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Homo- and hetero-complexes of exchangeable apolipoproteins in solution and in lipid-bound form

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Abstract

The self-association state of human plasma apolipoprotein E (apoE) in solution and in complexes with dimyristoylphosphatidylcholine (DMPC) varying in stoichiometry was studied in sub-micromolar concentration range by gel filtration, fluorescence anisotropy, fluorescence quenching and energy transfer measurements with apolipoprotein labeled with lysine-specific fluorescent dyes. Together, these results confirm the equilibrium scheme for various apoE structures in solution: oligomer (in aged preparations) ⇆ 'closed' tetramer ⇆ 'open' tetramer ('molten globule' state) ⇆ native or partially denatured monomer ⇆ fully denatured monomer. Within DMPC:apoE discoidal complex (125:1) the apolipoprotein association state seems to be intermediate between that in solution and in larger vesicular complex (1000:1); for both complexes, the degree of exposure of fluorescein chromophores into water phase decreased. Hetero-associates of apoA-I and apoC-III-1 in solution and in the complexes with DMPC appear to behave similarly to apoE. When extrapolated to native HDL particles, 'molten globule' state seems to be a structure responsible for the interaction of exchangeable apolipoproteins with phospholipid. For a first time, the location of various apolipoprotein molecules on disc periphery was confirmed. The lysine residue(s) seems to locate closely to reacting residue(s) within apolipoprotein molecules in associates, however, with different package constraints for discoidal versus vesicular complexes with phospholipid.

Keywords: Apolipoprotein A-I; Apolipoprotein C-III; Apolipoprotein E; Protein–protein interactions; Non-radiative energy transfer

Abbreviations: Alkyl-apoE, apoE with SH-group(s) blocked by iodoacetamide; apoE, apolipoprotein E; apoE/F, fluorescein-labeled apoE; apoE/D, dansyl-labeled apoE; Buffer *A*-0.02 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1 mM EDTA; Buffer *B*-0.05 M phosphate, pH 7.0, 0.15 M NaCl, 1 mM EDTA; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; *f*, fractional chromophore accessibility; Gdn-HCl, guanidine hydrochloride; K_{S-V}, Stern–Volmer quenching constant; LDL, low density lipoproteins; PMSF, phenylmethyl sulfonylfluoride; TG, triglyceride; VLDL, very low density lipoproteins.

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1. Introduction

Apolipoprotein E (apoE) is a key protein constituent of human plasma chylomicrons, very low and high density lipoproteins that determines the metabolic fate of these particles through the interaction with LDL-receptor and apoE-receptor [1–4]. The supramolecular organization of apoE on the surface of TG-rich particles may be principally important in the reaction with the receptor as follows from low interaction efficiency for VLDL particles from normolipidemics [5] and its prominent increase for hypertriglyceridemic particles [3,4] and from the involvement of several apoE molecules on one particle into the interaction [6]. Lipid-associated and free apoE molecules are in dynamic equilibrium each other [6]. The rate of lipid binding may depend on the structure and self-association state both of apoE conformers and hetero-associates of apoE with other apolipoprotein molecules and on the rate of exchange of individual apolipoprotein molecules between different structures in water phase. The associated state was suggested not to contribute to apoE content on the surface of lipoprotein particle [7]. ApoE self-association in solution has been previously described [8–11] and we proposed an equilibrium scheme for various apoE structures in solution [8]: oligomer (in aged preparations) ⇆ tetramer ⇆ native or partially denatured monomer ⇆ fully denatured monomer. The structure of apoE in solution might also reflect the conformational transition(s) at apoE dissociation from the surface of TG-rich particles during lipolytic degradation [12]. It is not clear whether these conformational transition(s) are relatively fast process(es), i.e. can apolipoprotein in dissociated state exist in non-equilibrium state in measurable amounts. Various non-equilibrium structures could include 'reaction-like' structure of apoE. On the other hand, apolipoprotein–phospholipid recombinants behave as stable structures [13], and apolipoprotein conformational change(s) from a free to a lipid-bound structure should be a fast process(es).

The other exchangeable apolipoproteins which are found both in VLDL and HDL—apoA-I, apoA-II, apoC-I, apoC-II, apoC-III—also exist in dynamic equilibrium between water and lipid phases. Recently, the intermediate 'molten globule' state has been suggested for apoA-I [14,15], apoA-II [16], apoE [3] and apolipophorin-III [17]. The lipid-binding activity of apolipoproteins seems to correlate with the formation of a partially folded conformation [17]. Despite of many efforts in an attempt to describe both the degree and mode of apolipoprotein self-association in solution and apolipoprotein–lipid interaction [18–23], there are only a few non-coincident studies on the self-association of amphipatic apolipoproteins in the lipid phase: the prominent peptide–peptide interactions has been suggested for apoA-I [24] and apoC-II [25] but not for apoE [26]. The existence of several non-coincident models for apoA-I alignment in discoidal complex with phospholipid [27–29] gives an additional uncertainty to the problem of apolipoprotein–apolipoprotein interactions in the lipid.

