Interaction of α-Tocopherol with Model Human High-Density Lipoproteins

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ABSTRACT The effects of α-tocopherol on the properties of model high-density lipoproteins (HDLs), composed of human apolipoprotein A-I and dimyristoylphosphatidylcholine, were investigated by physicochemical methods. The intrinsic fluorescence of α-tocopherol and its effects on the polarization of fluorescence of 1,6-diphenyl-1,3,5-hexatriene, which probes the hydrocarbon region of the lipids, and 4-heptadecyl-7-hydroxycoumarin, which is a probe of lipid surfaces, suggest that α-tocopherol is located at the lipid-water interface. Relative to cholesterol, α-tocopherol in lipid surfaces is virtually inert physicochemically. Incorporation of α-tocopherol into HDLs induces only a modest increase in particle size, no change in the transition temperature, and little change in lipid polarity and lipid-lipid interactions. Moreover, α-tocopherol has only a negligible effect on the kinetic parameters of the lipophilic enzyme lecithin:cholesterol acyltransferase, which binds to phosphatidylcholine surfaces and forms cholesteryl esters. However, α-tocopherol has a dramatic inhibitory effect on the rate of association of apolipoprotein A-I with dimyristoylphosphatidylcholine, a process that occurs through the insertion of the protein into preformed defects in the lipid surface. It is proposed that α-tocopherol inhibits the rate of association of apolipoprotein A-I with dimyristoylphosphatidylcholine by inserting into defects within the lipid surface, thereby reducing the size and/or number of sites for insertion of apolipoprotein A-I.

INTRODUCTION

Vitamin E, an antioxidant that occurs largely as α-tocopherol, is the only significant lipid-soluble, chain-breaking antioxidant present in human blood (Burton and Ingold, 1986; McCay, 1985). In blood, α-tocopherol transfers reversibly among blood cells and its major carrier, lipoproteins (Kayden and Trayber, 1987). Additionally, α-tocopherol can be delivered to cells by receptor-mediated uptake of lipoproteins that contain it (Traber and Kayden, 1984) and by a poorly defined mechanism involving the hydrolysis of chylomicrons by lipoprotein lipase (Traber et al., 1985). The transport of α-tocopherol among lipoproteins is important because of its putative protective effects within a variety of cells that have been implicated in the development of atherosclerosis. These effects include preservation of endothelial function and inhibition of monocyte-endothelial cell adhesion, smooth muscle cell proliferation, monocyte-reactive oxygen species and cytokine release, and platelet adhesion and aggregation (Devaraj and Jialal, 1998).

Although α-tocopherol transport is closely connected to lipid and lipoprotein transport, detailed mechanisms of the connection are not known. Through interactions with polyunsaturated fatty acid residues of phospholipid molecules, α-tocopherol modifies cell membrane stability and rigidity and the lateral mobility of membrane lipids (Lucy, 1978; Lai et al., 1985; Wasall et al., 1986; Villalain et al., 1986; Ortiz et al., 1987; Massey et al., 1982; Ekiel et al., 1988). The phytyl side chain appears to suppress the transfer of α-tocopherol between liposomal membranes (Niki et al., 1985; Castle and Perkins, 1986; Massey, 1984; Murphy and Mavis, 1981) and to modify the lateral distribution of α-tocopherol in a membrane (Ortiz et al., 1987; Massey et al., 1982; Ekiel et al., 1988). Additionally, α-tocopherol may affect the activities of lipid-metabolizing enzymes (Napoli and Beck, 1984; Douglas et al., 1986) and inhibit some lipid-protein interactions (Halks-Miller et al., 1985). Studies of α-tocopherol in membranes and membrane models have led to our broad hypothesis that α-tocopherol modulates the structure and function of the lipid-protein systems that make up the plasma lipoproteins.

To address this hypothesis, we studied the effects of α-tocopherol on the assembly, structure, and function of model HDLs consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and either apolipoprotein (Apo)A-I or ApoA-II. Model HDLs are a well characterized system that has been widely used to study the effects of cholesterol in plasma lipoprotein structure and function (Pownall et al., 1978, 1979, 1985, 1987; Massey et al., 1985a,b,c; Matz and Jonas, 1982; Reijngould and Phillips, 1984) and offer a simple system with which to investigate its effects on lipoprotein structure and lipid-protein interactions. Although there have been several studies of the effects of α-tocopherol on phospholipid bilayers (Lai et al., 1985; Wasall et al., 1986; Villalain et al., 1986; Ortiz et al., 1987; Massey et al., 1982; Ekiel et al., 1988), none has addressed the effects of α-tocopherol within a lipid system containing a lipid-associating protein. Such information may aid our understanding of the role of HDLs in the lipoprotein-dependent transport of vitamin E (Kayden, 1981).

