Effects of Polymorphism on the Lipid Interaction of Human Apolipoprotein E*

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Apoe exists as three common isoforms, apoE2, apoE3, and apoE4; apoE2 and apoE3 preferentially bind to high density lipoproteins, whereas apoE4 prefers very low density lipoproteins (VLDL). To understand the molecular basis for the different lipoprotein distributions of these isoforms in human plasma, we examined the lipid-binding properties of the apoE isoforms and some mutants using lipid emulsions. With both large (120 nm) and small (35 nm) emulsion particles, the binding affinity of apoE4 was much higher than that of apoE2 and apoE3, whereas the maximal binding capacities were similar among the three isoforms. The 22-kDa N-terminal fragment of apoE4 displayed a much higher binding capacity than did apoE2 and apoE3. The apoE4(E255A) mutant, which has no electrostatic interaction between Arg61 and Glu255, showed binding behavior similar to that of apoE3, indicating that N- and C-terminal domain interaction in apoE4 is responsible for its high affinity for lipid. In addition, the apoE3(P267A) mutant, which is postulated to contain a long α-helix in the C-terminal domain, had significantly decreased binding capacities for both sizes of emulsion particle, suggesting that the apoE4 preference for VLDL is not due to a stabilized long α-helical structure. Isothermal titration calorimetry measurements showed that there is no significant difference in thermodynamic parameters for emulsion binding among the apoE isoforms. However, fluorescence measurements of 8-anilino-1-naphthalenesulfonic acid binding to apoE indicated that apoE4 has more exposed hydrophobic surface compared with apoE3 mainly due to the different tertiary organization of the C-terminal domain. The less organized structure in the C-terminal domain of apoE4 leads to the higher affinity for lipid, contributing to its preferential association with VLDL. In fact, we found that apoE4 binds to VLDL with higher affinity compared with apoE3.

Human apoE, a 34-kDa protein composed of 299 amino acids, plays an important role in lipoprotein metabolism and neurobiology through its interaction with the low density lipoprotein (LDL) receptor family and cell-surface heparan sulfate proteoglycans (1–4). ApoE exists in three major isoforms, apoE2, apoE3, and apoE4, each differing by cysteine and arginine at positions 112 and 158. ApoE3, the most common form, contains cysteine and arginine at these positions, respectively, whereas apoE2 contains cysteine and apoE4 contains arginine at both sites (5). These differences have profound effects on the biological functions of apoE. Both apoE3 and apoE4 bind to the LDL receptor with high affinity, whereas apoE2 exhibits defective binding to the LDL receptor and is associated with type III hyperlipoproteinemia (6). ApoE4 is associated with high plasma cholesterol level and an increased risk for both coronary heart disease and Alzheimer’s disease (7–9). The apoE isoforms are further distinguished by their preferential distribution among lipoprotein classes: apoE4 prefers very low density lipoproteins (VLDL), whereas apoE2 and apoE3 prefer high density lipoproteins (HDL) (10).

ApoE contains two independently folded functional domains: a 22-kDa N-terminal domain (residues 1–191) and a 10-kDa C-terminal domain (residues 222–299) (11, 12). The N-terminal domain exists in the lipid-free state as a four-helix bundle of amphipathic α-helices and contains the LDL receptor-binding region (residues 136–150 in helix 4) (13). The C-terminal domain has a high affinity for lipid and is responsible for lipoprotein binding (2, 14). In apoE4, these two domains interact in a unique manner unlike in the other isoforms: Arg112 causes a rearrangement of the Arg61 side chain in the N-terminal domain of apoE4, allowing it to interact with Glu255 in the C-terminal domain (15, 16). This domain interaction in human apoE4 is responsible for the preferential association with VLDL and has been suggested to contribute to the accelerated catabolism of this isoform and, consequently, the increased cholesterol and LDL levels in plasma (9, 16).

Association of apoE with lipid is required for its high affinity binding to the LDL receptor (17). A number of recent studies carried out to understand the molecular basis for this phenomenon indicated that the four-helix bundle in the N-terminal domain undergoes a conformational opening upon lipid binding, leading to the receptor-active conformation of apoE (18–21). In this conformation, the positive electrostatic potential in the receptor-binding region of apoE is enhanced, probably allowing its high affinity binding to the LDL receptor (22, 23). In addition, we have recently shown that the two domains in

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* The abbreviations used are: LDL, low density lipoprotein; VLDL, very low density lipoprotein(s); HDL, high density lipoproteins; PC, phosphatidylcholine; ANS, 8-anilino-1-naphthalenesulfonic acid; ITC, isothermal titration calorimetry.
apoE4 lead to two different lipid-bound conformations (open or closed four-helix bundle) on emulsion particles (24), providing a structural rationale for the variable receptor-binding activity displayed by lipoprotein-associated apoE (25).