The present study is concerned with: (1) the investigation of the structure formation at apoE refolding in solution after denaturation by guanidine hydrochloride or sodium cholate, that further confirmed the existence of intermediate 'molten globule' state at apoE folding; (2) the study of self-association of lipid-bound apoE alone or the mixture of apoA-I and apoC-III-1. For a first time, the existence of homo- (apoE) and hetero- (apoA-I/apoC-III-1) associates within phospholipid phase of discoidal and vesicular particles was observed.

2. Materials and methods

2.1. Apolipoprotein isolation and labeling

ApoA-I [30], apoC-III-1 [31] and apoE [32] were isolated as described. The purity of each apolipoprotein checked by 5–20% SDS-PAGE was greater than 95%. The reduction of disulfide bonds and blockage of thiol groups of apoE by iodoacetamide (alkylation) was performed [33]. Native or alkylated apoE (5.6 μM) was labeled with either dansyl chloride (Serva) or with fluorescein isothiocyanate (Isomer 1, Sigma) in 0.1 M $NaHCO_3$, pH 9.2 at a dye/protein molar ratio around 10:1.

Extent of labeling was varied by varying the incubation time which never exceeded 14 h. Unreacted dye was separated by gel-filtration on a PD-10 column (Pharmacia/LKB) equilibrated with 0.02 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1 mM EDTA (buffer *A*) and by subsequent dialysis against a 100-fold excess of buffer *A*. In the case of native apoE, 1 mM dithiothreitol was added. Samples were filtered through a 0.45 μ filter, HV type (Millipore, USA) and the protein concentration in the labeled samples was determined [34] with native or alkylated apoE as a standard. A molar extinction coefficient of $\varepsilon_{280} = 43\,376\ M^{-1}\ cm^{-1}$ was calculated based upon the amino acid content of native apoE. The labeling stoichiometry was calculated using coefficients of $\varepsilon_{340} = 3400\ M^{-1}\ cm^{-1}$ for bound dansyl [35] and $\varepsilon_{495} = 42\,500\ M^{-1}\ cm^{-1}$ for bound fluorescein [36]. ApoA-I and apoC-III-1 were labeled with fluorescent dyes in an analogous manner.

2.2. ApoE denaturation and refolding in solution

Three different procedures were used.

a) A mixture of dansyl-labeled (apoE/D) and fluorescein-labeled apoE (apoE/F), in a total volume of 500 μl (donor–acceptor, DF-sample), was incubated for 2 h at 24 °C at varying donor:acceptor ratios. Three other samples prepared similarly contained either apoE/D (donor, D-sample), apoE/F (acceptor, F-sample) or unlabeled apoE (blank). The apoE concentration in all four samples was kept constant and equal to 0.6–0.8 μM.
b) Four samples were pre-denatured by incubation in 4 M Gdn-HCl for 3 h at 24 °C and subsequently dialyzed at 4 °C with two changes against a 500-fold excess of buffer *A*.
c) The third approach consisted in solubilizing the four samples (total volume 200 μl) with 7.5 mM sodium cholate (above the critical micellar concentration) for 2 h at 24 °C and subsequent incubation overnight at 4 °C. The samples were diluted with buffer *A* up to 500 μl and dialyzed in a Microdialyzer System 500 (Pierce) for 48 h at 24 °C with three changes of buffer. Buffer *A* in this case contained 0.1 mM PMSF and 0.5 g of the bile-acid adsorbent XAD-2 (Sigma). The dialysis chamber containing 100 ml of buffer was connected with a reservoir containing 200 ml of buffer and the entire content of this system was continuously stirred by means of peristaltic pump with a flow rate of 50 ml h^{-1}. The cholate removal was also done by two sequential incubations for 3 h, at different temperatures with bile acid adsorbent Bio Beads [30].

The gel filtration was performed on Superose 6 PG column (1.4 × 47.5 cm) with the elution rate 0.5 ml min^{-1}. The profiles were monitored by apoE/F fluorescence with excitation and emission wavelengths as 490 and 520 nm, respectively. Multiple peak alignment was done by decomposition of elution profiles with Origin software. The column was calibrated with ferritine (450 kDa), aldolase (158 kDa) and ovalbumine (45 kDa).

2.3. The formation of apoE–phospholipid complexes

The procedure was essentially the same as for apolipoprotein refolding in solution (Section 2.2c) but only alkylated apoE, both labeled and unlabeled, was used. Dimyristoylphosphatidylcholine (DMPC) previously solubilized by cholate was added to a protein to obtain different DMPC:apoE ratios. The total apoE concentration was 0.84 μM, the molar donor:acceptor ratio was 1:1, and the labeling stoichiometries for apoE/D and apoE/F were 5.1:1 and 1.9:1, respectively. Dialysis was performed at 24 °C, close to DMPC phase transition temperature.