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EXPERIMENTAL PROCEDURES

Materials

ApoA-I, ApoA-II, and lecithin:cholesterol acyl transferase (LCAT) were isolated from human plasma as previously described (Pownall et al., 1985;

Massey et al., 1985a). DMPC was obtained from Avanti Polar Lipids (Birmingham, AL). [^{3}H]Cholesterol was from New England Nuclear (Boston, MA), and [^{3}H]DMPC was synthesized as previously described (Pownall et al., 1978). α-Tocopherol (95% pure) was a gift from the Henkel Corp. and was further purified by chromatography over alumina using a 0–4% gradient of ethyl acetate in hexane. The product was detected and quantified on the basis of the spectral absorption at 290 nm and by reaction with 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Bellemare and Fragata, 1980). The resulting purity was >99% as determined by thin-layer chromatography on silica gel, using a solvent of ethyl acetate/hexane (10:90 v:v) and identification by iodine staining, spraying with DPPH, or spraying with acid and then charring. DPPH was from Aldrich Chemical Co. (Milwaukee, WI). Prodan, 4-heptadecyl-7-hydroxycoumarin (HC), and 1,6-diphenyl-1,3,5-hexatriene (DPH) were from Molecular Probes (Grand Junction, OR).

Methods

Kinetics of ApoA-I/DMPC association

The rate of ApoA-I association with multilamellar liposomes of DMPC containing various concentrations of α-tocopherol was determined by measuring the rate of decrease of liposomal turbidity (Pownall et al., 1978, 1979). DMPC/α-tocopherol liposomes were prepared by co-solubilizing the desired quantity of each lipid in chloroform, removing the solvent under a stream of nitrogen, and lyophilizing. The dried lipids were dispersed by vortexing for a few minutes above the DMPC transition temperature (>23.9°C) in a buffer of 100 mM NaCl, 1 mM sodium azide, 1 mM ethylenediaminetetraacetic acid, and 10 mM Tris, pH 7.4; this buffer was used throughout. The liposomes and ApoA-I were preincubated at the appropriate temperature and then mixed for a final concentration of liposomes (0.5 mg of phospholipid) and ApoA-I (0.2 mg) in a final volume of 3 ml. Rates of association were measured by following the reduction in right-angle light scattering versus time in an SLM 8000 spectrofluorimeter (Urbana, IL) with excitation and emission wavelengths of 325 nm. The samples were continuously stirred to prevent settling of the liposomes. The data were analyzed according to pseudo-first-order kinetics, where the rate constant $k_{1/2} = 1/t_{1/2}$, with $t_{1/2}$ representing the time for disappearance of 50% of the light-scattering intensity.

Preparation of model HDLs

Model HDLs were formed by spontaneous association or by dialysis from mixed micelles of cholate. In the first method, liposomes containing various amounts of α-tocopherol were prepared by dissolving DMPC and α-tocopherol in chloroform, removing the solvent under a stream of nitrogen, and suspending the dried lipids in buffer by vortexing above the transition temperature (>24°C). ApoA-I (1 mol % DMPC) was added and incubated with the liposomes for 24 h at 24°C (Massey et al., 1985b; Pownall et al., 1978). In the second method, DMPC was solubilized in cholate until the solution was transparent; ApoA-I was then added and the detergent was removed by extensive dialysis at room temperature (Massey et al., 1985b). The model HDLs were isolated by chromatography over Sepharose CL-4B (1.6 × 40 cm) at ambient temperature. The column effluent was monitored by scintillation counting of [^{3}H]DMPC and absorbance at 280 nm. Pooled fractions were analyzed for phospholipid (Barlett, 1959) and protein (Lowry et al., 1951). The α-tocopherol concentration was determined from its absorbance after extraction into organic solvents.