Because the three isoforms of apoE exhibit different thermal and chemical stabilities (apoE4 < apoE3 < apoE2) (12, 26), the lipid-binding activity of these isoforms is expected to be different. Indeed, the reactivity to dimyristoylphosphatidylcholine that thiodicholoroliposomes of the 22-kDa N-terminal fragments of the three isoforms tends to vary inversely with the stabilities of these fragments (27). In this study, we examined further the lipid interaction of the three isoforms of apoE using lipoprotein-like emulsion particles to understand the molecular basis for the different lipoprotein distribution of apoE isoforms. In addition, to test the hypothesis that the domain interaction in apoE4 stabilizes an extended helical structure in the C terminus that targets a less curved VLDL surface (16, 28), the lipid-binding properties of two apoE mutants, apoE3(P267A) and apoE4(E255A), were determined.

EXPERIMENTAL PROCEDURES

Materials—Egg yolk phosphatidylcholine (PC) and trisolein were purchased from Sigma, and stock solutions were stored in chloroform/methanol (2:1) under nitrogen at −20 °C. [14C]Formaldehyde (40–60 Ci/mol) in distilled water was purchased from PerkinElmer Life Sciences. NaCNBH3 (Aldrich) was recrystallized from methylene chloride before use. 8-Aminolo-1-naphthalenesulfonic acid (ANS) was purchased from Molecular Probes, Inc. (Eugene, OR). Ultrapure guanidine hydrochloride was from ICN Pharmaceuticals (Costa Mesa, CA). Bacteriological media were obtained from Fisher. The prokaryotic expression vector pET32a was from Novagen (Madison, WI), and the competent Escherichia coli strains BL21(DE3) and DH5α were obtained from Invitrogen. PCR supplies and DNA purification kits were from Qiagen Inc. (Chatsworth, CA). Restriction enzymes were purchased from Promega (Madison, WI). Isopropl-β-D-thiogalactopyranoside, β-mercaptoethanol, aprotinin, and ampicillin were from Sigma. Oligonucleotides were from IDT (Coralsville, IA). All other salts and reagents were analytical grade.

Lipoprotein and Apolipoproteins—VLDL was isolated from fasting normolipidemic human plasma by ultracentrifugation at a density cut of 1.006 g/ml. Examination by agarose gel electrophoresis showed that the VLDL had pre-mobility and that it was not contaminated with either chyomicrons or LDL. SDS-PAGE showed the expected presence of full-length human apoE2, apoE3, and apoE4 and their 22- and 10-kDa fragments described (24, 27).

Isothermal Titration Calorimetry (ITC) Measurements—Heats of dilution were determined in the corresponding apoE/emulsion binding experiments. The decay rate constants for the heats of binding were determined in control experiments by injecting either apoE solution or emulsions into buffer, and these heats were subtracted from the heats determined in the corresponding apoE/emulsion binding experiments. The decay rate constants for the heats of binding were obtained by fitting the titration curves to a one- or two-phase exponential decay model.

RESULTS

Binding of ApoE Isoforms to Emulsion Particles—Previously, we determined the parameters for binding of apoE4 and its 22- and 10-kDa fragments to emulsion particles using an ultracentrifugal separation (24). In this study, we applied this method to compare the lipid-binding properties of the three isoforms. As shown in Fig. 1, both full-length apoE2 and apoE3 displayed saturable binding to large emulsions, similar to the behavior of full-length apoE4, whereas the 22-kDa fragments of apoE2 and apoE3 hardly bound to the emulsion surface. The dissociation
constant \( (K_d) \) and the maximal binding capacity \( (B_{max}) \) for small and large emulsion particles are listed in Table I. In the case of the full-length proteins, apoE2 and apoE3 displayed much lower binding affinity for both emulsions compared with apoE4, whereas the binding capacities of the three isoforms were similar for both emulsion particle sizes. The binding parameters for full-length apoE3 were comparable to the previously reported data for human apoE3 (34, 35), and the higher affinity of apoE4 compared with apoE3 was also observed for VLDL-size emulsion particles (16, 36). In contrast to the 22-kDa fragment of apoE4, the 22-kDa fragments of apoE2 and apoE3 displayed negligible binding capacities for both emulsions.

