor binding region of human apoE3 (1D7, provided by Dr. Ross Milne, University of Ottawa Heart Institute, and 2E8, provided by Dr. Karl Weisgraber, Gladstone Foundation, San Francisco, CA) were used to probe the hybrid apolipophorin III for presence of epitopes located within residues 131–151 of human apoE, both at dilutions of 1:5,000.

**Analytical Methods—** Electrospray ionization mass spectrometry analyses were performed using a VG quattro electrospray mass spectrometer (Fisons Instruments, Manchester, United Kingdom). Protein masses were determined as the mean value of several multiply charged ions within a coherent series. The instrument was calibrated with horse heart myoglobin (16,951 Da). Isoelectric focusing gel electrophoresis was performed using the PHAST gel apparatus (Amersham Biosciences). CD spectroscopy was performed using a Jasco J-720 spectropolarimeter (Jasco Inc., Easton, MD) interfaced with an Epson Eq. 386/25 computer equipped with Jasco software. Near and far UV CD spectra were obtained as described earlier (28), and deconvolution was performed using the Contin program, version 1.0, of Provencher and Gloeckner (29). Denaturation experiments were performed by observing the effect of increasing concentrations of urea on the molar ellipticity at 222 nm. The denaturation curve was fit to a sigmoidal shape to approximate the midpoint of urea denaturation. Sedimentation equilibrium experiments were carried out in 50 mM Tris, pH 8.0, at 20 °C with Beckman XL-A analytical ultracentrifuge using absorbance optics following the procedures described by Laue and Stafford (30). 110-μl aliquots of sample solution were loaded into six-sector charcoal-filled Epon sample cells, allowing three concentrations of sample to be run simultaneously. Runs were performed at 14,000 and 18,000 rpm, and each speed was maintained until there was no significant difference in r² versus absorbance scans taken 2 h apart to ensure that equilibrium was achieved.

The sedimentation equilibrium data were evaluated using the NONLIN program, which incorporates a nonlinear least-squares curve-fitting algorithm described by Johnson et al. (31). This program allows for analysis of both single and multiple data files. Data can be fit to either a single ideal species model or models containing up to four associating species, depending on which parameters are permitted to vary during the fitting routine. The protein's partial specific volume and the solvent density were estimated using the SEDNTERP program, which incorporates calculations detailed by Laue et al. (32).

**Heparin Binding Studies—** Hybrid apolipoprotein, *M. sexta* apoLp-III, and apoE3-NT were dissolved in buffer (20 mM sodium phosphate, pH 7.2) and applied to a HiTrap Heparin column (Amersham Biosciences). After loading, the column was washed with buffer, and bound proteins were eluted with a linear gradient of NaCl from 0 to 1.0 M.

**Lipoprotein Binding Assays—** Human LDL (150 μg protein) in 10 mM sodium phosphate, pH 7.5, was incubated with 80 milliunits of *B. cereus* PL-C at 37 °C in the absence or presence of 30 μg of WT apoLp-III or hybrid apolipoprotein. Sample absorbance was measured at 340 nm in a Spectromax 250 microtiter plate reader (Molecular Devices, Sunnyvale, CA) as a function of time.

**Phospholipid Vesicle Transformation Assays—** Bilayer vesicles of DMPC were prepared by extrusion as described elsewhere (33). Protein
apoE3-NT (top right) are shown. The C-terminal 21 amino acids of apoLp-III (part of the fifth molecular architecture. The chimera was created by replacing the apoE3 includes three acidic and eight basic residues (plus one pH 7.2, 150 mM NaCl, 0.5 mM EDTA using 250 /H9262 presence of 5 lino-8-naphthalenesulfonate (ANS) were carried out in 20 mM Tris-HCl, monochromators set at 612 nm using a slit width of 3 nm. LS 50B luminescence spectrometer, with the excitation and emission light scattering intensity was measured on a PerkinElmer Life Sciences C. Sample apoE3-NT; lane 3 apoLp-III; lane 2, WT apoLp-III; lane 3, hybrid apolipoprotein.

was dissolved in 20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5 mM EDTA. DMPC (125 μg) and an equal amount of protein were mixed in a final volume of 250 μl using a microcuvette equilibrated at 24 °C. Sample light scattering intensity was measured on a PerkinElmer Life Sciences LS 50B luminescence spectrometer, with the excitation and emission monochromators set at 612 nm using a slit width of 3 nm.

