Lysines in apolipoprotein (apo) E are key factors in the binding of apoE to the low density lipoprotein receptor, and high affinity binding requires that apoE be associated with lipid. To gain insight into this effect, we examined the microenvironments of the eight lysines in the 22-kDa fragment of apoE3 (residues 1-191) in the lipid-free and lipid-associated states. As shown by 1H,13C heteronuclear single quantum coherence nuclear magnetic resonance, lysine resonances in the lipid-free fragment were poorly resolved over a wide pH range, whereas in apoE3-dimyristoyl phosphatidylcholine (DMPC) disc, the lysine microenvironments and protein conformation were significantly altered. Sequence-specific assignments of the lysine resonances in the spectrum of the lipidated 22-kDa fragment were made. In the lipid-free protein, six lysines could be resolved, and all had pK_a values above 10. In apoE3-DMPC complexes, however, all eight lysines were resolved, and the pK_a values were 9.2-11.1. Lys-143 and Lys-146, both in the receptor binding region in helix 4, had unusually low pK_a values of 9.5 and 9.2, respectively, likely as a result of local increases in positive electrostatic potential with lipid association. Shift reagent experiments with potassium ferricyanide showed that Lys-143 and Lys-146 were much more accessible to the ferrocyanide anion in the apoE3-DMPC complex than in the lipid-free state. The angle of the nonpolar face of helix 4 is smaller than the angles of helices 1, 2, and 3, suggesting that helix 4 cannot penetrate as deeply into the DMPC acyl chains at the edge of the complex and that its polar face protrudes from the edge of the disc. This increased exposure and the greater positive electrostatic potential created by interaction with DMPC may explain why lipid association is required for high affinity binding of apoE to the low density lipoprotein receptor.

Human apolipoprotein (apo)E serves a critical function in cholesterol and lipoprotein metabolism by modulating the lipolysis and clearance of plasma lipoproteins and the production of very low density lipoprotein triglyceride (5). Humans (6, 7) and mice (8-10) lacking apoE cannot clear remnant lipoproteins from the plasma and are at increased risk for atherosclerosis. ApoE is a high affinity ligand for the low density lipoprotein receptor (LDLR) family and for cell surface heparan sulfate proteoglycans (1, 4). Defective binding of apoE to receptors causes cholesterol-rich lipoprotein particles to accumulate in the plasma and is the mechanism of type III hyperlipoproteinemia, a genetic disorder characterized by elevated plasma cholesterol and triglyceride levels and accelerated coronary artery disease (11).

ApoE is a single polypeptide chain of 299 amino acids (M_r = 34,200) (1) consisting of two independently folded functional domains (2, 3). The C-terminal domain contains the major lipid binding region. The N-terminal domain exists in the lipid-free state as a four-helix bundle of amphipathic a-helices and contains the LDLR binding region (amino acids 138-150 in helix 4), which coincides with a heparin binding site (2).

The binding of apoE to the LDLR is thought to involve ionic interaction between acidic residues in the lipid binding domain of the LDLR and a cluster of basic residues in the receptor binding region of apoE. Replacing these basic residues with neutral residues reduces receptor binding affinity (12). High resolution x-ray crystallography of the 22-kDa N-terminal thrombolytic fragment of apoE in the lipid-free state has provided detailed information about molecular features of the a-helices (see Fig. 1). However, apoE must be lipidated to bind with high affinity to the LDLR (13). When the 22-kDa fragment of apoE is complexed with dimyristoyl phosphatidylcholine (DMPC) as a simple model of lipoprotein, the amphipathic a-helices open, exposing their hydrophobic faces (2, 14). This conformational change is associated with receptor binding activity.

The receptor binding region of lipid-associated apoE has not been examined directly, and the conformational change it undergoes upon binding to lipid is poorly understood. Neither X-ray crystallography nor multidimensional nuclear magnetic resonance (NMR) can be applied readily to plasma lipoprotein particles to solve protein structure. Lipoprotein particles are too big for sufficient resolution by NMR, and lipoprotein crystals of adequate quality have not been obtained. In this study, we used multidimensional NMR to characterize the microenvironments of the eight lysines in the 22-kDa fragment of apoE3 in the lipid-free and lipid-associated states.