To account for the higher affinity of apoE4 for VLDL compared with apoE3, it has been proposed that a N- and C-terminal domain interaction in apoE4 stabilizes an extended helical structure in the C terminus, thereby promoting its binding to VLDL (16). To explore whether this hypothesis can be applied to the different lipid-binding behaviors of apoE3 and apoE4, we examined the lipid-binding properties of two apoE mutants, apoE3(P267A) and apoE4(E255A). ApoE3(P267A) is postulated to have a long \( \alpha \)-helix in its C terminus because the mutation P267A is likely to remove the interruption or kink between predicted helices 225–266 and 268–289 (28). The mutation E255A in apoE4 is known to alter the apoE4 preference from VLDL to HDL by disrupting the domain interaction (16). Fig. 2 shows the binding isotherms of these mutants for small emulsions in comparison with the isotherms of the respective wild-type proteins; the binding parameters for both sizes of emulsion particles are summarized in Fig. 3. The mutation E255A in apoE4 reduced the lipid affinity without changing the binding capacity for both emulsions; and, as a result, the apoE4(E255A) mutant bound in a similar manner compared with wild-type apoE3 rather than apoE4, indicating that the domain interaction in apoE4 is responsible for its high affinity for lipid. In contrast, the apoE3(P267A) mutant displayed a much lower binding capacity compared with wild-type apoE3 regardless of the emulsion particle size, suggesting that helical length in the C terminus is not responsible for the different lipid-binding behavior of apoE3 and apoE4.

**ITC Measurements**—To obtain thermodynamic information about the lipid interaction of apoE isoforms, we performed ITC measurements of apoE binding to emulsions (24). Fig. 4 shows the injections of full-length proteins and the 22-kDa fragments of apoE isoforms into large emulsions. Using the binding constants given in Table I and Fig. 3, the thermodynamic parameters for binding of apoE isoforms and mutants to small and large emulsions were obtained (Table II). As previously reported for apoE4 (24), binding of apoE isoforms and mutants to large particles was an exothermic process, but binding to small particles was much less exothermic or rather endothermic. As a result, the binding to large particles is enthalpically driven, whereas that to small particles is entropically driven. There was no significant difference in the thermodynamic binding parameters among the full-length apoE isoforms. In contrast, the enthalpies of binding of the 22-kDa fragments of apoE2 and

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**Table I**

| Protein          | 35-nm emulsion | 120-nm emulsion |
|------------------|----------------|-----------------|
|                  | \( K_d \) (\( \mu \text{g/ml} \)) | \( B_{max} \) (amino acids/mol PC) | \( K_d \) (\( \mu \text{g/ml} \)) | \( B_{max} \) (amino acids/mol PC) |
| ApoE2            | 21.8 ± 4.5     | 0.84 ± 0.07     | 18.8 ± 2.9     | 0.74 ± 0.05     |
| ApoE3            | 17.3 ± 2.5     | 0.88 ± 0.05     | 19.3 ± 2.3     | 0.79 ± 0.04     |
| ApoE4            | 4.2 ± 0.6      | 0.82 ± 0.03     | 8.1 ± 1.3      | 0.77 ± 0.03     |
| ApoE2 22-kDa fragment | 26.2 ± 16.9   | 0.05 ± 0.02     | 21.4 ± 22.6    | 0.03 ± 0.01     |
| ApoE3 22-kDa fragment | 27.7 ± 38.5   | 0.02 ± 0.01     | 38.6 ± 22.1    | 0.06 ± 0.03     |
| ApoE4 22-kDa fragment | 39.2 ± 12.9   | 0.32 ± 0.05     | 28.2 ± 7.1     | 0.27 ± 0.03     |

* Data are from Saito et al. (24).
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Fig. 4. Isothermal titration calorimetry of full-length apoE2, apoE3, and apoE4 (A) and their corresponding 22-kDa fragments (B) injected into large emulsions. Each peak corresponds to the injection of 10 μl aliquots of a 0.8 mg/ml solution of protein. The PC concentration in the emulsions was 8.0 mM.