To determine the Stoke's radius (R_o), pooled fractions from the Sepharose CL-4B column were concentrated using Amicon Centricon microconcentrators (Amicon, Beverly, MA) and analyzed by analytical gel filtration on two Superose HR6 columns (Pharmacia, Piscataway, NJ) connected in tandem; 200-μl aliquots were collected and monitored for absorbance at 280 nm. The Stoke's radius was determined by comparison with the following standards: thyroglobulin (R_o = 85 Å), ferritin (R_o = 61 Å), catalase (R_o = 52 Å), aldolase (R_o = 48 Å), bovine serum albumin (R_o = 36 Å), ovalbumin (R_o = 31 Å), chymotrypsin (R_o = 21 Å), and ribonuclease A (R_o = 16 Å). Sonicated DMPC single-bilayer vesicles ($R_o \approx 125$ Å) and human low-density lipoprotein (LDL) ($R_o \approx 105$ Å) were also run as standards. The column parameter K_{AV} was calculated as $K_{AV} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the model HDLs, V_0 is the void volume determined by using [^{3}H]DMPC liposomes, and V_t is the salt peak determined using [^{14}C]phosphorylcholine (Massey et al., 1981).

Fluorescence methods

Fluorescence polarization of DPH (Massey et al., 1985b) and HC (Pal et al., 1985) in model lipoproteins was measured on an SLM 8000 spectrofluorimeter equipped with Glan-Thompson prisms. DPH was chosen to measure the mobility of the acyl chain region of the bilayer, and HC was chosen because it is sensitive to the motion at the interfacial head group regions of a phospholipid bilayer (Pal et al., 1985). The fluorescence probes were introduced by injection of microliter aliquots of the probe in ethanol into the samples. Final concentrations were <0.1% by volume of ethanol and contained <1 mol of probe per 500 mol of phospholipid. The sample chamber was maintained at a constant temperature with a thermostat-controlled water bath, and the temperature in the cuvette was recorded with a Bailey Instruments digital thermometer (model Bat 8). The respective excitation wavelengths were 375 nm and 350 nm for for HC and DPH. The intensity of the emitted light was measured after passing through a Corning 3–144 cutoff filter. The Prodan fluorescence spectra were measured and analyzed for spectral width and the wavelength of maximal intensity (Massey et al., 1985c). On the basis of the partition coefficient previously determined for Prodan with model HDLs (Massey et al., 1985c), conditions were selected so that >99% of the probe was associated with phospholipid.

LCAT measurements

The effect of α-tocopherol on the reactivity of model HDLs to the reaction catalyzed by LCAT was determined (Pownall et al., 1985; Massey et al., 1985a). Model HDLs composed of ApoA-I (1 mol %), DMPC (97 mol %), and [^{3}H]cholesterol (2 mol % cholesterol) were prepared by using the cholate dialysis method described above. Other model HDLs containing α-tocopherol in amounts up to 8 mol % of the DMPC were prepared similarly except that α-tocopherol was added to the DMPC before lyophilization and hydration. These HDL models were used as substrates for LCAT according to previously described experimental procedures (Pownall et al., 1985; Massey et al., 1985a). Enzymatic activity was determined as a function of model HDL phospholipid concentration, and the maximal enzymatic velocities (V_{MAX}) and apparent Michaelis–Menten constants (K_M) were determined.

Microenvironment and reactivity of α-tocopherol in model HDLs

Fluorescence spectra of α-tocopherol in model HDLs containing ApoA-II were recorded on an SLM 8000 spectrofluorimeter. The microenvironment and reactivity of α-tocopherol were determined in previously characterized (Massey, 1984; Massey et al., 1981) model HDLs composed of ApoA-II and DMPC (1/75 molar ratio of lipid to protein (M/M)). These models were used because ApoA-II has a spectral window at 290 nm where α-tocopherol has an absorption maximum. This window permits the environment of α-tocopherol to be directly examined from the fluorescence spectrum of the chromanol ring in α-tocopherol. To determine the accessibility of the chromanol ring system to the aqueous solvent, D_2O was used as a perturbant (Massey and Pownall, 1989).

To assess the reactivity of α-tocopherol in model HDLs, the kinetics of reductive bleaching of DPPH by α-tocopherol were determined by a previously described method (Bellemare and Fragata, 1980). In essence, the rate of DPPH bleaching was followed by measuring the absorbance at 516 nm as a function of time. A Cary model 15 spectrophotometer was used with the temperature of the cell holder maintained by a Neslab water

bath and monitored by a Bailey Instruments digital thermometer (model Bat 8) with thermocouple.

