Self-association of Human Apolipoprotein E3 and E4 in the Presence and Absence of Phospholipid*

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Human apolipoprotein E (apoE) exists as three main isoforms, differing by single amino acid substitutions, with the apoE4 isoform strongly linked to the incidence of late onset Alzheimer’s disease. We have expressed and purified apoE3 and apoE4 from Escherichia coli and compared their hydrodynamic properties by gel permeation liquid chromatography, capillary electrophoresis, circular dichroism, and sedimentation methods. Sedimentation velocity experiments, employing a new method for determining the size distribution of polydisperse macromolecules in solution (Schuck, P. (2000) Biophys. J. 78, 1606–1619), provide direct evidence for the heterogeneous solution structures of apoE3 and apoE4. In a lipid-free environment, apoE3 and apoE4 exist as a slow equilibrium mixture of monomer, tetramer, octamer, and a small proportion of higher oligomers. Both sedimentation velocity and equilibrium velocity experiments indicate that apoE4 has a greater propensity to self-associate. We also demonstrate that apoE3 and apoE4 oligomers dissociate significantly in the presence of dihexanoylphosphatidylcholine micelles (20 mN) and to a lesser extent at submicellar concentrations (4 mN). The α-helical content for both isoforms was almost identical (50%) in the presence and absence of dihexanoylphosphatidylcholine. These results reveal that apoE oligomers undergo phospholipid-induced dissociation to folded monomers, suggesting the monomeric form prevails on the lipoprotein surface in vivo.

Apolipoprotein E (apoE)† is a 299-residue glycoprotein found associated with most lipoprotein classes, including chylomicrons, very low density lipoprotein, intermediate density lipoprotein, and subclasses of high density lipoprotein (1–4). It functions primarily as a ligand for cell surface receptors, namely the apoB/E (low density lipoprotein) receptor and the low density lipoprotein receptor-like protein (4). Three main isoforms of apoE exist, apoE2, apoE3, and apoE4, differing by single amino acid substitutions involving cysteine-arginine replacements at positions 112 and 158 (5–7). ApoE2 (Cys112, Arg158) displays defective receptor binding and is associated with type III hyperlipoproteinemia (4, 8). ApoE3 (Cys112, Arg158) is the most common isoform, whereas apoE4 (Arg112, Arg158) has been strongly linked with the incidence of late onset Alzheimer’s disease (9).

ApoE comprises two domains, referred to as the amino-terminal (residues 20–165) and carboxyl-terminal domain (residues 225–299) (10). The NH2-terminal domain mediates cell surface receptor recognition and forms an elongated four-helix bundle (11, 12). In comparison to apoE3, the apoE4 NH2-terminal structure has an additional salt bridge between arginine 112 and glutamate 109, causing a displacement of the arginine 61 side chain (13). Unlike the NH2-terminal domain, the structure of the COOH-terminal domain of apoE is unknown, although a proteolytic fragment comprising residues 223–272 has recently been crystallized (14). Studies employing a thrombolytic fragment of apoE3 (residues 216–299) or COOH-terminal truncated forms have shown that the COOH-terminal region of apoE mediates lipoprotein attachment and self-association (15, 16). Further work has demonstrated that an amphipathic peptide spanning residues 263–286 of apoE binds dimyristoylphosphatidylcholine vesicles with similar affinity to full-length apoE (17) and forms a helix-turn-helix structure in the presence of SDS micelles (18). This peptide also forms a tetramer in the presence of trifluoroethanol (19).

Analytical ultracentrifugation, cross-linking, and gel permeation liquid chromatography studies demonstrate that human apoE of unspecified phenotype self-associates primarily to a tetramer in the absence of lipid (20). Tetramer formation was also observed in another study employing apoE purified from the plasma of a homozygous E3/3 human (15). However, a significant difference in this latter study was the observation that apoE3, in a time-dependent manner, self-associated to a mixture of tetramer (Stokes radius = 6.6 nm) and a species with a Stokes radius of 8.5 nm or larger (15). This larger species is suggested to be an octamer on the basis of sedimentation equilibrium analysis (15). More recently, fluorescence anisotropy measurements have revealed that the kinetics of apoE denaturation are heterogeneous, consistent with a monomer-tetramer-higher oligomer equilibria (21). The higher oligomer was noted to be present primarily in aged preparations.