2.4. The formation of apoA-I / apoC-III-1-phospholipid complex

The DMPC liposomes were prepared by sonication [30]. The apolipoproteins, both labeled and unlabeled, were sequentially added to liposomes at the desired DMPC:apolipoprotein ratio and after each addition the incubation was performed for 2 h at 24 °C. The competition between apoA-I/D and apoC-III-1 and between apoC-III-1/D and apoA-I for DMPC binding was studied using the

increase of the quantum yield of dansyl-labeled apolipoprotein at the lipid binding. The percent of bound labeled protein was calculated as:

$$\text{Bound} = \frac{I_{+\text{competitor}} - I_{\text{solution}}}{I_{-\text{competitor}} - I_{\text{solution}}} \times 100 \quad (1)$$

where $I_{+\text{competitor}}$, $I_{-\text{competitor}}$ and I_{solution} are fluorescence intensities in the presence and absence of second protein and in solution, respectively.

2.5. Fluorescence measurements

Fluorescence measurements were performed either on a Shimadzu RF-540 or an Aminco-500 spectrofluorimeters at 24 °C. The spectrum of the blank sample was subtracted from those of labeled samples. The energy transfer efficiency E is given by:

$$E = \frac{100(I_{345}^{530}(\text{DF}) - I_{345}^{485}(\text{DF})(I_{345}^{530})(\text{D})/I_{345}^{485}(\text{D})) - I_{490}^{530}(\text{DF})(I_{345}^{530}(\text{F})/I_{490}^{530}(\text{F})))/I_{490}^{530}(\text{DF})}{A_{345}(\text{D})/A_{490}(\text{F})} \quad (2)$$

where I and A are the fluorescence intensity and absorbance values and subscripts and superscripts correspond to excitation and emission wavelengths, respectively. The fluorescence intensity values of the donor and acceptor, excited at 345 nm, are subtracted from the fluorescence intensity of the donor–acceptor sample, excited at the donor absorption band (345 nm) and measured at a wavelength of 530 nm where the intensity is enhanced due to acceptor [37].

The measured values of fluorescence anisotropy r were corrected for instrumental polarization using the G-factor [38]. All measurements were corrected for light scattering and excitation and emission wavelengths were 490 and 520 nm, respectively.

To monitor the temperature-induced apoE/F anisotropy changes, the sample was continuously heated at a rate 0.5 °C min^{-1}. The data are given in double reciprocal plots according to Perrin equation:

$$\frac{r_o}{r} = 1 + \frac{\tau RT}{\eta M(\bar{v} + h)} \quad (3)$$

where r_o and r are the maximum and the measured values of anisotropy, τ is fluorescence lifetime, η is viscosity, M is molecular mass, $\bar{v}$ is partial protein volume and h is the degree of protein hydration [38].

ApoE/F fluorescence was quenched by acrylamide and the fraction of chromophores f accessible to quencher and Stern–Volmer quenching constant K_{S-V} were determined from modified Stern–Volmer equation [38]:

$$\frac{I_o}{I_o - I} = \frac{1}{f[Q]K_{S-V}} + \frac{1}{f} \quad (4)$$

where I_o and I are fluorescence intensities in the absence and in the presence of quencher at the concentration Q.

2.6. ApoE cross-linking

Cross-linking of apoE at the concentration of 0.72 μM with the water-soluble bifunctional reagent 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (20 mM) was performed at 24 °C in a buffer containing 0.05 M phosphate, pH 7.0, 0.15 M NaCl, 1 mM EDTA (buffer *B*). Aliquots were taken at different reaction times and the reaction was stopped by the addition of sample buffer used for SDS-PAGE [39]. After separation of the reaction products by 5–20% SDS-PAGE the proteins were visualized by silver staining [40].

3. Results

3.1. Self-association of apoE in solution

The self-association of apoE was investigated by fluorescence energy transfer measurements. The

Forster's radius, corresponding to the distance at which the energy transfer value E is equal to half from the maximum, is 3.3–4.1 nm for dansyl–fluorescein pair [41]. We studied the exchange between mixed native self-associated forms of dansyl- and fluorescein-labeled apoE with 1:3 molar ratio and with labeling stoichiometries 4.1 and 5.1 mole dye per mole protein for donor and acceptor, respectively. The donor fluorescence intensity was quenched and the intensity at the fluorescein emission band was higher than the sum of the individual donor and acceptor emission spectra (Fig. 1); the E value was 6.2%. It should be pointed out that all measurements were done at pH 7.5 that is one pH unit higher than pK of fluorescein. Therefore, both pH influence on the spectral properties of fluorescein and local shifts in ionization state of functional group(s) close to labeling sites were neglected.