RESULTS

Gel filtration chromatography

The size and extent of formation of model HDLs formed by the association of ApoA-I and DMPC were analyzed by gel filtration chromatography over Sepharose CL-4B. The criterion for the formation of model HDLs is the co-elution of the lipid and protein; size was assessed on the basis of elution volume. At 0, 1, 3, and 6 mol % α-tocopherol, the ApoA-I and DMPC eluted as a single, homogeneous peak, but at 12, 18, and 24 mol % α-tocopherol, most of the liposomes eluted in the void volume (data not shown). To "catalyze" the formation of model HDLs (Massey et al., 1985b), mixtures of ApoA-I, DMPC, and α-tocopherol were solubilized in cholate and the cholate was removed by dialysis. The range of α-tocopherol concentrations in DMPC extended from 0 to 30 mol %. Representative data in Fig. 1 show that at 0, 12, 24, and 30 mol % α-tocopherol, nearly all of the lipid and protein co-eluted as a single product with no lipid appearing in the void volume, indicating that the association of ApoA-I and DMPC is nearly quantitative. Pooled samples from incubations containing a wide range of mol % α-tocopherol were analyzed by Superose 6 gel filtration chromatography. In mixtures containing up to 12 mol % α-tocopherol in DMPC liposomes, homogeneous model HDLs having similar Stoke's radii were formed by spontaneous association and by cholate dialysis. At higher mol % α-tocopherol in DMPC, the distribution of size among the model HDLs was bimodal (Fig. 2). The chromatographic data showed that with the cholate dialysis method, the α-tocopherol and DMPC were quantitatively incorporated into model HDLs identical in composition to the initial mixtures (Figs. 2 and 3 *A*). Furthermore, these data show that as the mol % α-tocopherol in DMPC was increased the DMPC to ApoA-I ratio decreased slightly (Fig. 3 *B*), and the size of the model HDLs decreased (Figs. 2 and 3 *C*).

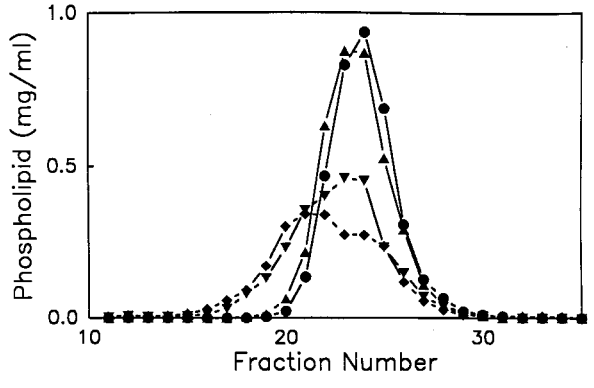

FIGURE 1 Sepharose CL-4B gel filtration of model HDLs of ApoA-I and α-tocopherol/DMPC liposomes. DMPC (5 mg) and different amounts of α-tocopherol were incubated with ApoA-I (2 mg) and 10% sodium cholate for 24 h at 24°C. The samples were exhaustively dialyzed against a buffer and applied to a column of Sepharose CL-4B. The elution profile was determined by collecting fractions and analyzing an aliquot for [^{3}H]D-PMC by liquid scintillation counting. Elution profiles are for model HDLs formed from ApoA-I and DMPC containing 1, 12, 24, and 30 mol % α-tocopherol. Pooled samples were formed as follows: ●, 0 mol %, fractions 22–26; ▲, 12 mol %, fractions 22–26; ▼, 24 mol %, fractions 19–22 (pool A) and fractions 23–27 (pool B); ♦, 30 mol %, fractions 19–22 (pool A) and fractions 23–27 (pool B).

Fluorescence polarization

The phase behavior of the lipid matrix in the model HDLs formed by the cholate dialysis method was investigated by measuring the temperature dependence of the fluorescence polarization of DPH and HC, which are sensitive to changes in mobility within the hydrocarbon and surface regions, respectively, of lipids and lipoproteins (Massey et al., 1985b; Pal et al., 1985). The temperature of the midpoint of the change in the fluorescence polarization, which corresponds to the gel-to-liquid crystalline transition (T_C = 28°C) of the model HDLs, was invariant with respect to an α-tocopherol content between 0 and 24 mol %. According to the polarization of fluorescence of DPH, α-tocopherol modulates the phase behavior of DMPC in the model HDLs