Most other exchangeable apolipoproteins also self-associate in the absence of lipid (22). However, little is known about the state of association of lipid-bound apolipoproteins. This is primarily because of experimental difficulties in studying the solution properties of apolipoproteins bound to large native lipoproteins, synthetic lipid bilayers, or lipid emulsions. A possible way of overcoming this experimentally is to use a short chain phospholipid, such as dihexanoylphosphatidylcholine (DHPC), which forms micelles rather than bilayers in solution. DHPC has the chemical structure of a phospholipid and exists...
as monomers in equilibrium with micelles comprising approximately 40 molecules (CMC, 12.7–14.3 m\text{m}). (23, 24).

In the present study, we have firstly compared the self-association of lipid-free apoE3 and apoE4 by native gel permeation chromatography, capillary electrophoresis and sedimentation studies. We have utilized sedimentation velocity experiments to resolve the heterogeneous subunit structures of apoE3 and apoE4, employing a new method for determining the continuous size distribution of polydispersity macromolecules in solution. We also report the effects of the short chain phospholipid, DHPC, on the state of association of apoE3 and apoE4 oligomers.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Apolipoprotein E3 and E4—**pGEX-3X plasmids containing human cDNA for full-length apoE3 and apoE4 were kindly provided by Dr. Karl Weisgraber (Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, CA). The apoE-containing vectors were transformed into the UT6600 strain of E. coli Deaveragen, Madison, or Luria broth (100 µg/ml) containing 50 µg/ml ampicillin (Anresco) was inoculated with a single colony and grown overnight at 37 °C. This culture was then used to inoculate 3 × 2 liters of the same media and grown to late-log phase (\(A_{570} = 1.0\)), before induction with 1.0 mM isopropyl-\(\beta\)-D-thiogalactopyranoside (Astral, Sydney, Australia) for 30 min at 37 °C. Maximal induction of protein expression occurred at 30 min, as determined by GST enzymatic activity using the GST Detection Module (Amersham Pharmacia Biotech). Cells were pelleted by centrifugation at 3,800 × \(g\) for 5 min at 4 °C, resuspended in 10 ml of 15% (w/v) sucrose, 50 mM Tris, 50 mM EDTA, 1 mM DTT, and 0.5 mM PMSF, pH 7.4; and lysed by sonication using a 9.5-mm titanium probe (MSE Soniprep 150). Cell lysate was centrifuged at 26,900 \(\times g\) to remove debris and dialyzed against phosphate-buffered saline containing 1 mM EDTA and 1 mM PMSF, pH 7.4 (Buffer A). ApoE-GST fusion proteins were batch purified by incubating dialyzed cell lysate with 10 ml of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) at room temperature for 1 h. Bound material was pelleted by low speed centrifugation (2,230 \(\times g\)) at 4 °C for 5 min and washed thoroughly with 3 × 40 ml of 1% (w/v) (Buffer A). The apoE moiety was cleaved directly from bound GST by incubating with 100 µg of factor Xa (New England Biolabs) in 20 mM Tris, 100 mM NaCl, and 2 mM CaCl\(_2\), pH 8.0, for 16 h at room temperature. The supernatant, collected after centrifugation, was dialyzed against 2,230 \(\times g\) for 5 min at 4 °C, was applied to a Sephacryl S-300 HR 10/30 column (Amersham Pharmacia Biotech) pre-equilibrated in 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, and 7.4, and eluted at a flow rate of 1.0 ml/min. Fractions containing apoE (as assessed by SDS-PAGE) were pooled and concentrated onto a Source Q HR 5/5 column (Amersham Pharmacia Biotech) and eluted at 1.0 ml/min using a 0–1.0 M NaCl gradient. apoE3 eluted at 0.53 M NaCl, and apoE4 at 0.52 M NaCl. Purity of apoE3 and apoE4 (as assessed by SDS-PAGE) was estimated to be >98%. The molar masses of purified apoE3 and apoE4 (34,245 and 34,297, respectively) determined by electrospray mass spectrometry agree well with theoretical values and known differences between the amino acid compositions of the isoforms. Typical yields of purified apoE3 and apoE4 were 0.5–1.0 mg/liter of culture.