**EXPERIMENTAL PROCEDURES**

**Materials**—DMPC was purchased from Avanti Polar Lipids (Pelham, AL), and stock solutions were stored in chloroform/methanol (2/1) under nitrogen at -20 °C. Its purity was assayed by thin layer chromatogra-
phy on Silica Gel G plates (Anatech, Newark, DE) in chloroform/methanol/water (65/25/4, v/v). Lipids were visualized by spraying developed thin layer plates with a 50% sulfuric acid solution and charring at 200 °C for 15 min; 100-μg quantities gave a single spot by charring. D_2O (Cambridge Isotope Laboratories, Andover, MA) was routinely deuteriumated and adjusted to 100% deuterium under nitrogen. As a topic enrichment as a 20% solution in water was also obtained from Cambridge Isotope Laboratories. [14C]Formaldehyde (40–60 Ci/mol) in distilled water was purchased from PerkinElmer Life Sciences. Anionic surfactants and reagents were analytical grade.

Aldrich strain DH5α was transformed. The lipid was incubated with 5 ml of buffer (150 mM NaCl, 1 mM methanol solution under nitrogen to a thin film on the walls of a 15-ml amide gel electrophoresis showed that the apoE was 1% of the total protein. The apoE was isolated by gel filtration chromatography. The complex (10 mg of apoE3 22-kDa in a 5-ml volume) was applied to a calibrated Superdex 200 Pregrade column with a fast protein liquid chromatography system. Fractions (1 ml each) were collected, and the absorbance of each fraction was monitored at 280 nm to locate the protein peak and estimate the particle diameter. Fractions containing the complexes were pooled and dialyzed against saline/EDTA before characterization and reductive methylation.

Reductive Methylation of the 22-kDa Fragment—The [14C] label was introduced into the DMPC-associated 22-kDa fragment by reductive methylation of lysines with [14C]formaldehyde as described elsewhere (20). The reductive methylation was done at 5–10-fold molar excess over the lysines, whereas the molar ratio of formaldehyde to lysine was kept at 10:1. This allowed maximal labeling of the lysines, which was reproducible to ±3%. The level of incorporation of label was monitored by dopping the [14C]formaldehyde with [14C]formaldehyde and determining the [14C] specific activity of the mixture. The degree of labeling estimated from [14C] incorporation (NMR) agreed closely with the value from [14C] incorporation determined by liquid scintillation counting. This labeling procedure did not cause protein degradation, as determined by SDS-polyacrylamide gel electrophoresis. No labeling of lipid occurred because no [14C] radioactivity could be extracted into chloroform/methanol.

NMR Measurements—The 1H,13C heteronuclear single quantum coherence (HSQC) two-dimensional NMR spectra of apoE3 22-kDa DMPC complexes were obtained with a Bruker DMX400 wide bore spectrometer equipped with a SGI 02 computer and a 5-mm inverse broad band probe. The spectra were correlated by using double INEPT transfer (21) and gradient pulses for coherence selection. The temperature for the two-dimensional NMR spectra was set at 310 K. The two-dimensional 1H,13C HSQC spectra were recorded with carbon dioxide in the sample as an internal pH reference. The time proportional phase incrementation method (22) was used to obtain phase-sensitive spectra. Chemical shifts and line widths for lipid-protein complexes were measured as described elsewhere (18, 23–25). The pseudocount shifts observed when K_2Fe(CN)_6 was added to the aqueous phase were used to explore the exposure of (13CH_3)2 lysines to the aqueous medium (18). The chemical shifts of (13CH_3)2 lysine and (13CH_2)2-terminal amino residues of (13CH_2)2-DMPC complex were determined as a function of pH. Reductively methylated apoE3 22-kDa-DMPC complexes (sample volume was typically 2.0 ml, 2.0 mg of apoE3 22-kDa/ml) were introduced into a 5-mm NMR tube. The pH of the solution was adjusted before each experiment by adding micromolar amounts of concentrated NaOH or HCl. Two samples were used, one for titration from pH 10.0 to 12.5 and the other for titration from pH 5.5 to 10.0. At pH 5.0, and before each two-dimensional NMR experiment, a one-dimensional 1H spectrum was obtained with proton composite pulse decoupling. The pK_a values of the 13C-labeled dimethyl lysines were obtained by nonlinear regression fitting of the chemical shifts at different pH values to the Henderson-Hasselbalch equation with the GraphPad Prism computer program (GraphPad Software). The sigmoidal equation is y = (U + W × (X – Xc))/(10(–X – Xc)) + 1, where y is the chemical shift, U is the lowest limit of the shift, W is the upper limit, X is pH, and Xc is pK_a.