ApoE is a polymorphic protein, and its three major isoforms (apoE2, apoE3, and apoE4) differ only by a single amino acid substitution; yet these changes have profound functional consequences on lipoprotein metabolism and neurobiology (2, 4, 9). Variations in interactions between the N- and C-terminal domains appear to be a major contributing factor to the isoform-specific effects such as lipoprotein-binding preference; apoE2 and apoE3 bind preferentially to HDL, whereas apoE4 prefers VLDL (15, 16). This suggests that these isoforms may interact with lipid differently; and, in fact, we found recently that apoE4 forms dimyristoylphosphatidylcholine discs more rapidly compared with the other isoforms (27). However, the molecular basis for the different lipid interactions of apoE isoforms has not been elucidated.

In this study, we employed an equilibrium binding assay using lipoprotein-like lipid particles of different sizes (24), allowing us to compare the lipid-binding properties of apoE isoforms quantitatively. We found that apoE4 has much a higher lipid affinity compared with the other isoforms regardless of particle size (Table I), consistent with previous reports that apoE4 displays preferential binding to VLDL-like emulsion particles compared with apoE3 (16, 36). As shown in Figs. 2 and 3, this higher affinity of apoE4 for lipid was diminished by the E255A mutation, indicating that the N- and C-terminal domain interaction involving a salt bridge between Arg61 and Glu255 (16) modulates the apoE4 preference for lipid. In addi-
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Table II

| ApoE Isoform                  | 35-nm Emulsion | 120-nm Emulsion |
|-------------------------------|----------------|-----------------|
|                               | ΔH/ΔG/K         | ΔH/ΔG/K         | ΔH/ΔG/K         | ΔH/ΔG/K |
| ApoE2                         | -5.2 ± 1.9      | -10.8 ± 0.2     | 54 ± 7          | -64.3 ± 5.5 |
| ApoE3                         | -0.9 ± 0.5      | -11.0 ± 0.1     | 34 ± 2          | -66.6 ± 7.1 |
| ApoE4                         | -1.7 ± 1.2      | -11.8 ± 0.1     | 34 ± 4          | -68.7 ± 3.0 |
| ApoE2 22-kDa Fragment         | 0.4 ± 0.8       | -10.5 ± 0.3     | 36 ± 4          | -13.9 ± 2.5 |
| ApoE3 22-kDa Fragment         | 3.1 ± 0.5       | -10.4 ± 0.4     | 45 ± 3          | -3.7 ± 1.2  |
| ApoE4 22-kDa Fragmentα        | -2.4 ± 0.7      | -10.2 ± 0.2     | 26 ± 3          | -43.0 ± 4.6 |
| ApoE3(P267A)                  | -2.4 ± 1.9      | -10.9 ± 0.2     | 29 ± 7          | -7.6 ± 3.4  |
| ApoE4(E255A)                  | -4.6 ± 2.1      | -11.1 ± 0.1     | 22 ± 7          | -34.0 ± 3.8 |

* Free energy was calculated according to ΔG = -RT ln 55.5(S/Kc) using the binding constants given in Table I and Fig. 3.
* The entropy of binding was calculated from ΔG = ΔH - TΔS.
* Data are from Saito et al. (24).

![Fig. 5. Binding enthalpies of full-length apoE2, apoE3, and apoE4 for large emulsions obtained under two limiting conditions.](image)

![Fig. 6. ANS fluorescence spectra in the presence of apoE4 (trace a), apoE4(E255A) (trace b), apoE3 (trace c), the apoE4 22-kDa fragment (trace d), the apoE3 22-kDa fragment (trace e), and free ANS in buffer (trace f). The inset shows ANS fluorescence spectra in the presence of apoE3(P267A) (trace g), the apoE 10-kDa fragment (trace h), apoE3 (trace i), and free ANS (trace j). a.u., arbitrary units.](image)

![Fig. 7. Binding isotherms of full-length apoE3 (●) and apoE4 (○) to human VLDL. a.a., amino acids.](image)

The large decrease in the binding capacity of the apoE3(P267A) mutant compared with wild-type apoE3 for both sizes of emulsion particles demonstrates that an extended helical structure in the C-terminal domain is not responsible for the lipid preference of apoE. Rather, the likely alteration in helix organization due to removal of the proline residue interrupts lipid interaction even on the large emulsion particles, which have a less curved surface. The ITC result showing that the P267A mutation largely eliminated the favorable enthalpy of binding of apoE3 to emulsions (Table II) further confirms the unfavorable consequences of the P267A mutation on lipid binding. Presumably, the conformational restriction in the C-terminal domain caused by the putative long α-helix spanning residues 225−289 (~10 nm) in this mutant hinders the stable interaction of the apolipoprotein molecule with the lipid surface (27).