**Analytical Gel Permeation Liquid Chromatography—**Superose 6 HR 10/30 (Amersham Pharmacia Biotech) fast performance liquid chromatography was conducted at room temperature in 100 mM NaCl and 10 mM \(\text{NH}_2\text{HCO}_3\), pH 7.8, at a flow rate of 1.0 ml/min. The column was calibrated with individual aliquots (1.0 mg/ml) of blue dextran (mass = 2,000 kDa), thyroglobulin (mass = 669 kDa), ferritin (mass = 440 kDa), aldolase (mass = 158 kDa), ovalbumin (mass = 43 kDa), and cytochrome c (mass = 12.4 kDa) (Amersham Pharmacia Biotech). Partition coefficients (\(K_p\)) were calculated from the relationship: \(K_p = \left(V_r - V_c\right) / V_c\), where \(V_r = \) elution volume of sample, \(V_c = \) void volume, and \(V_c = \) total column volume. (32)

**Capillary Electrophoresis—**Experiments were conducted at 500 V/cm with normal polarity and 23 °C using a Beckman PACE system 5010, equipped with a Laser Module 488, UV absorbance detector, and Beckman PACE station software (version 1.0). Capillaries were of fused silica (Beckman) with inner diameter of 75 µm and total length of 50 cm. Liquid borate capillary performance run buffer, pH 8.35 (Beckman), was employed as electrolyte. The capillary was equilibrated at 20 p.s.i. in 1 N HCl for 5 min, deionized water for 2 min, 0.1 N NaOH capillary generator solution (Beckman) for 10 min, deionized water for 2 min, followed by a 5-min wash in run buffer. Samples (30 µl) were injected in the absence or presence of 1% (w/v) SDS for 5 s at 0.5 p.s.i., and electropherograms were monitored at 214 nm.

**Combined Sedimentation Equilibrium—**A Beckman Optima model XL-A analytical ultracentrifuge equipped with a photoelectric absorbance optical detection system was used for all sedimentation experiments. Samples (100 µl) and reference (120 µl) solutions were loaded into a conventional double-sector filled epon centapiece (pathlength 1.2 cm) with quartz windows and mounted in a Beckman An-60 Ti rotor. Data was collected at 230 rpm with five averages, at time intervals of 240 min and radial intervals of 0.001 cm, until sedimentation equilibrium was achieved. Experiments were conducted at 20 °C with rotor velocities of 10,000 rpm and 16,000 rpm. For calculation of weight average molar masses, base-line offsets were obtained by high speed depletion at 50,000 rpm for at least 3 h. Solvent densities were measured at 20 °C in an Anton Paar model DMA 02 C precision density meter equipped with a water bath, or computed using the program SEDNTPERP (25), kindly supplied by David Hayes (Magdalen College, Warner, NH), Tom Laue (University of New Hampshire, Durham, NH), and John Philo (Alliance Protein Laboratories, Thousand Oaks, CA). Molar extinction coefficients at 280 nm (44,500 m\(^{-1}\) cm\(^{-1}\)) and partial specific volumes (\(\nu\)) of apoE3 and apoE4 (0.732 ml/g) were calculated from amino acid composition (25). Global nonlinear least square best-fits were obtained by fitting sedimentation data at two speeds or concentrations to a mixture of up to three species and a base-line offset using the program MATLAB. Estimates of the weight-average molar mass (\(M_w\)) were obtained from the nonlinear least squares best-fits to a single species according to Equation 1.

\[
A(r) = A(r_0) \exp \left(-\frac{r^2}{2R^2} \right) + E
\]

\(A(r)\) is the absorbance at radius \(r\), \(A(r_0)\) is the absorbance at the reference radius \(r_0\), \(\rho\) is the rotor angular velocity, \(R\) the gas constant, \(T\) the temperature, \(M_w\) the weight-average molar mass, \(\nu\) the partial specific volume of the solute, \(\rho\) is the solvent density, and \(E\) the baseline offset.