Analytic Methods—Protein concentrations were determined by the Lowry procedure (26). Phospholipid content was monitored by phosphorus analysis (27). Negative staining electron microscopy (28) was used to measure the size of the apoE3 22-kDa DMPC discs. [13C] radioactivity was measured by standard liquid scintillation procedures. Polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate (SDS) was used to examine the absence of SDS was performed using a Pharmacia Phast Electrophoresis System to monitor the purity of the apoE3 22-kDa fragment or determine the size of DMPC discs. Circular dichroism spectra were obtained on a Jasco J600 spectropolarimeter equipped with a temperature-controlling device and interfaced with the computer. The α-helical content of each apoE 22-kDa complex was calculated by using a computer program (29)
kDa–DMPC preparation was derived from the molar ellipticity at 222 nm by established procedures (20, 29).

RESULTS

The apoE3 22-kDa fragment (residues 1–191) rather than the full-length apoE3 was employed to evaluate the microenvironments of the lysines because there are only 8 lysines in the fragment compared with 12 in the intact molecule, simplifying the resolution and assignment of the resonances in the NMR spectrum. It is important to note that the apoE3 22-kDa fragment adopts a receptor-active conformation when complexed with DMPC and binds to the LDLR with an affinity similar to that of full-length apoE3 (30). The structural motif adopted by the apoE3 22-kDa fragment in the lipid-free state is the four-helix bundle (31), and it has been demonstrated that the bundle undergoes a conformational change in which it opens, exposing the hydrophobic faces of the helices when binding to DMPC (14). The x-ray structure of the lipid-free apoE3 22-kDa molecule (Fig. 1) shows that the receptor binding region in helix 4 contains a cluster of basic amino acids including Lys-143 and Lys-146 located on the polar face.

To gain insight into the conformational change that gives rise to high affinity binding of apoE to the LDLR, we used multidimensional 1H,13C HSQC NMR to examine individual lysines in the 22-kDa fragment of apoE3 in the lipid-free state and in a complex with DMPC, as applied by Zhang et al. (32) to calmodulin. In our previous 13C NMR studies of apoE (20) and amphipathic α-helical peptides (33), methylation had no effect on lipid binding affinity (20) or on the pK<sub>a</sub> values of the lysines (15). Dimethylation of lysines in apoE does decrease receptor binding activity (34) because some of the lysines are directly involved in the receptor binding domain. However, this decrease does not result from a structural perturbation of apoE induced by the dimethylation or a significant change in the pK<sub>a</sub> values of the lysines. Rather, it probably reflects the ~10% reduction in ion pair energy caused by lysine dimethylation (35), the reduced hydrogen bonding capability of dimethyl lysine, or both.

The spectrum of the 22-kDa fragment of apoE3 in the lipid-free state showed a well resolved N-terminal α-helical group with a 13C chemical shift of 41.0 ppm at pH 10.0 (Fig. 2). The 1<sup>13</sup>CCH<sub>3</sub> resonances from the eight lysines were relatively poorly resolved. Six resonances with 13C chemical shifts of 43.1–43.9 ppm and 1H chemical shifts of 2.1–2.5 ppm were detected. The existence of multiple (1<sup>3</sup>CH<sub>3</sub>)<sub>2</sub> lysine resonances reflects ordered structures because only a single resonance is obtained when apoE is present in a disordered state in 8 M urea solution (20). The spectrum of the apoE3–DMPC complex showed striking differences in the lysine resonances, indicating significant changes in the lysine microenvironments and in protein conformation (Fig. 2). The 13C resonances from the DMPC molecules in the lipid-protein complex have been described before (20). The apoE3 22-kDa–DMPC discs were homogeneous in size, with a hydrodynamic diameter of ~16 nm as judged by their elution from a calibrated Superdex 200 gel filtration column. Volumetric calculations indicated that each disc contained about 750 DMPC molecules and seven apoE3 22-kDa molecules.