ANS Dye Binding—Fluorescent dye binding experiments with 1-ani- lino-8-naphthalenesulfonate (ANS) were carried out in 20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5 mM EDTA using 250 μM ANS alone or in the presence of 5 μM apoE3-NT, M. sexta apoLp-III, or hybrid apolipoprotein. Excitation was set at 395 nm, and emission was monitored between 400 and 800 nm using a slit width of 6 nm.

LDL Receptor Binding Assay—Human skin fibroblasts were grown to ~60% confluence in the presence of DMEM with 10% fetal bovine serum. Fibroblasts were then grown to 100% confluence in DMEM with 10% lipoprotein-deficient serum. At confluence, cells were cooled on ice for 30 min, washed twice with phosphate-buffered saline containing 1 mg/ml fatty acid-free albumin (FAFA), and then incubated with DMEM containing 1 mg/ml FAFA, 2 μg/ml 125I-LDL, and different amounts of receptor binding competitor for 2 h at 4 °C. The medium was removed, and the cells were washed five times with chilled phosphate-buffered saline-FAFA and two times with chilled phosphate-buffered saline. Cells were released from the surface of the dishes by incubation with 0.1 N NaOH for 1 h at 24 °C, and cell-associated radioactivity was measured on a Cobra II Auto-Gamma Counter. Cell protein was determined by the bicinchoninic acid assay (Pierce) using bovine serum albumin as the standard.

RESULTS

Design of the Hybrid Apolipoprotein—The three-dimensional structures of M. sexta apoLp-III (23, 24) and human apoE3-NT (10, 11) were utilized in the design of an apolipoprotein chimera. apoLp-III is a 166-amino acid protein composed of five amphipathic α-helices that adopt a globular helix bundle molecular architecture. The chimera was created by replacing the C-terminal 21 amino acid residues of apoLp-III (part of the fifth helix) with 21 residues from helix 4 of human apoE (Fig. 1). Whereas residues 146–166 of apoLp-III include five acidic and four basic residues (plus two histidine), residues 131–151 of apoE3 includes three acidic and eight basic residues (plus one histidine). The hydrophobic face of the apoLp-III segment is composed primarily of alanine and valine, subtending an angle of ~120 degrees. On the other hand, the hydrophobic face of the apoE segment is composed entirely of leucine residues, subtending an angle of ~100 degrees. The hydrophobic/hydrophilic interface in this peptide is clearly defined although the hydrophilic face contains multiple hydrophobic residues (e.g. Val-135, Ala-138, and Leu-149). Given these differences, it is expected that replacement of residues 146–166 of apoLp-III by apoE residues 131–151 will 1) introduce an increase in net positive charge, 2) increase the general hydrophobic character of this segment of the protein, 3) decrease the angle subtended by the hydrophobic face of this helix, and 4) increase the presence of hydrophobic residues on the hydrophilic face. Despite these differences, the hybrid apolipoprotein should provide a suitable protein scaffold for helix 4 of apoE such that the biological function of this helix segment can be studied outside of the context of the apoE protein.