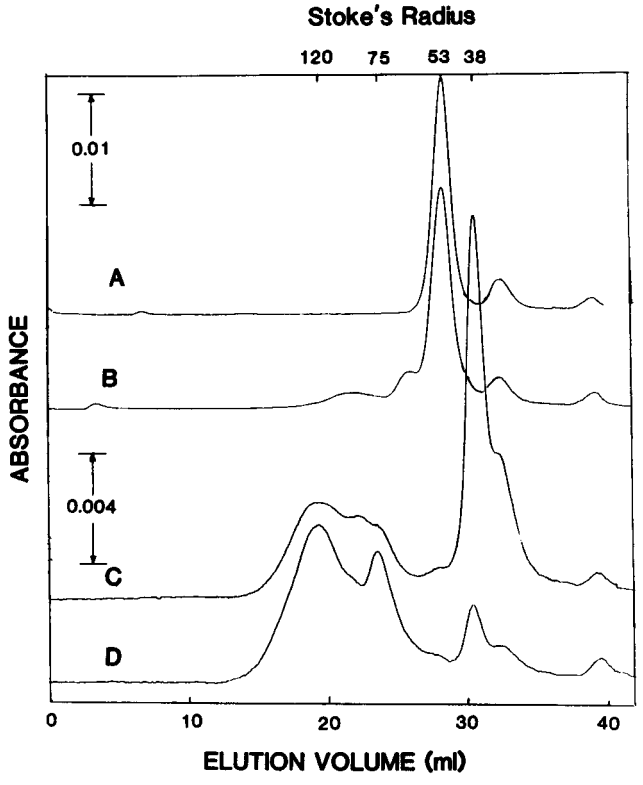

FIGURE 2 Superose 6 gel filtration chromatography of model HDLs containing α-tocopherol. Pooled samples as described in Figure 1 were applied to a Superose 6 column, and the effluent was monitored by measuring the absorbance at 280 nm. (*A*) 0 mol % α-tocopherol; (*B*) 12 mol % α-tocopherol; (*C*) 30 mol % α-tocopherol, pool A; (*D*) 30 mol % α-tocopherol pool B. The elution volumes of standards are shown at the top of the figure with their respective Stoke's radii (Å).