**Sedimentation Velocity—**Sedimentation velocity experiments were performed using a Beckman model XL-A analytical ultracentrifuge. Prior to centrifugation, samples at either 35 or 150 µg/ml were exhaustively dialyzed (>20 h) into 50 mM sodium phosphate, pH 7.4. Samples (300–400 µl) and reference (320–420 µl) solutions were loaded into a conventional double-sector quartz cell and mounted in a Beckman An-60 Ti rotor. Experiments were conducted at 20 °C and a rotor speed of 16,000 rpm. Data were resolved into single scattering functions at a single wavelength, time interval of 300 s, and a step size of 0.003 cm without averaging. Multiple scans at different time points were fitted to a single species, a monomer-tetramer-octamer rapid self-association, or to a continuous size distribution (see below) using the program SEDFIT (which is available from the authors on request).

**Rapid Monomer-Tetramer-Octamer Self-association Model—**The sedimentation velocity analysis with a model for rapid reversible monomer-tetramer-octamer self-association was performed by calculating finite element solutions of the Lamm equation according to methods described by Claverie (26), combined with local weight-average sedimentation coefficients and gradient-average diffusion coefficients (27), and a two-step propagation scheme as described by Schuck (28). For each simulation, a table for the concentration dependence of the sedimentation coefficients and diffusion coefficients (\(D(\omega_c)\)) was calculated (27) with monomer concentrations in 1000 logarithmically spaced increments between \(10^{-5}\) and \(10^{2}\). During the simulations, linear interpolation between the table entries at the calculated total concentrations was used. For testing the numerical precision of this approach, the approach of sedimentation equilibrium at low speed was simulated, for which the concentration profiles converged within \(10^{-4}\) to the thermodynamically predicted distributions, with association constants within \(10^{-3}\) of the correct values. Although the procedure used is conceptually very similar to those outlined by Claverie (26) and Cox (27), because of the larger time intervals enabled by the propagation scheme and because of enhanced computational power of a current desktop PC, the algorithm is fast enough to allow modeling of experimental data by nonlinear regression with the Simplex routine, treating the association constants and the species sedimentation coefficients as floating parameters.

**Continuous Size Distribution Analysis—**The continuous size (mass or sedimentation coefficient) distribution of non-interacting polymers can be determined using finite element solutions of the Lamm equation combined with size distribution analysis techniques by regularization (29). In brief, the experimentally observed sedimentation profiles are
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FIG. 1. Fast protein native gel permeation liquid chromatography of apoE3 and apoE4. Absorbance at 280 nm was plotted as a function of retention time (min). ApoE3 (solid line) and apoE4 (dotted line) were loaded at a starting concentration of 100 μg/ml. Horizontal arrows correspond to peak elution positions of 1 mg/ml molecular weight standards as follows: 1, blue dextran; 2, thyroglobulin; 3, ferritin; 4, aldolase; 5, ovalbumin; 6, cytochrome c.

FIG. 2. High performance fused-silica capillary electrophoresis of apoE4. The absorbance at 214 nm was plotted as a function of elution time (min). ApoE4 (30 μl) at an initial protein concentration of 250 μg/ml in 50 mM sodium phosphate, pH 7.4, was loaded in the absence (solid line) and presence (dashed line) of 1% (w/v) SDS.

described as a superposition of the contributions of each subpopulation $c(M)$ of particles with masses between $M$ and $M + dM$ by the integral equation, Equation 2.

$$a(r,t) = f c(M) I(M, D, r, t) dM$$  (Eq. 2)

$a(r,t)$ represents the experimentally observed signal at radius $r$ and time $t$; $I(M, D, r, t)$ denotes the sedimentation profile of an ideally sedimenting monodisperse species of molar mass $M$ and diffusion coefficient $D$, calculated as the solution to the Lamm equation (30). Analogously, the size distribution can be calculated as a sedimentation coefficient distribution, $a(s)$, according to Equation 3.

$$a(r,t) = f c(s) I(s, D, r, t) ds$$  (Eq. 3)