Bacterial Expression and Identification of the Hybrid Apolipoprotein—The hybrid apolipoprotein/pTYB2 vector was transformed into E. coli for expression of a hybrid apolipoprotein-intein-chitin binding domain fusion protein. Recombinant hybrid apolipoprotein was liberated from the fusion via an in-column, intein-mediated, cleavage reaction. Analysis of the recombinant hybrid apolipoprotein product by SDS-PAGE indicated a relative migration that was comparable with WT apoLp-III (Fig. 2). Isoelectric focusing yielded an apparent pI of 9.0 for the hybrid apolipoprotein (compared with 6.0 for WT apoLp-III; see Ref. 34), consistent with an increased net positive charge in the hybrid apolipoprotein. Electrospray ionization mass spectrometry analysis of the hybrid apolipoprotein yielded a molecular mass of 18,777, in very good agreement with the predicted mass of 18,775 for this chimera. An immunoblot probed with rabbit polyclonal antibodies directed against M. sexta apoLp-III (Fig. 2, B) revealed that WT apoLp-III and hybrid apolipoprotein were recognized by the antibody, whereas apoE3-NT was not. In other immunoblot experiments, employing two mAbs specific for the receptor binding region of apoE (1D7 and 2E8), reactivity toward the hybrid apolipoprotein was not detected (data not shown).
Characterization of the Hybrid Apolipoprotein—Far UV CD spectroscopy of the hybrid apolipoprotein in buffer (Fig. 3) indicated a high content of $\alpha$-helix secondary structure (61%). Inclusion of 50% TFE (v/v) resulted in an induction of $\alpha$-helix (77%). Near UV CD analysis (Fig. 3, inset) revealed a pattern of peaks and troughs representing the environments of the aromatic residues (seven phenylalanines and one tyrosine) in the hybrid apolipoprotein. The CD spectral data demonstrate that the hybrid apolipoprotein is folded in solution. To determine the relative stability of the hybrid apolipoprotein, the effect of urea on the molar ellipticity at 222 nm was determined. A single native $\rightarrow$ unfolded transition with a midpoint at 1.05 M urea was observed (Fig. 4). Sedimentation equilibrium experiments revealed that, unlike WT apoLp-III or apoE3-NT, the hybrid apolipoprotein self-associates in solution. A plot of apparent molecular weight versus concentration showed a range of molecular weights that could be fitted to a monomer-dimer-tetramer model. The calculated apparent average molecular weight from a global fit of the data was 30,309, a value that is between the monomer and dimer molecular weight.

Heparin Binding Studies—A well characterized property of apoE is its interaction with heparan sulfate proteoglycans. Using heparin as a model for heparan sulfate proteoglycans, two heparin binding sites have been identified in apoE, one in the NT domain and one in the CT domain (35, 36). The NT domain binding site has been localized to residues 142–157 and therefore is present within the apoE peptide (residues 131–151) employed in construction of the hybrid apolipoprotein. Thus, if the apoE peptide folds correctly in the context of the hybrid apolipoprotein, it should manifest heparin binding affinity. In control experiments, it was demonstrated that WT apoLp-III lacks heparin binding ability. On the other hand, apoE3-NT and the hybrid apolipoprotein bound to heparin-Sepharose (Fig. 5). Application of a linear gradient of NaCl revealed that the ionic strength required for elution of both apoE3-NT and hybrid apolipoprotein from the column was similar, indicating that heparin binding activity has been conferred to the hybrid apolipoprotein as a result of helix swapping.

Lipid Interaction Properties of the Hybrid Apolipoprotein—A hallmark feature of exchangeable apolipoproteins is an ability to transform DMPC bilayer vesicles into discoidal complexes, resulting in a marked decrease in sample light scattering intensity (37). In control incubations lacking apolipoprotein, no changes in DMPC vesicle light scattering were observed (Fig. 6). In other control incubations, M. sexta apoLp-III induced a $\sim$65% decrease in light scattering intensity after 30 min whereas apoE3-NT induced a corresponding 15% decrease over the same time period. Examination of the hybrid apolipoprotein revealed dramatically increased vesicle solubilization activity, eliciting a $\geq$90% decrease in light scattering intensity after 200 s. Subsequent preparative scale experiments permitted analysis of hybrid apolipoprotein-DMPC complexes by native gradient PAGE. The size of the particles was $\sim$600 kDa, similar to that reported for disc complexes containing WT apoLp-III (38).