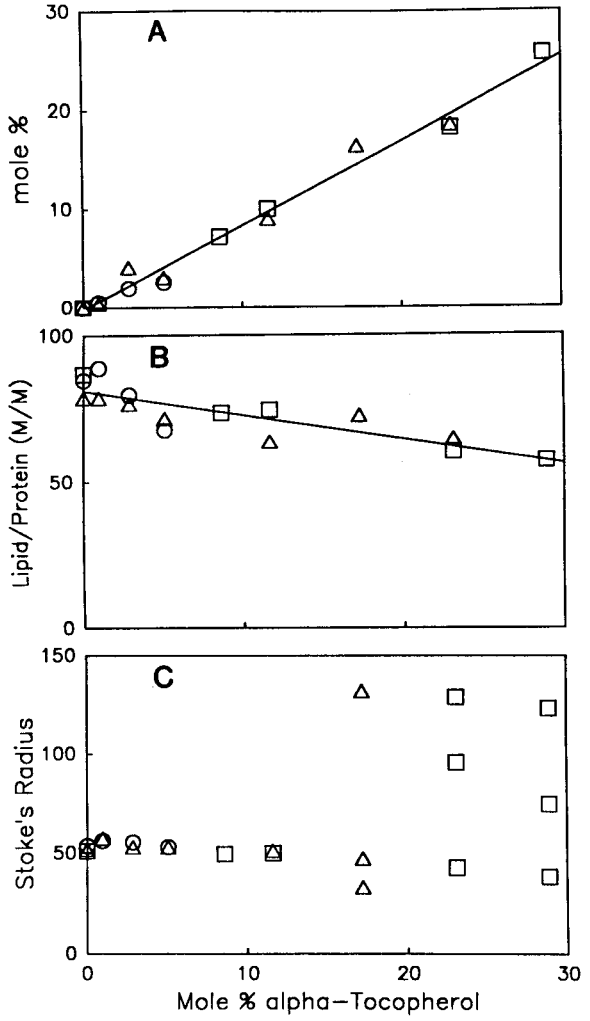

FIGURE 3 Effects of different amounts of α-tocopherol on the physicochemical properties of model HDLs. Model HDLs were prepared by spontaneous association (○ and △) and by the cholate dialysis method (□), and the major subfractions were isolated by Superose 6 chromatography. The isolated samples were analyzed for α-tocopherol, DMPC, and ApoA-I. On the basis of these analyses, the mol % α-tocopherol in DMPC and the DMPC/ApoA-I ratios were calculated. These values and the Stoke's radii in Å were plotted against the mol % α-tocopherol in the starting mixture. (*A*) mol % α-tocopherol in DMPC; (*B*) DMPC to ApoA-I molar ratio; (*C*) Stoke's radius. Spontaneous association experiments 1 (□) and 2 (△) were conducted on different days.

(Fig. 4 *A*). Below the transition temperature, incorporation of α-tocopherol into the model HDLs decreased the polarization of the fluorescence of DPH, whereas above the transition temperature the opposite was observed. This is consistent with α-tocopherol's increasing the fluidity below the transition temperature and decreasing it above the transition temperature. A similar effect was observed with HC except that below the transition temperature, α-tocopherol had no effect on the polarization of HC fluorescence (Fig. 4 *B*).

Prodan fluorescence

The fluorescence spectrum of Prodan is sensitive to the quality of its microenvironment (Massey et al., 1985c). The

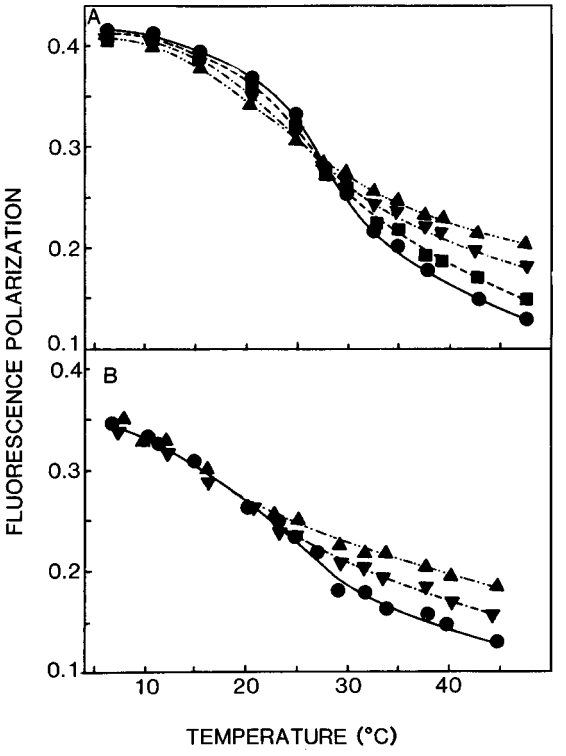

FIGURE 4 Temperature dependence of the fluorescence polarization of DPH and HC in model HDLs containing different amounts of α-tocopherol. (*A*) DPH polarization; (*B*) HC polarization. The model HDLs contained 0 (●), 6 (■), 12 (▼), or 24 (▲) mol % α-tocopherol.

spectral location of the fluorescence maximum reflects the polarity of the microenvironment and the spectral half-width gives an indication of the heterogeneity of the microenvironment. The change in the phase state of the phospholipid elicited a dramatic change in the emission maximum and the spectral half-width of the fluorescence (Fig. 5). In

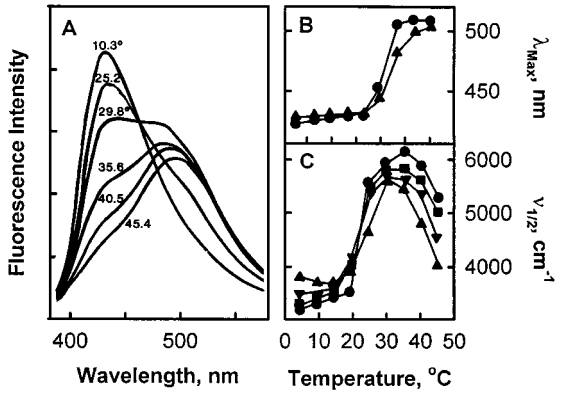

FIGURE 5 Temperature dependence of the fluorescence behavior of Prodan in model HDLs containing different amounts (*n*) of α-tocopherol. Model HDLs were composed of ApoA-I/DMPC (1/100). (*A*) Fluorescence spectra of Prodan in model HDLs at different (denoted) temperatures; (*B*) Wavelength maximum (α-tocopherol content in the model HDLs was 0 mol % (▲) or 24 mol % (●)); (*C*) Spectral half-width ($\nu_{1/2}$) (0 mol % (▲), 12 mol % (▼), 18 mol % (■), and 24 mol % (●))