The distributions were calculated using maximum entropy regularization, selecting the most parsimonious distribution within a predefined confidence level $P$ for the quality of the fit. A fractional ratio $f_0$ representing the ratio of the frictional coefficient to that of an anhydrous sphere was employed, together with the Stokes-Einstein and the Svedberg equation, for estimating the diffusion coefficients $D$ for all species. Unless stated otherwise all size distributions were solved on a radial grid of 300 radius values between the meniscus and bottom, a confidence level of $p = 0.95$, an anhydrous fractional ratio of $f_0 = 1.0$, and a resolution $N$ of 200 sedimentation coefficients between 0.2 and 25 S, respectively. For Monte Carlo statistical analysis, 1,000 synthetic data sets were generated, based on the best-fit size distribution, each with different normally distributed noise. For each point in the distribution, the mean and the quantiles enclosing 95% of the values from the analyses of the simulated distributions were determined.

Circular Dichroism Spectroscopy—Spectra were obtained using an Aviv 62DS circular dichroism spectrometer equipped with a Peltier cell water bath. Spectra of sample dissolved in 50 mM sodium phosphate, pH 7.4, were recorded at 20 °C using 1-mm quartz cuvettes with a wavelength range of 190–250 nm at a step size of 0.5 nm. Signal averaging time was 1.0 s, and the slit bandwidth was 1.5 nm. Data were smoothed by nonlinear least squares fitting to a third order polynomial in a physical environment, both apoE3 and apoE4 elute as similar, relatively broad peaks. Taken from the ordinate maximum of the chromatograms, the partition coefficients of apoE3 and apoE4 are calculated to be 0.52 and 0.53, respectively, corresponding to molar masses of approximately 110,000, relative to standard proteins. Given that the monomeric molar mass of apoE is approximately 34,250, this suggests that both apoE3 and apoE4 exist as trimers and/or tetramers. Furthermore, the broadness and structure of the peaks suggest the presence of a mixture of oligomers, which are stable on the time scale of the experiment. A small proportion of high molar mass aggregate is also observed eluting near the void volume of the column in both the apoE3 and apoE4 chromatograms.

Capillary Electrophoresis—Recombinant apoE3 and apoE4 isoforms were also studied in solution by high performance capillary electrophoresis on fused silica. Fig. 2 shows the electropherogram of apoE4 in the absence and presence of SDS. In the absence of the anionic detergent, the electropherogram of apoE4 is relatively broad, consistent with the fast performance liquid chromatography results (Fig. 1). The addition of 1% (w/v) SDS to the apoE4 sample causes considerable retention time shifts and peak narrowing, attributed to the binding of SDS to apoE4, changing the charge state of the protein and causing dissociation of higher oligomers to monomers (Fig. 2, dashed lines). The small peaks at approximately 3.5 and 16 min are attributed to base-line components present in the sample buffer, whereas the peaks at 6–7 min may indicate residual self-association in the presence of SDS. Identical results were obtained with the apoE3 isoform (data not shown).

Despite evidence of heterogeneity in gel permeation chromatograms and capillary electropherograms, these techniques were unable to resolve the oligomeric structures of apoE3 and apoE4 because of the diffusional broadening of the retention time profiles. To learn more about the oligomeric structures of apoE3 and apoE4, we conducted sedimentation studies in the analytical ultracentrifuge, employing a new method for determining the size distribution of polydisperse macromolecules, which can deconvolute the effects of diffusion (29).