To examine its ability to bind spherical lipoproteins, human LDL was incubated with PL-C in the presence or absence of hybrid apolipoprotein. PL-C-induced hydrolysis of LDL phosphatidylcholine generates diacylglycerol moieties that destabilize LDL structural integrity, leading to particle aggregation and sample turbidity development. In studies of this phenomenon Liu et al. (39) showed that exchangeable apolipoproteins, including WT apoLp-III, bind to PL-C-modified LDL and prevent aggregation. In control incubations lacking exogenous apolipoprotein PL-C induced a rapid increase in LDL sample...
turbidity. Under the conditions employed WT-apoLp-III and hybrid apolipoprotein prevented PL-C-induced LDL aggregation, consistent with formation of a stable binding interaction (data not shown).

Fluorescent Dye Binding Experiments—To examine the hypothesis that enhanced DMPC vesicle transformation activity of the hybrid apolipoprotein may be because of increased exposure of hydrophobic sites on the protein surface as a result of helix swapping, dye binding experiments with the fluorescent probe, ANS, were performed (Fig. 7). Whereas ANS displays low fluorescence in buffer alone (curve a), its fluorescence is dramatically enhanced (together with a blue shift in emission max) upon interaction with hydrophobic sites on proteins (40, 41). Thus, ANS fluorescence can be used to measure the amount of exposed hydrophobic surface on a given protein. At pH 7 WT M. sexta apoLp-III had little effect on ANS fluorescence (curve b), indicating a lack of solvent-exposed hydrophobic pockets in the globular five-helix bundle. Likewise, apoE3-NT induced a similar, albeit slightly larger, increase in ANS fluorescence intensity (curve c). By contrast, the hybrid apolipoprotein induced a 7–10-fold increase in ANS fluorescence intensity compared with the parent proteins (curve d).

Receptor Binding Activity of the Hybrid Apolipoprotein—To examine whether the hybrid apolipoprotein is a functional ligand for LDL receptor binding, 125I-LDL competition binding assays were performed. Human skin fibroblasts were grown to confluence in lipoprotein-deficient serum, transferred to 4 °C, and incubated with 125I-LDL in the absence or presence of competitor ligands. 125I-LDL binding in the absence of competitor is taken as 100% binding (Fig. 8). Addition of a 50-fold excess of unlabeled LDL (cold LDL; 100 μg of protein) resulted in a marked decrease in 125I-LDL binding. Lipid-free WT apoLp-III, apoE3-NT, or hybrid apolipoprotein induced only slight decreases in 125I-LDL binding (data not shown). Likewise, WT apoLp-III-DMPC discs competed poorly. However, addition of hybrid apolipoprotein-DMPC discs gave rise to a concentration-dependent reduction in 125I-LDL binding. The level of reduction observed with hybrid apolipoprotein discs at 50 μg/ml was similar to that seen with apoE3-NT-DMPC at the
same concentration. Thus, the data indicate that hybrid apolipoprotein-DMPC discs possess LDL receptor binding activity.

**DISCUSSION**

In the present study an apolipoprotein chimera was designed and generated wherein residues 131–151 of human apoE were substituted for the 21 C-terminal residues of insect apoLp-III. We hypothesized that providing a helix bundle scaffold for apoE residues 131–151 would allow for proper alignment and orientation of the hydrophobic face of this amphipathic helix, a necessary requirement for maintaining and stabilizing helix-lipid and helix-helix interactions, as well as productive interaction with receptors of the LDL receptor family (18).