In the next set of experiments the self-association of dansyl- and fluorescein-labeled apolipoprotein was studied by denaturation of protein mixture with 4 M Gdn-HCl and subsequent renaturation by dialysis. The energy transfer value increased from 0 to 29% when the molar fraction of the acceptor increased from 0 to 0.75 (Fig. 2). The dependence of energy transfer upon the molar fraction of the acceptor deviated from linearity at high apoE/F content. Similar results were obtained for renaturation of apoE pre-treated by sodium cholate. In this case, the energy transfer was studied as a function of the average number of fluorescein groups per apoE molecule with a fixed molar ratio of dansyl- to fluorescein-labeled apolipoprotein 1:1. The E values were 29.2±2.6% (mean±S.E.M. of three experiments) and 54.2% (mean of two experiments) for labeling stoichiometries 1.9 and 4.9 mole fluorescein per mole protein, respectively. For both preparations, apoE/F fluorescence anisotropy r values (0.14–0.15) were identical, i.e. fluorescence depolarization due to energy transfer between several fluorescein groups present on the same apoE molecule was negligible. The sensitivity of the energy transfer to the distribution of the acceptor groups close to the donor emitting sphere was thus demonstrated by two independent approaches. The self-association of apoE in solution visualized by high energy transfer and anisotropy values was confirmed by cross-linking experiments within the same protein concentration range (0.5–0.8 μM). The kinetics of apoE cross-linking by water-soluble bifunctional reagent EDC was characterized by the appearance of intermediate dimer and

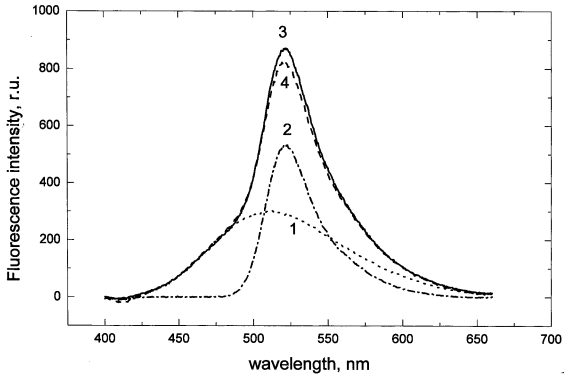

Fig. 1. Emission spectra of apoE/D (1), apoE/F (2), (apoE/D+apoE/F) mixture (3) and the sum of the individual spectra of apoE/D and apoE/F (4). Fluorescence was excited at 345 nm, acceptor molar fraction was 0.75; apoE/D and total apolipoprotein concentrations were 0.16 and 0.64 μM, respectively. The spectra were taken at 24 °C after 2 h incubation at 24 °C in buffer A.

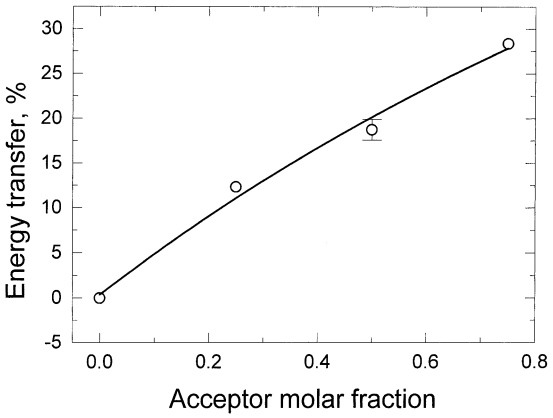

Fig. 2. The concentration dependence of the efficiency of energy transfers E between apoE/D and apoE/F. The donor–acceptor mixture was pre-denatured by 4 M Gdn-HCl and renaturation was performed by dialysis. The energy transfer measurements were performed at 24 °C immediately after dialysis. The mean values of E parameter are given for acceptor molar fractions 0.25 and 0.75 ($n=2$), while mean±S.E.M. ($n=4$) is given for acceptor molar fraction 0.5.

trimer forms and of tetramers as final reaction products (data not shown).

The data obtained earlier [3] suggest the occurrence of slow conformational change(s) of apoE during the last phase(s) of refolding, when the apolipoprotein self-associates already. To verify this, both hydrodynamic dimensions and temperature profile of anisotropy were studied. At gel filtration of the mixture of apoE/F and apoE predenatured by Gdn-HCl and subsequently folded by extensive dialysis at 4 °C, the best fit of profile included five peaks (Fig. 3) which corresponded to oligomer (269±1 kDa, 15.7%), tetramer (125±7 kDa, 20%), dimer (66±4 kDa, 3.7%) and monomer (31±1 kDa, 60.0%) forms of apolipoprotein according to their hydrodynamic dimensions (the fifth wide peak can originate from the non-specific apoE/F sorbtion to column). The short programmable heating of the sample up to 40 °C, followed by gel filtration, resulted in a profound decrease of oligomer and tetramer (Fig. 3) up to 8.4 and 10.9%, respectively.