Sequence-specific assignments of the eight lysine resonances in the spectrum of the lipidated 22-kDa fragment of apoE3 were made by producing a series of recombinant variants in which each lysine was individually mutated to glutamine. To ensure that these mutations did not affect the properties of the apoE3 22-kDa fragment, we compared the properties of the mutants and the wild-type molecule. As shown by circular dichroism, the α-helical content of each mutant protein in the lipid-free state was ~55 ± 5%, which is similar to that of the wild-type fragment (36, 37). When the mutants were complexed with DMPC, their α-helical content increased by 10–15%, in good agreement with the increase exhibited by the wild-type protein. Furthermore, the mutations did not affect the lipid binding capacity of the protein, and the mutants interacted with DMPC to form complexes identical in size and lipid-to-protein ratio to those formed with the wild-type fragment.

Fig. 3 demonstrates how the resonance from Lys-143 was assigned. Comparison of the two spectra reveals that the resonance at 13Cδ = 43.30 ppm and 1Hδ = 2.26 ppm in the spectrum of the wild-type protein is missing in the spectrum of the Lys-143 → Gln mutant. Some of the other lysine resonances are not at the same chemical shifts in the two spectra in Fig. 3 because of a slight difference in pH. However, when compared at the same pH, the chemical shifts of the remaining seven lysine resonances (obtained from the pH titration curves of the individual lysine resonances) were identical. This finding confirms that mutation of the lysines does not change the global conformation of the 22-kDa fragment of apoE3 and that the lysines do not interact with one another. The other lysine resonances shown in the spectra of Fig. 3 were identified in similar fashion. In each case, the lysine-to-glutamine mutation led to a loss of a single resonance, as expected. Consistent with surface locations of the lysines and high segmental motions, the 13C line widths of all the lysine resonances were similar (20–30 Hz). For comparison, the 13C line width of the −N(CH<sub>3</sub>)<sub>2</sub> resonance from the DMPC polar group is about 10 Hz.

To characterize further the lysine microenvironments in apoE3 22-kDa–DMPC discs, we investigated the ionization behavior of the lysines at different pH values. The pK<sub>a</sub> value for each lysine was obtained by monitoring the chemical shift as a function of pH over a range of pH values (5.5–12.5). The titration curves were fully reversible across the pH range studied. The pH dependence of the 13C chemical shifts for Lys-143, Lys-146, and Lys-157 is shown in Fig. 4. The derived pK<sub>a</sub> values for the eight lysines in apoE3 22-kDa–DMPC discs were 9.2–11.1, with Lys-143 and Lys-146 having unusually low values of 9.5 and 9.2, respectively (Table I). The normal pK<sub>a</sub> value for a fully hydrated, noninteracting lysine is 10.5. Because of the loss of resolution of the lysine resonances in the spectrum of lipid-free apoE3 22-kDa molecule (Fig. 2), pK<sub>a</sub> values for all of the lysines could not be obtained. However, titration curves
showed that all of the lysines had a p\(K_a\) > 10.0. Examination of the intensities of the resonances in the spectra of the Lys-143 \(\rightarrow\) Gln and Lys-146 \(\rightarrow\) Gln variants, in the lipid-free and DMPC-associated states, gave an estimated assignment for these two lysines. The p\(K_a\) values of these resonances were 10.1 and 10.4, respectively, in the lipid-free molecule. Thus, comparison of the receptor binding domain in the 22-kDa fragment of apoE3 in the lipid-free and lipidated states showed that the
The resonances from selected linear regression fitting to the Henderson-Hasselbalch equation, as $5.5-12.5$. The $pK_a$ of the proton chemical shifts led to similar $pK_a$ values for lysines were obtained by nonlinear regression fitting to the Henderson-Hasselbalch equation, as described under "Experimental Procedures.

The $pK_a$ values for lysine residues in an apoE3 22-kDa · DMPC disc were derived from titration curves obtained by running NMR spectra of the type shown in Fig. 3 at $pH$ values of 5.5-12.5. The $pK_a$ values were derived from $^{13}C$ chemical shifts; the $pK_a$ values for selected $^N$-dimethyllysyl residues of apoE3 22-kDa · DMPC discoidal complexes. The chemical shifts were obtained from NMR spectra of the type shown in Figs. 2 and 3. The $pH$ titration curves and $pK_a$ values for lysines were obtained by nonlinear regression fitting to the Henderson-Hasselbalch equation, as described under "Experimental Procedures.