Hybrid apolipoprotein was expressed in *E. coli* as part of a ternary intein/chitin binding domain fusion protein. Immunoblot experiments showed that, whereas antibodies directed against *M. sexta* apoLp-III recognized the hybrid apolipoprotein, mAbs specific for the receptor binding region of apoE did not. Although the epitopes of mAb 1D7 and mAb 2E8 include the sequence introduced into the hybrid apolipoprotein, recognition may require an additional sequence that is not present in the chimera. For example, mAb 2E8 showed reduced reactivity toward human apoE2, whereas arginine 158 had been substituted by cysteine (42). Likewise mAb 1D7 has been shown to react with residues 150–160 of human apoE. Thus, it is reasonable to speculate that the inability of these antibodies to recognize the hybrid apolipoprotein may be because of either the presence of an incomplete epitope or altered structural organization of the epitope in the engineered helix bundle setting.

Far UV CD spectra of the hybrid apolipoprotein showed distinct troughs at 208 and 222 nm, indicative of the presence of $\alpha$-helical secondary structure. Hybrid apolipoprotein $\alpha$-helix content, as predicted from deconvolution of the spectra, was 61%, compared with 60% for WT apoLp-III (28). In the presence of 50% TFE, hybrid apolipoprotein $\alpha$-helix content increased to 77%, consistent with results observed for WT apoLp-III,apoE3-NT domain, and other helix bundle exchangeable apolipoproteins (26, 27, 43, 44). Moreover, CD spectra in the absence and presence of TFE reveal differences in the ratio of ellipticity at 222 and 208 nm. In other $\alpha$-helical peptides, a 222/208 nm ellipticity ratio of $-1.0$ is indicative of interhelical contacts, such as those present in coiled coil or helix bundle structures (45–47). By contrast, values in the range of 0.90 indicate an elongated helix with little or no interhelical contacts (46–48).

The ratio of 0.95 observed for the hybrid apolipoprotein in buffer is consistent with an $\alpha$-helix bundle organization whereas the observed decrease in the ratio of ellipticity at 222/208 nm to 0.91 in the presence of 50% TFE ($\delta\psi$) suggests replacement of helix-helix contacts for helix-TFE contacts.

Near UV CD spectra revealed extrema corresponding to discrete environments of the aromatic residues present in the hybrid apolipoprotein (seven Phe, one Tyr, and zero Trp). The presence of such extrema is consistent with a folded tertiary structure. Comparison of the near UV CD spectrum of the hybrid apolipoprotein and WT apoLp-III (eight Phe, one Tyr, and zero Trp) showed similar patterns (27, 28), indicating that the overall environment of the aromatic residues was maintained in the chimera, presumably in a helix bundle conformation. Denaturation studies of the hybrid apolipoprotein yielded a urea transition midpoint of 1.05 M. In comparison with the urea midpoint value of WT apoLp-III (1.01 M; see Ref. 27), the hybrid apolipoprotein and WT apoLp-III display similar overall stability. These data provide support for the concept that helix swapping did not disrupt the global secondary structure stability of the protein. It is known from x-ray crystallography (10, 11) ofapoE3-NT and multidimensional NMR studies of apoLp-III (23, 24) that these proteins adopt unique helix bundle molecular architectures. Spectroscopic and stability studies of the hybrid apolipoprotein indicate it retains a helix bundle conformation, establishing that interapolipoprotein helix swapping, with retention of tertiary structure, is feasible.

As a functional test to assess whether apoE residues 131–151 fold correctly when placed in the context of an unrelated helix bundle protein, we determined the ability of the hybrid apolipoprotein to bind heparin-Sepharose. The finding that both the hybrid apolipoprotein and apoE3-NT bound to heparin, whereas WT apoLp-III did not, provides evidence that the hybrid apolipoprotein possesses functional abilities present in the apoE parent protein and demonstrates that, in this study, helix swapping has resulted in preservation of structure and function.