Sedimentation Velocity—Sedimentation velocity data of apoE3 at 360-s intervals show a time-dependent broadening of the sedimentation boundary (Fig. 3). The solid lines in panel A represent the best-fit sedimentation profiles assuming a single component, corresponding to an apparent molar mass (Mr) of 29,400, sedimentation coefficient ($s$) of 7.75 S, and diffusion coefficient ($D$) of $24.1 \times 10^{-7}$ cm²/s. The poor distribution of residuals (Fig. 3A, inset) and the unphysical combination of the
relative high value for \( s \) and low value for \( M \) (or high value for \( D \)) are clearly results of the broadening of the sedimentation boundary due to underlying heterogeneity. For comparison, the best-fit sedimentation profiles to a continuous size distribution calculated in the absence of regularization (Equation 3) are shown in Fig. 3B (solid lines). The fit to the continuous size distribution model (r.m.s.d. = 0.00459) is significantly better than the fit to the single species model (r.m.s.d. = 0.0366). This is also reflected in the more random distribution of residuals (Fig. 3B, inset), compared with the residuals from the single-component best-fit (Fig. 3A, inset). As previously reported (29), calculated size distribution without regularization can be governed by the noise in the data and produce unreliable levels of detail and artifacts.

To investigate the effect of regularization, the solution to the continuous sedimentation coefficient distribution of apoE3 was also determined with regularization on a 0.683 and 0.950 confidence level (Fig. 4A). Fits with maximum entropy regularization (\( p = 0.683 \) or \( p = 0.950 \)) provide a more robust and simpler distribution, while leading to fits of the data that are of statistically indistinguishable quality on the respective confidence level. This analysis consistently reveals the presence of five major species with sedimentation coefficients of approximately 2.2, 6.0, 9.1, 12.9, and 16.6 S (Table I). Similarly, the effect of different discretization of the sedimentation coefficients on the distribution was studied. As demonstrated in Fig. 4B, at low resolution (\( n = 20 \)) relatively coarse distributions were obtained and a fine discretization (\( n = 200 \)) led to smooth distributions. However, no qualitative differences in the results were obtained. Consequently, all subsequent continuous size distribution calculations were solved on a grid of \( n = 200 \) sedimentation coefficients (or molar masses) with a confidence level of \( p = 0.950 \). Finally, the effect of the average anhydrous frictional ratio \( (f/f_0) \) employed in the calculation of the continuous size distribution for estimating diffusion coefficients was also investigated (Fig. 4C). Minor shifts in the ordinate maximum of each peak are evident, although the most significant change observed with increasing \( f/f_0 \) is the broadening of the peaks.

The best fit of the data was obtained with \( f/f_0 = 1.2 \), which for apoE3 at an initial concentration of 150 \( \mu \)g/ml led to an r.m.s.d.
To compare and resolve the oligomeric structures of apoE3 and apoE4, the sedimentation coefficient distributions were determined from sedimentation velocity experiments under identical solution conditions (Fig. 5). Distributions obtained for both isoforms at initial protein concentrations of 150 µg/ml reveal five major peaks. Monte Carlo statistical analysis (Fig. 5, A and B, insets) demonstrates that the peaks are robust against noise, indicating that they are not due to reported oscillations due to noise-induced artifacts in the regularization (29). Additionally, from closer inspection of the raw sedimentation data, the presence of the different subpopulations can be qualitatively verified (Fig. 3): a slow sedimenting part of the boundary at absorbances below approximately 0.2, a rapidly sedimenting part of the boundary at absorbances below approximately 0.2, and a strong depletion of the solution plateau indicating larger species. As for apoE3, the c(s) distribution best fit for apoE4 at 150 µg/ml was obtained using an average f/f₀ = 1.2, yielding an r.m.s.d. = 0.00392 (Fig. 5B). Taken from the ordinate maximum of each peak, the sedimentation coefficients for apoE4 are estimated to be 2.2, 5.9, 9.3, 12.6, and 16.4 S, similar to apoE3 (Table I).

Importantly, the observed structure in the sedimentation profiles is not consistent with the presence of a rapid reversible equilibrium between the species, instead indicating stability of the species on the time scale of the sedimentation experiment. This is illustrated in Fig. 6, which shows the data with best-fit sedimentation profiles of a monomer-tetramer-octamer rapid self-association model. When using the association constants estimated from equilibrium studies (see below, Table II) the fit converges to unphysical, negative sedimentation coefficients for the tetramer. All fits with sets of physically meaningful parameters lead to a poor description of the data (Fig. 6).