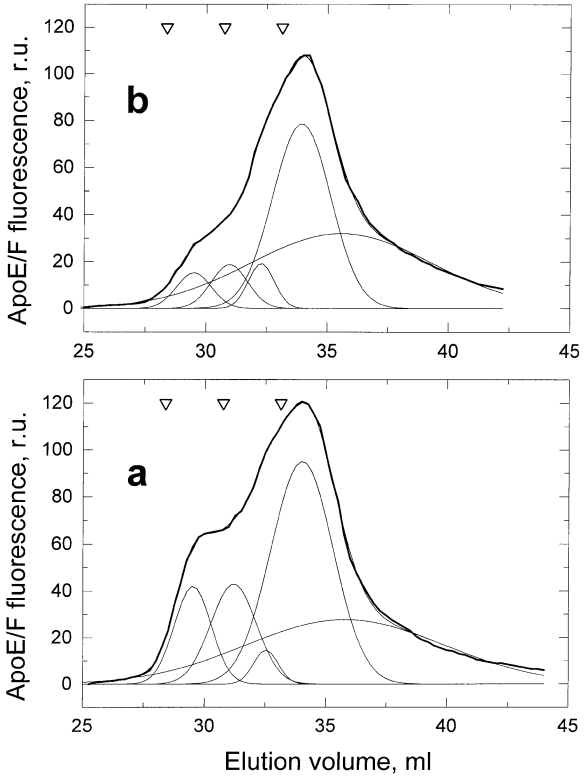

Fig. 3. The self-association of apoE studied by gel filtration. The mixture of 0.24 μM apoE/F and 0.78 μM apoE were predenatured by 5.2 M Gdn-HCl and denaturant was removed by dialysis at 4 °C. After dialysis, the aliquots without any treatment (a) or additionally heated to 40 °C at a rate 0.5 °C min^{-1} (b) were separated on Superose 6 PG column (1.4 × 47.5 cm). The elution positions of ferritine (450 kDa), aldolase (158 kDa) and ovalbumine (45 kDa) are indicated by tingles. The labeling stoichiometry was 3.62 mole fluorescein per mole of protein.

As evident from temperature-induced changes of apoE/F fluorescence anisotropy for the sample pre-denatured by cholate and folded by dialysis and additionally incubated at 4 or 37 °C (Fig. 4), the folding at elevated temperature resulted in an appearance of well-structured form(s) of apolipo-

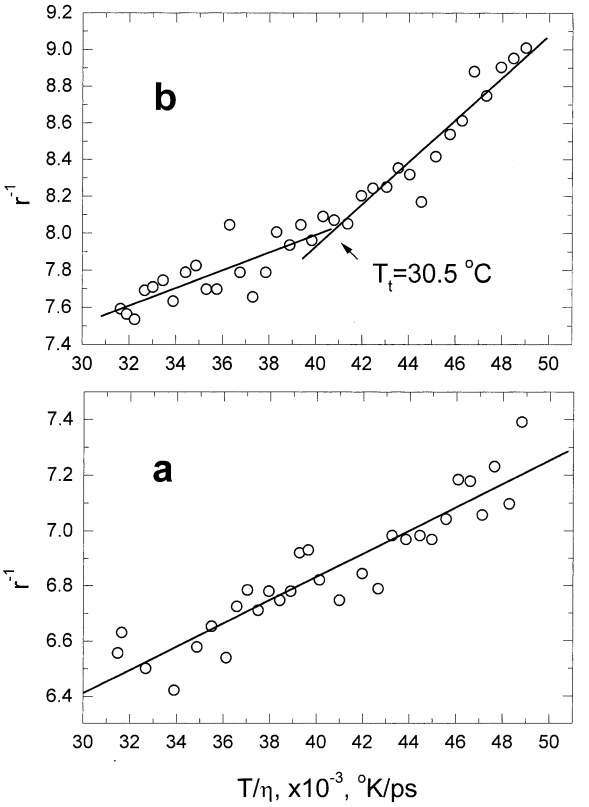

Fig. 4. The induction of apoE structure by temperature. The mixture of 0.48 μM apoE/F, 0.48 μM apoE/D and 1.08 μM apoE was pre-solubilized by cholate and detergent was removed by Bio-Beads treatment at 4 (a) or 37 °C (b). The data are given in double reciprocal plots. The labeling stoichiometries were 3.62 and 9.62 mole dye per mole protein for fluorescein and dansyl, respectively.

protein with the transition temperature 30.5 °C. The major contribution of τ in observed anisotropy changes may be excluded as the expected monotonous decrease of fluorescence lifetime with temperature would result in a monotonous anisotropy growth (Eq. (3)). The folding at low temperature resulted in an accumulation of less structured self-associated form. Its elution profile coincided with that for structured form (Fig. 3). Therefore, the self-associated structure of apolipoprotein changed from an 'open molten globule' to a more condensed state with the formation of a hydrophobic structured 'core' shielded from the water phase.