Unlike WT apoLp-III or apoE3-NT, sedimentation equilibrium experiments with the hybrid apolipoprotein showed evidence of self-association, apparently a result of the helix swapping process. If the substituted helix is unable to align precisely with the structural scaffold provided by helices 1–4 of *M. sexta* apoLp-III, then it is possible that either apoE residues 131–151 or helices in apoLp-III will reorient with respect to their neighbors resulting in some degree of exposure of hydrophobic elements. The helices of apoE3-NT have been assigned as class G$^*$ helices (including the fragment used here) whereas the helices of apoLp-III are class A (49). Thus, differences in the arrangement of charged residues and/or the angle subtended by the hydrophobic face of these helices are likely to be determinants of the relative exposure of hydrophobic elements, as well as other aspects of helix packing and bundle tertiary structure organization. Alternatively, it is conceivable that hybrid apolipoprotein self-association is affected by solution pH. The present studies were conducted at pH 8.0, 1 unit lower than the apparent pI of 9.0. Further studies over a broader pH range...
may reveal differences in solubility and/or self-association behavior of the hybrid apolipoprotein. Nonetheless, self-association is a well known property of lipid-free exchangeable apolipoproteins (e.g. apolipoprotein A-I) and likely provides a mechanism to sequester and protect otherwise exposed hydrophobic lipid binding surfaces on the protein. Support for the concept that the present helix interchange produces an alteration in helix bundle tertiary fold that results in increased exposure of hydrophobic surface can be seen in fluorescent dye binding experiments. ANS is a dye whose intrinsic fluorescence increases upon binding to a hydrophobic surface or cavity (50). Whereas both WT apoLp-III and apoE3-NT present few ANS binding sites, the hybrid apolipoprotein manifests a large increase in ANS fluorescence intensity and a blue shift in ANS wavelength of maximum fluorescence emission, characteristic of its interaction with hydrophobic binding sites on proteins. The increase in ANS binding to the hybrid apolipoprotein, compared with either parent protein, is similar to the effect seen when point mutations are introduced into Locusta migratoria apolipoprotein-III (51) or when the tertiary structure of apoE3-NT is altered by exposure to buffer at pH 3.0 (52).

A known property of both WT apoLp-III and apoE3-NT is their ability to associate reversibly with lipid surfaces by undergoing a significant structural alteration. Several lines of evidence support the view that loop regions that connect helical segments in the bundle conformation can function as “hinges,” about which the bundle opens to expose its hydrophobic interior to potential lipid surface binding sites (4, 53). Whereas this structural adaptation is critical to the lipid interaction properties of both proteins, it is also an essential step for apoE to adopt a receptor competent state. A convenient assay to evaluate the lipid interaction properties of exchangeable apolipoproteins is based on their ability to solubilize bilayer vesicles of DMPC (37), transforming them into discoidal complexes wherein the apolipoprotein circumscribes the periphery of the bilayer disc with the hydrophobic faces of amphipathic α-helices in contact with the phospholipid fatty acyl chains around the perimeter of the disc (13, 54, 55). In DMPC vesicle solubilization kinetic assays the hybrid apolipoprotein was significantly more active than WT apoLp-III or apoE3-NT. From other studies it is known that perturbation of the tertiary structure organization of exchangeable apolipoproteins, either by decreasing the solution pH (52, 56), introducing chaotropic agents (57) or site-directed point mutations (33), increases DMPC vesicle solubilization activity. The present results, wherein helix swapping resulted in a hybrid apolipoprotein with increased exposure of hydrophobic elements that manifest enhanced lipid binding activity, is consistent with the view that the helix bundle tertiary structure of the hybrid apolipoprotein does not achieve optimal sequestration of its hydrophobic content. Some degree of solvent exposure of hydrophobic elements in lipid-free hybrid apolipoprotein is likely responsible for the observed self-association, increased ANS binding, and enhanced DMPC solubilization kinetics. Interestingly, the apparent exposure of hydrophobic elements in lipid-free hybrid apolipoprotein does not affect its ability to resist urea-induced denaturation. This result is consistent with the recent observation that apoE-NT isomeric stability was largely unchanged at pH levels of 7.0 and 3.0, despite a dramatic enhancement in DMPC vesicle solubilization activity at the lower pH (58).