Furthermore, the calculated profiles of the rapid reversibility model indicate patterns in the upper part of the boundary that are qualitatively different from the experimental data. Although the experimental profiles exhibit a slowly decreasing slope, the rapid association model produces increasing slopes. This qualitative difference is consistent with the results from Cox (27), who found bimodal gradient curves also in several other rapidly reversibly self-association schemes. Therefore, it appears that the population of rapidly sedimenting oligomers in the experimental profiles are not coupled (in the time scale of the sedimentation) to the slower sedimenting components. This result also appears consistent with the structured shape of the elution profile from gel filtration (Fig. 1).

The stability of the apoE3 and apoE4 oligomers was investigated as a function of time (Fig. 7). Samples were studied by sedimentation velocity at an initial concentration of 150 µg/ml. At t = 0, 24, and 96 h, the calculated sedimentation distributions for apoE3 are very similar (Fig. 7A). Likewise, the distributions obtained for apoE4 are time-independent (Fig. 7B). Both sets of data reveal the presence of five oligomeric components, which are reproducible when the samples are stored over a period of 3 days. Sedimentation velocity experiments were also conducted at lower initial protein concentrations. The c(s) distributions obtained for apoE3 and apoE4 at 35 µg/ml reveal the presence of two broad peaks (Fig. 5, dashed lines). The reduction in peak number and broadening could indicate the slow dissociation of the oligomers when diluted, but can be attributed also in part to the loss of resolution due to the smaller signal/noise ratio at the lower concentration. Accordingly, the first and major peak in the distribution may represent...
a mixture of monomers, dimers, and tetramers (or monomers, dimers, and trimers). Likewise, the smaller peak possibly corresponds to tetramers and octamers (or trimers and hexamers).

In view of these data, although the number and relative concentration of individual apoE oligomers can be achieved by employment of the continuous size distribution method, it is, however, difficult to assign the oligomeric identity of each peak. This is highlighted by the broadening of the distribution at 35 g/ml and further complicated by the well known difficulty of assigning molar mass values to only hydrodynamically defined species. Furthermore, although the calculated best-fit frictional ratios can be used to transform the c(s) distribution into a molar mass distribution c(M), because of the possible errors introduced in this transformation (29), the resulting maxima in c(M) are not consistent with the theoretical molar mass of apoE oligomers (Table I). Given that apoE has previously been shown to self-associate from monomers to predominantly tetramers and possibly octamers and possibly octamers and higher oligomers (15, 20, 21), it is likely that the distributions represent a population of monomers, tetramers, and octamers, and also a small proportion of dodecamers and hexadecamers. However, we cannot rule out a monomer-trimer-hexamer-higher oligomer model from the sedimentation velocity analysis.

Sedimentation Equilibrium—To further study the mode of oligomerization, sedimentation equilibrium experiments were performed at initial apoE3 and apoE4 concentrations of 35 and 150 g/ml. Initially, sedimentation equilibrium data at 10,000 and 16,000 rpm were globally fitted to a single species using Equation 1. Weight-average molar masses obtained from this analysis are concentration-dependent (Table I), suggesting that the distributions represent a population of monomers, tetramers, and octamers, and also a small proportion of dodecamers and hexadecamers. However, we cannot rule out a monomer-trimer-hexamer-higher oligomer model from the sedimentation velocity analysis.

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changes coincide with oligomer dissociation in the presence of submicellar and micellar DHPC. First, we determined the circular dichroism spectra of apoE3 and apoE4 in the presence of micellar SDS (35 mM), given its established role as a lipid mimetic (18, 31, 32). Similar spectra were recorded for apoE3 and apoE4 in the absence of SDS and in the presence of SDS micelles (Fig. 10), resulting in a small change in estimated α-helical content (Table III). Likewise, in the presence of 4 mM or 35 mM DHPC, almost identical spectra were recorded for both apoE3 and apoE4, relative to spectra recorded in a lipid-free environment (Fig. 10). As summarized in Table III, there is little change in α-helical content when DHPC, below or above the critical micelle concentration, is added to apoE3 and apoE4. These data suggest, therefore, that binding of DHPC and the consequent dissociation of higher oligomers do not affect apoE3 and apoE4 at the level of secondary structure.