**Table I**

| Lysine Residue | $pK_a$ values of lysine residues in an apoE3 22-kDa · DMPC disc |
|---------------|---------------------------------------------------------------|
| Lys-1         | 10.5                                                          |
| Lys-69        | 10.4                                                          |
| Lys-72        | 10.0                                                          |
| Lys-75        | 10.1                                                          |
| Lys-95        | 10.1                                                          |
| Lys-143       | 9.5                                                           |
| Lys-146       | 9.2                                                           |
| Lys-157       | 11.1                                                          |

*The $pK_a$ values were derived from titration curves obtained by running NMR spectra of the type shown in Fig. 3 at $pH$ values of 5.5-12.5. The $pK_a$ values were derived from $^{13}C$ chemical shifts; the $pK_a$ values for selected $^N$-dimethyllysyl residues of apoE3 22-kDa · DMPC discoidal complexes. The chemical shifts were obtained from NMR spectra of the type shown in Figs. 2 and 3. The $pH$ titration curves and $pK_a$ values for lysines were obtained by nonlinear regression fitting to the Henderson-Hasselbalch equation, as described under "Experimental Procedures.

The current NMR data provide insights into how such a conformational change affects the microenvironments of the lysines in the 22-kDa fragment of apoE3. The local environments of the lysines in helices 2 and 3 (Fig. 1) were not greatly altered by the interaction with DMPC. The formation of apoE3 22-kDa · DMPC discoidal complexes involves opening of the four-helix bundle to allow the nonpolar amino acid side chains to interact with the acyl chains of the DMPC molecules (36, 37). The opening is probably initiated at the flexible end, where there is a loop region near residue 80 (40). Helices 1 and 2 and helices 3 and 4 preferentially remain paired upon exposure of their hydrophobic faces (14, 39), at least in the initial stages of DMPC disc formation.

In contrast to the relatively small DMPC-induced changes in the microenvironment of the lysines in the 22-kDa fragment of apoE3, the local environments of the lysines in the LDLR binding domain (Fig. 1) were solvent-exposed and not involved in salt bridges (2), but the lipid-free molecule does not bind to the receptor with high affinity. The changes in the microenvironments of the LDLR binding domain revealed in this study strongly suggest that the intramolecular reorganization of the four-helix bundle structure induced by lipid is critical for functionality.

**Table II**

| $^{13}C$ resonance | $^{13}C$ NMR chemical shift$^a$ | Change in chemical shift$^a$ |
|-------------------|-------------------------------|----------------------------|
| Without $K_3Fe(CN)_6$ | With $K_3Fe(CN)_6$ | $ppm$ |
| DMPC-(CH$_2$)$_3$ | 54.00 | 54.20 | 0.20 |
| $\alpha$-N-terminal | 41.14 | 41.17 | 0.03 |
| Lys-1 | 43.10 | 43.22 | 0.12 |
| Lys-69 | 44.50 | 44.53 | 0.03 |
| Lys-72 | 43.91 | 43.93 | 0.11 |
| Lys-75 | 44.49 | 44.52 | 0.03 |
| Lys-95 | 43.30 | 43.42 | 0.12 |
| Lys-143 | 43.23 | 43.42 | 0.19 |
| Lys-146 | 43.69 | 43.94 | 0.25 |
| Lys-157 | 42.70 | 42.81 | 0.11 |

$^a$ The $pK_a$ values were derived from titration curves obtained by running NMR spectra of the type shown in Fig. 3 at $pH$ values of 5.5-12.5. The $pK_a$ values were derived from $^{13}C$ chemical shifts; the $pK_a$ values for selected $^N$-dimethyllysyl residues of apoE3 22-kDa · DMPC discoidal complexes. The chemical shifts were obtained from NMR spectra of the type shown in Figs. 2 and 3. The $pH$ titration curves and $pK_a$ values for lysines were obtained by nonlinear regression fitting to the Henderson-Hasselbalch equation, as described under "Experimental Procedures.