Receptor binding properties of the hybrid apolipoprotein were examined in 125I-LDL competition binding assays. apoE3-NT-DMPC discs are known to compete with 125I-LDL under the conditions of this assay (8, 26). The basis of the assay relies on the ability of a competitor ligand to bind LDL receptors on the surface of human skin fibroblasts in place of 125I-labeled LDL. It is known that apoE must be bound to a lipid surface to effectively compete for LDL receptor binding (8), and consistent with this finding, lipid-free hybrid apolipoprotein was a poor competitor. However, hybrid apolipoprotein-DMPC complexes effectively competed with 125I-labeled LDL for LDL receptor binding. Increased amounts of hybrid apolipoprotein-DMPC discs led to decreased 125I-LDL binding. These results provide support for the concept that apoE residues 131–151 retain a native-like conformation in the context of the hybrid apolipoprotein in the lipid-bound state. When compared at the same concentration (50 μg/ml), the relative ability of hybrid apolipoprotein-DMPC discs and apoE3-NT-DMPC discs to compete with 125I-LDL for receptor binding sites on the surface of cultured fibroblasts was similar. This result was unexpected in view of the report by Morrow et al. (59) that Arg-172 is necessary for optimal apoE receptor interaction. It is conceivable that residues from M. sexta apoLp-III align in a manner on the disc particles wherein they substitute for the function of Arg-172 of apoE. Indeed, residues 1–145 of M. sexta apoLp-III possess 21 lysine + arginine, one or more of which could align on the disc particle in a conformation that permits alignment with the receptor in a context similar to that imparted by Arg-172 in apoE.

In conclusion, hybrid apolipoprotein, an apoLp-III-apoE chimera, is biophysically and functionally similar to WT apoLp-III, yet is capable of binding to heparin and the LDL receptor. Thus, in the context of a distinct helix bundle scaffold, human apoE residues 131–151 are sufficient to retain key functional abilities. The present report, wherein protein engineering has been used to create a helix bundle apolipoprotein chimera using high resolution structure data available for both parent proteins, extends the application of apolipoprotein chimeras as tools to investigate exchangeable apolipoprotein structure and function. This general approach has been used previously to create hybrid apolipoproteins using elements of human apoA-I and apoA-II. For example, replacement of the C-terminal region of human apoA-I (residues 190–243) by residues 12–77 of apoA-II generated an apolipoprotein chimera that displayed defective lipoprotein affinity (60). Using a similar strategy, Dhoest et al. (61) found that an apolipoprotein chimera, wherein residues 125–166 of human apoA-I were replaced by apoA-II residues 12–75, manifests a markedly reduced ability to activate lecithin cholesterol acyltransferase. In a similar manner, transgenic expression of this chimera in apoE knockout mice indicated that the central region of apoA-I is critical for high density lipoprotein-mediated inhibition of macrophage homing and early atherosclerosis (62). Reschly et al. (63) constructed a human/mouse apoA-I chimera in which helix segments in human apoA-I were substituted for their counterparts from mouse apoA-I. These authors obtained evidence that helices 7 and 8 of human apoA-I serve to modulate high density lipoprotein subclass distribution. Taken together, these studies demonstrate the potential use of apolipoprotein chimeras to dissect their functional and biological properties. By utilizing the growing body of detailed structural knowledge for this protein family, it will be possible to construct new “designer” apolipoproteins, providing valuable molecular tools to dissect structure and function properties. Knowledge gained from this approach should yield insight for development of strategies to treat or prevent atherosclerosis and neurodegenerative disease.

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Structure-guided Protein Engineering Modulates Helix Bundle Exchangeable Apolipoprotein Properties

Robert S. Kiss, Paul M. M. Weers, Vasanthy Narayanaswami, Jenny Cohen, Cyril M. Kay and Robert O. Ryan

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