3.2. The influence of phospholipid on apoE self-association

The apoE/DMPC complexes with initial lipid:-protein ratios as 1000:1 (large vesicular complexes) and 125:1 (small discoidal complexes) were prepared by cholate dialysis procedure. Compared with apoE in solution, the efficiency of energy transfer did not change significantly for small complexes, but decreased 3.4 times for large ones (Table 1). The fluorescein group(s) of apoE/F in large complexes with DMPC became significantly (ten times at a mean level) more shielded from water phase as measured by acrylamide quenching (Table 1).

3.3. The influence of phospholipid on apoA-I–apoC-III-1 association

Initially the range of concentrations for both apolipoproteins where they did not interfere each other at the binding to DMPC liposome was established by competition study (Fig. 5a). ApoA-I efficiently displaced apoC-III-1/D from lipid surface in the concentration range studied while apoC-III-1 weakly competed. The ratio of concentrations of the second protein required for 50% inhibition of the maximal binding of the first protein ED_{50}(C-III-1)/ED_{50}(A-I) was 135 reflecting much higher affinity of apoA-I/D to DMPC compared with apoC-III-1/D. Under conditions chosen (DMPC:apoA-I/D > 500, apoC-III-1/F:apoA-I/D = 5:1), the apolipoproteins behave

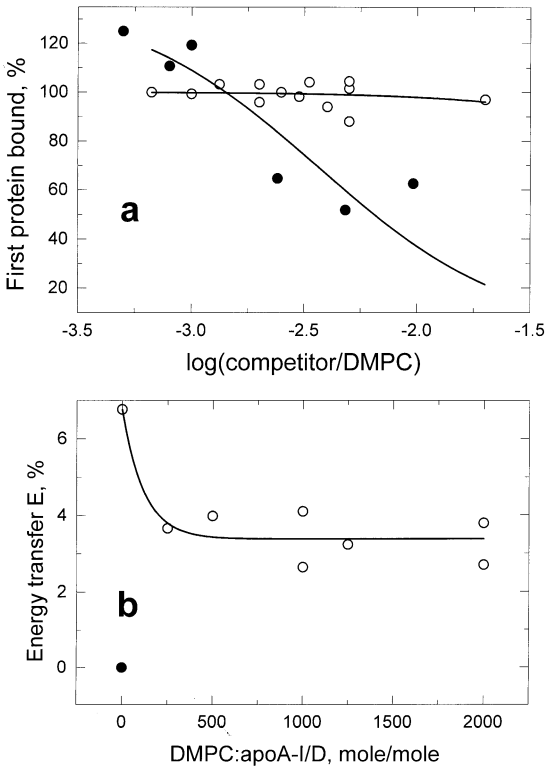

Fig. 5. The interaction of apoA-I and apoC-III-1 with DMPC liposomes. (a): the competition between apoA-I/D and unlabeled apoC-III-1 (open symbols) and between apoC-III-1/D and unlabeled apoA-I (closed symbols) for the binding. The dansyl-labeled apolipoprotein was added to DMPC liposomes at a fixed lipid:protein ratio (usually 1000:1 or 1500:1), the mixture was incubated for 60 min at 24 °C and the second unlabeled apolipoprotein was added at increasing concentrations. After second incubation for 60 min, the percent of labeled protein still bound to DMPC was measured. The concentration of unlabeled protein ED_{50} required for 50% inhibition of maximal binding of the labeled protein was calculated by non-linear curve fitting in one site competition model. (b): the dependence of energy transfer between apoA-I/D and apoC-III-1/F as a function of DMPC added. The measurements were done after two sequential additions of apolipoproteins followed by two incubations for 2 h at 24 °C. The apoA-I/D:apoC-III-1 ratio was 1:5 and the labeling stoichiometries were 10 moles dansyl per mole apoA-I and 1 mole fluorescein per mole apoC-III-1. The zero value of energy transfer for apolipoprotein mixture in solution (closed symbol) was obtained when 2% SDS were added.

independently at the binding. However, association between them evident in solution ($E = 6.7\%$) still remained to exist in small and large complexes (Fig. 5b): the efficiency of energy transfer between

Table 1
Different apoE structures in solution and in the complex with phospholipid