The current NMR data provide insights into how such a conformational change affects the microenvironments of the lysines in the 22-kDa fragment of apoE3. The local environments of the lysines in helices 2 and 3 (Fig. 1) were not greatly altered by the interaction with DMPC. The formation of apoE3 22-kDa · DMPC discoidal complexes involves opening of the four-helix bundle to allow the nonpolar amino acid side chains to interact with the acyl chains of the DMPC molecules (36, 37). The opening is probably initiated at the flexible end, where there is a loop region near residue 80 (40). Helices 1 and 2 and helices 3 and 4 preferentially remain paired upon exposure of their hydrophobic faces (14, 39), at least in the initial stages of DMPC disc formation.

In contrast to the relatively small DMPC-induced changes in the microenvironments of the lysines in helices 2 and 3, the lysines in helix 4 were significantly affected by the reorganization of the four-helix bundle. The $pK_a$ values of Lys-143 and Lys-146 decreased by 0.6 and 1.2 $pH$ units, respectively. Shift reagent experiments indicated that these lysines, which are three amino acids apart on the polar face of the helix, become much more acces-
sible to the ferricyanide anion after binding of the 22-kDa fragment to DMPC. Lys-157, which is three turns along the helix from Lys-146, is not as accessible to the ferricyanide anion (Table II). Lys-157 had an unusually high $pK_a$ value of 11.1 (Table I), perhaps because the intrahelical salt bridges with Asp-153 and Asp-154 (2) are maintained after the four-helix bundle reorganizes upon interaction with DMPC.

What is the basis for the striking DMPC-induced changes in the microenvironments of Lys-143 and Lys-146 (and, by inference, the neighboring arginines)? The $pK_a$ of a lysine decreases if the local environment becomes more hydrophilic. However, this seems an unlikely explanation for the decreases in the $pK_a$ values of Lys-143 and Lys-146 because they became more accessible to the ferricyanide anion upon binding to DMPC. Most likely these decreases resulted from increases in the local positive electrostatic potential, which favors deprotonation of the lysine $\varepsilon$-amino groups. One contribution to such an effect could involve the interactions of Arg-147. In the lipid-free, four-helix bundle this residue is involved in interhelical salt bridges with Asp-107 and Asp-110 in helix 3 (2). If these and similar interactions are disrupted by the interaction with DMPC, then the net positive charge potential on the polar face of helix 4 could increase, thereby decreasing the $pK_a$ values of Lys-143 and Lys-146. This effect would be expected to be greater for Lys-143, as observed experimentally.

The amphiphilic $\alpha$-helices in the four-helix bundle (Fig. 1) are all of the G* type (41). In these helices, the acidic and basic residues are distributed across the polar face, whereas the nonpolar face does not contain any charged residues. One difference in helix 4 is that the angle subtended by the nonpolar face is 100°, which is less than the angles in helices 1, 2, and 3, which are in the range of 120–180°. Consequently, the nonpolar face of helix 4 cannot penetrate as deeply among the DMPC acyl chains at the edge of the discoidal complex. For this reason, the polar face of helix 4 is expected to protrude at the edge of the DMPC disc. This domain may be relatively sequestered in the lipid-free state because of the close helix juxtaposition in the bundle structure (Fig. 1).

The enhanced exposure of the polar face of helix 4 and the greater positive electrostatic potential created by interaction with DMPC probably explain why the apoE3 22-kDa-DMPC binds with high affinity to the LDLR, whereas the lipid-free molecule does not. The fact that binding is improved by greater exposure of the basic residues in helix 4 suggests that this helix has to fit into a complementary structure in the LDLR. The LDLR binds apoE via tandemly repeated, structurally independent modules that are cysteine-rich and contain an acidic region that binds calcium (42). The modules have little recognizable secondary structure, and the details of the ligand binding site are not known although the presence of a patch of negative surface electrostatic potential is consistent with ligand binding being mediated by electrostatic complementarity of the LDLR and apoE (42). The electrostatic interaction with acidic residues in the LDLR would be improved by the DMPC-induced increase in positive electrostatic potential around the domain containing Lys-143 and Lys-146. Hydrogen bonding is an important component of the salt bridges formed between the basic residues on apoE and the acidic residues on the LDLR (12). The ability of Lys-143 and Lys-146 to form ion pairs with acidic residues would be decreased because, as reflected in their lower $pK_a$ values, a greater fraction of their $\varepsilon$-amino groups are deprotonated at neutral pH. Nonetheless, the lysine $\varepsilon$-amino groups contribute significantly to the free energy of binding of apoE to the LDLR because replacement of these residues with neutral amino acids decreases the binding significantly (12).