**DISCUSSION**

Since the discovery that apoE4 gene dose is associated with a higher risk to develop late onset Alzheimers’ disease (9), many studies have compared human apoE3 and apoE4, primarily to determine differences in their ability to bind β-amyloid (33–37). Other studies have been successful in highlighting differences in the tertiary structures of the amino-terminal domains of apoE3 and apoE4 (12, 13) and also demonstrated an isoform-specific preference to the binding of high density lipoprotein and very low density lipoprotein in vitro (13, 38).

However, a detailed comparison of the hydrodynamic properties and quaternary structures of apoE3 and apoE4 has previously not been addressed.

In this study, we initially employed the techniques of native size exclusion liquid chromatography and capillary electrophoresis to demonstrate the similar polydisperse solution structures for apoE3 and apoE4. This is not surprising considering that the primary structure of the two isoforms differ only by a single amino acid substitution. Unfortunately, these techniques do not allow adequate facility for resolving the individual oligomers of apoE. Procedures for resolving the oligomeric forms of apoE3 and apoE4 were therefore sought. In the present paper, we achieved a better resolution through the application of sedimentation equilibrium combined with sedimentation velocity experiments employing a new method for determining the continuous size distribution of polydisperse macromolecules, which takes into account the effects of diffusion (29). We found that the sedimentation profiles were not consistent with the presence of a fast reversible self-association (Fig. 6). In contrast, we found that both apoE3 and apoE4 exist as five major species in a slow equilibrium, consistent with a
monomer-tetramer-octamer-higher oligomer scheme (Figs. 5 and 8, Table I). At low protein concentrations (35 μg/ml), there is also evidence to suggest the tetramers dissociate to dimers. Assuming a hydration value based on amino acid composition, estimates of the axial ratios (a/b) for each oligomeric component of the c(s) distribution can be calculated, providing evidence for the shape or asymmetry of each oligomer (Table IV). It is apparent from the systematic decrease of the axial ratios with increasing oligomer size (Table IV) that the self-association of apoE3 and apoE4 can be described as a side-by-side assembly of asymmetric monomers to more compact higher oligomers. The asymmetric structure of the apoE monomer is consistent with x-ray crystallography studies that show the amino-terminal domain structure of apoE to be elongated (12, 13).

Although we observed a similar oligomer association scheme for apoE3 and apoE4, we find small differences in the size distributions (Figs. 5 and 7) and in the calculated monomer-tetramer (K1,4) and monomer-octamer (K1,8) equilibrium constants (Table II). This indicates that the higher oligomers of the apoE4 isoform are more stable in solution, as compared with apoE3, suggesting that a single amino acid difference alters the propensity of lipid-free apoE to self-associate. Furthermore, this phenomenon may be accentuated in vivo, given the crowded macromolecular environment (39, 40).

In addition to comparing the solution properties of apoE3 and apoE4 in the absence of lipid, we were also interested in studying the state of association of these isoforms in the presence of phospholipid. To achieve this we adopted a novel approach in utilizing a short chain phospholipid (DHPC), which exists as monomers and small micelles in solution, thereby making it possible to solve the oligomeric structure of phospholipid bound apoE3 and apoE4 by sedimentation methods. One of the most striking findings of this investigation was that DHPC induced dissociation of apoE3 and apoE4 oligomers. Despite the observation that only minor variations in secondary structure occur in the presence of DHPC (Fig. 10, Table III), significant changes in the quaternary structures were demonstrated in the presence of the phospholipid above the CMC (Fig. 9B). Dissociation of higher oligomers was also evident below the CMC (Fig. 9A), suggesting that apoE3 and apoE4 interacts with single phospholipid molecules. This is likely to be mediated by amphipathic regions within the COOH-terminal domain of apoE that are also critical for oligomerization. For instance, residues 263–286 have previously been implicated in the roles of lipid binding and self-association (17–19). The demonstration that apoE oligomers dissociate significantly in the presence of phospholipid suggests that apoE may exist as a monomer when bound to lipoproteins in vivo. If this is the case, we propose that the monomeric form of apoE represents the biologically active form, providing the necessary flexibility for the amino-terminal domain to interact with cell surface receptors.

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