	DMPC:apoE mole ratio		
	0	125	1000
Energy transfer E (%)	29.2 ± 2.6 ($n = 3$)	26.2 ($n = 1$)	8.5 ± 3.6** ($n = 5$)
Accessibility degree f	0.91 ± 0.10 ($n = 2$)	0.56 ($n = 1$)	0.09 ± 0.02* ($n = 4$)
Quenching constant K_{S-V}, M^{-1}	6.5 ± 3.2 ($n = 2$)	5.6 (n = 1)	52 ± 18* ($n = 4$)

For complex formation, apolipoprotein and DMPC were solubilized by cholate and detergent was removed by dialysis. The donor and acceptor labeling stoichiometries were 5.1 and 1.9 mole of dye per mole of protein, respectively, and the donor:acceptor molar ratio before dialysis was 1:1. ApoE/F fluorescence was quenched by acrylamide. Results are given as mean ± S.E.M. The number of separate experiments is indicated in parenthesis. (*) and (**)—$P < 0.05$ and $P < 0.01$, respectively, at comparison of parameters for lipid-bound apoE vs. apoE in solution.

lipid-bound apoA-I/D and apoC-III-1 decreased two-fold for a first concentration point (DMPC:apoA-I/D = 250:1) and did not change further at the dilution with lipid. The apolipoprotein associates in solution fully disappeared in the presence of 2% SDS confirming the validity of the approach to visualize apolipoprotein association on the phospholipid surface.

4. Discussion

4.1. 'Molten globule' state of apoE in self-associated form(s)

The refolding and self-association of apoE towards its native structure after guanidine- or cholate-induced denaturation was studied. The efficient energy transfer process detected at apoE concentrations lower than 0.6 μM (Figs. 1 and 2) indicates apolipoprotein self-association, as diffusion-controlled transfer does not occur at such low acceptor concentration [42]. Self-association of apoE in solution as tetramers was demonstrated also by us [8] and by others [9,10,43] using apoE/F fluorescence anisotropy [8], gel filtration [8,9,43], cross-linking reagents [8,9] and sedimentation analysis [9].

We suggested before [3] the existence of partially disordered self-associated 'molten globule' state and the equilibrium scheme was introduced for apoE structure in solution: oligomer (in aged preparation) ⇆ 'closed' tetramer ⇆ 'open'tetramer ('molten globule' state) ⇆ native or partially denatured monomer ⇆ denatured monomer. In the present study, 'molten globule' state with characteristic features such as the presence of a pronounced secondary structure and a high compactness without a rigid packing inside a molecule was verified both by gel-filtration (Fig. 3) and fluorescence anisotropy (Fig. 4) studies. Indeed, the identity of peak position at the elution of 'closed' and 'open' conformers (Fig. 3) suggests the formation of a hydrophobic 'core' in apoE self-associated structure without a change in hydrodynamic dimensions of apoE tetramer that coincided with identical energy transfer values for both conformers [4] Both slow rotational diffusion of apolipoprotein molecule and fast fluorophor mobility may contribute to the observed anisotropy as r_o value measured experimentally (0.2) was much lower compared with theoretical value (0.4) [8]. We cannot estimate the relative contributions of two mobility types, however, a fast local mobility of fluorescein groups may be suggested is more important for 'closed' compared with 'open' conformer.

Based on the temperature dependence of hydrophobic interactions, both hydrophobic and electrostatic interactions may be suggested to be involved in the stabilization of the apoE supramolecular structure in solution as evidenced by temperature response of fluorescence anisotropy (Fig. 4) and apoE cross-linking with 'zero-length' cross-linker. Among two domains in apoE molecule described by us and others [8,10,11,44], C-terminal domain is responsible for lipid binding. The hydrophobic interactions between C-domains

can be suggested to result in fast apoE self-association followed by much slower transition from 'open' to 'closed' tetramer. The formation of the 'right' local structures with salt bridges between monomer chains may be a rate-limiting step in the overall process of apoE folding. Moreover, a similar folding pattern may exist for other exchangeable apolipoproteins as follows from the existence of 'molten globule' for apoA-I [14,15], apoA-I Milano [15] and apoA-II [16]. Thus, a combined use of fluorescence spectrocopy and gel-filtration experiments permitted as to visualize a subtle difference in tertinary and quaternary structure of varions apoE self-associated conformers that would be difficult to detect by circular dichroism experiments.