It has been proposed recently that apoE has two distinct lipid-bound states on spherical particles in which the four-helix bundle adopts either an open or closed conformation (24, 25), and this model has been extended to explain the apoE isoform-specific lipid release from astrocytes (41) and binding activity for size-fractionated lipid particles (42). However, our ITC results showing that the differences in binding enthalpies under the two limiting conditions of high and low surface concentrations are similar among apoE isoforms (Fig. 5), together with the fact that there is no difference in binding capacities of three isoforms (Table I), indicate that all apoE isoforms appear to have two lipid-bound conformations. Therefore, the isoform-specific lipid preference found in this study is unlikely due to the different lipid-bound conformations of apoE isoforms.

The difference in the tertiary structure (13, 15, 43) and unfolding stability (12, 26, 44) of the N-terminal fragments of apoE isoforms has been studied extensively. In contrast, the structural organization of the C-terminal domain is poorly defined. The C terminus of apoE is predicted to have three α-hel-
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A number of recent studies on the N-terminal fragments of apoE (40, 44) and apolipoprotein III (47, 48) have demonstrated that a less organized, molten globule-like tertiary structure of apolipoproteins is correlated with increased lipid-binding ability of proteins. The finding that the 10-kDa fragment contributes to most of the increased ANS fluorescence in full-length apoE (Fig. 6, inset) suggests that the organization of the C-terminal domain is critical for the lipid-binding behavior of apoE, consistent with the previous observations of the dominant role of this domain in the lipid binding of apoE (24, 27, 49).

In this regard, the higher lipid-binding ability of apoE4 compared with apoE3 appears to come from a more solvent-exposed, flexible or- ganized structure of the C-terminal domain in apoE4 compared with apoE3, indicating the key role of the conformational flexibility of the C-terminal domain in lipid binding.

The reason why the C-terminal domain is less organized in apoE4 than in apoE3 is not clear at this time. A reduction of the enhanced ANS fluorescence in the apoE4(E255A) mutant (Fig. 6) suggests that the domain interaction in apoE4 alters the tertiary organization of apoE, especially in the C-terminal domain. A recent fluorescence resonance energy transfer study of lipid-free apoE3 indicates that the N- and C-terminal domains are in a spatially proximate orientation with respect to each other, probably through weak hydrophobic interaction (46). Therefore, it is conceivable that the domain interaction in apoE4 causes reorientation of the tertiary disposition of the N- and C-terminal domains, facilitating lipid interaction of the C-terminal domain with the more solvent-exposed, flexible organization. Such a difference in the tertiary interaction between the two domains of apoE isoforms appears to affect the thermal stability (26) and lipid interaction (27) of the isoforms.

It has been hypothesized that the domain interaction in apoE4 stabilizes an extended helical structure in the C terminus and thereby promotes binding to VLDL (16). However, the results with the apoE3(P267A) mutant suggest that helical length in the C-terminal domain is not responsible for the lipoprotein preference of apoE isoforms. Instead, the results of the present study imply that the more flexible organization of the C-terminal domain in apoE4 compared with apoE3 is more favorable for lipid interaction and facilitates its VLDL binding in some way, as shown in Fig. 7. A comparative study of the surface properties of lipoproteins using fluorescent probes showed that the acyl chain region of VLDL is more fluid than that of HDL, whereas the interfacial region of HDL seems to be less hydrophobic and more heterogeneous than that of VLDL (50). The apoE isoforms may recognize such differences in the surface properties of lipoproteins and thereby display the isoform-specific binding preference for lipoproteins. Interestingly, recent fluorescence (51) and NMR (52) studies of lipoprotein model particles indicate that the structure of the interfacial region plays a determinant role in apolipoprotein binding to the lipid surface.

In summary, we have demonstrated that the domain interaction in apoE4 leads to the less organized structure in the C-terminal domain, contributing to its preferential binding to lipid. The C-terminal domain of apoE has been implicated to be responsible for many isoform-specific behaviors of apoE, including not only the lipoprotein preference, but also the propensity for self-association (53), interaction with β-amyloid (54), and induction of neurofibrillary tangle-like inclusions in neurons (55). Thus, our findings provide new insight into how apoE exerts its biological effects in an isoform-specific manner.

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