4.2. Complex shape and composition at different phospholipid content

The current study presents a first characterization of the association between apoE or apoA-I and apoC-III-1 molecules in phospholipid phase monitored by fluorescence energy transfer. The validity of the approach is confirmed, firstly, by the sensitivity of the distance measured to lipidation state of apolipoproteins (Table 1, Fig. 5) and, secondly, by zero value of energy transfer between apoA-I/D and apoC-III-1/F in the presence of SDS (Fig. 5). The wide range of lipid:protein ratio used covers both discoidal and spherical shape of apolipoprotein–phospholipid complexes [45,46]. Both apoA-I [47,48] and apoE [49,50] interacted differently with phospholipid in vesicular and discoidal complexes. Both types of complexes can co-exist [48]; however, at low protein concentration in the reaction mixture the vesicular complex prevailed and discoidal complex began to form at increasing protein above threshold value. In the case of DMPC as complex-forming phospholipid, the lipid:protein molar ratio was around 700 for apoA-I [45] and 150 for apoC-III [46]; for DPPC, a threshold parameter may be obtained as 1400–2100 mole DPPC per mole apoA-I [48]. The different threshold values is suggested to originate from greater binding affinity and/or capacity of apoA-I compared with apoC-III that followed from much higher potency of apoA-I to displace apoC-III-1 (compared with reverse titration) from the complex with DMPC (Fig. 5). It should be mentioned, however, that we did not concentrate on the final complex stoichiometry, rather we were interested on spatial organization of several apolipoprotein molecules within these particles (The corresponding apolipoprotein:particle molar ratio for vesicular and discoidal complexes may be 6–7 and 2–3 for apoA-I [45]; 21 and 6-7 for apoC-III [46]; 5-7 [7] and 2 [49] for apoE).

4.3. Apolipoprotein alignment on phospholipid surface

The common feature for exchangeable apolipoproteins is: 1) the increase of α-helix content at the lipid binding that was observed for apoA-I [28], apoE [49,50], apoC-I [18] and apoC-III [51]; and 2) the existence of intradomain helix bundle within the solution tertiary structure of apoA-I [29], apoE [52] and apolipophorin III [53]. Helix bundle 'opening' to a more extended conformation upon lipid binding was described for N-terminal domain of apoE [22,54,55] and apolipophorin III [56]. The transition from water-soluble to lipid-bound state of exchangeable apolipoprotein may be suggested to be controlled by the less structured 'molten globule' state which is more efficient, compared with native structure, for lipid binding as shown for apoA-I [14] and apolipophorin III [17,53]. The prominent decrease of the accessibility of fluorescein chromophore(s) to acrylamide observed in apoE-containing complexes, both discoidal and vesicular (Table 1), indicates that hydrophobic interactions between hydrophobic face of helices and phospholipid may be involved into apolipoprotein-phospholipid interaction.

The organization of α-helices in the discoidal and spherical complexes is still questionable. Parallel [57] or perpendicular [58] orientations of helices relative to the phospholipid acyl chains in discs and hairpin model [28] have been proposed. In our [59] and other [60] studies of apoA-I-containing discoidal complexes a varying tilt of helical blocks was suggested; the organization of helical regions of apoE [49] and apoC-III [61], regardless of their exact topology within discs, seem to be similar to apoA-I. In vesicular com-

plexes, the supramolecular organization of helices is not established yet.

Based on a high value of energy transfer for apoE-containing complexes with an initial DMPC:protein molar ratio 125:1, a close proximity of two apolipoprotein molecules in these discoidal particle may be suggested. However, in micelle-forming dihexanoylphosphatidylcholine no apoE self-association was observed [26]; the different apoE behavior may depend on the nature of phospholipid. For DMPC:apoA-I molar ratio lower than 250, the energy transfer values intermediate between that in solution and in large excess of phospholipid seem to exist (Fig. 5) that also implies the small distance between apoA-I and apoC-III-1 in discs. Thus, for a first time, a close proximity between two apolipoprotein molecules in discoidal complex, either between apoE in homogeneous protein mixture or between apoA-I and apoC-III-1 in heterogeneous mixture is described. The location of apoE and apoC-III-1 molecules on the disc periphery in a manner, analogous to apoA-I-containing complex [59] may be suggested. Moreover, the protein–protein contact(s) seem to exist also in spherical particles. However, the apolipoprotein location in two types of particles may differ as follows from energy transfer study (Table 1, Fig. 5) and different relative alignment of apolipoprotein helical segments and phospholipid acyl chains in apoE-containing discoidal and spherical complexes [49,62]. The lysine residue(s) seems to locate closely to reacting residue(s) within apolipoprotein molecules in associates as follows from the amino group specificity of the fluorescent dyes used.

The intimate contacts between apolipoprotein molecules on the surface of native plasma lipoprotein particles may exist. In particular, the self-association of apoE on VLDL surface suggested earlier by us from functional study [3,4] is confirmed now with structural approach. The prominent increase in LDL-receptor affinity of lipoprotein particles with several apoE molecules relative to those with one molecule [1,3] coincides with the present data. Moreover, apoE–apoC-III competitive relationship in the binding of VLDL with LDL-receptor [63] and apoC-II–apoC-III interaction involved in lipoprotein lipase activity regulation [64] may be reasonably explained now as well. It can be speculated further that apolipoprotein molecules contact each other within lipoprotein particles that contain either one (LpA-I) or several apolipoprotein types (LpA-I:A-II, LpA-I:C-III etc. [65]).

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