contrast, the fluorescence of Prodan in model HDLs was only slightly sensitive to the addition of up to 24 mol % α-tocopherol (Fig. 4); the fluorescence maxima were nearly the same at 0 and 24 mol % α-tocopherol (Fig. 5 *B*). Fluorescence half-width, which is a measure of microscopic heterogeneity, was modulated by the addition of α-tocopherol (Fig. 5 *C*). Below the transition temperature, where small values of $\nu_{1/2}$ were observed, incorporation of α-tocopherol into the model HDLs increased the fluorescence half-width; above the transition temperature, where large values of $\nu_{1/2}$ were observed, the addition of α-tocopherol decreased the value of $\nu_{1/2}$.

Spectral and chemical behavior of α-tocopherol in model HDLs

To identify the effects of the phase transition of DMPC on the microenvironement of α-tocopherol, the fluorescence properties of the chromanol ring of α-tocopherol were monitored in model HDLs of ApoA-II and DMPC (Massey, 1984; Massey et al., 1981; Massey and Pownall, 1989). There was only a slight red shift in the fluorescence maximum of α-tocopherol in liquid crystalline (T = 50°C) compared with gel-phase DMPC (T = 10°C; Fig. 6 *A*). Replacement of H_2O with D_2O increased the fluorescence quantum yield and induced a slight blue shift in the fluorescence maximum. As replacing H_2O with D_2O alters the spectral properties of the chromanol moiety, α-tocopherol must be accessible to water. On the other hand, the chromatographic data show that α-tocopherol is bound to the lipids. Moreover, the discontinuity in the ratio of the fluorescence intensity of D_2O versus H_2O with respect to temperature, observed at the phase-transition temperature (28°C) of DMPC in model HDLs suggests that α-tocopherol interacts with the hydrocarbon region of DMPC. These data suggest that the chromanol ring of α-tocopherol is in the aqueous-lipid interfacial region of the bilayer, where it is accessible to the aqueous phase. The higher quantum yield in D_2O above 28°C suggests that the accessibility of the chromanol ring to the aqueous solvent is greater above the phase transition (Fig. 6 *B*). The rate of reaction of α-tocopherol with DPPH (Bellemare and Fragata, 1980) was measured as a function of temperature. Below the phase transition (T < 20°C), the rate was slow, and as the temperature increased the rate increased gradually until the DMPC transition temperature was reached, when the rate increased dramatically (Fig. 6 *B*).

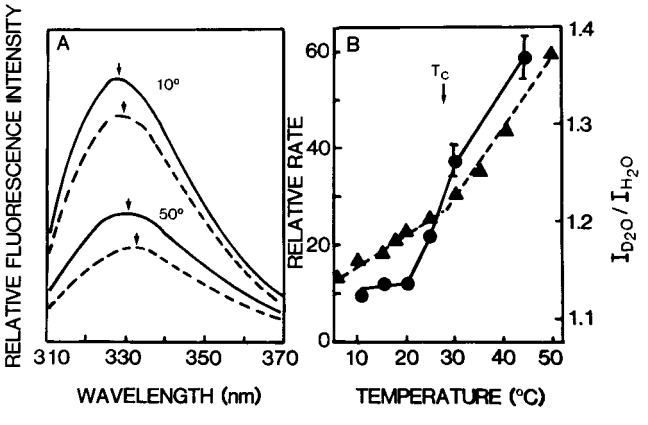

FIGURE 6 Spectral behavior and chemical reactivity of α-tocopherol in model HDLs. Model HDLs were composed of ApoA-II, DMPC, and α-tocopherol (1/75/1 M/M/M). (*A*) Fluorescence spectra of α-tocopherol at 10°C and 50°C in H_2O (- - -) and 95% D_2O (——). Arrows indicate the wavelength maximum for each spectrum. (*B*) The ratio of the maximal fluorescence intensity in D_2O (I_{D2O}) versus that observed in H_2O (I_{H2O}) was calculated and plotted as a function of temperature (▲). A discontinuity is observed at T_c = 28°C, the DMPC phase transition in model HDLs (Massey et al., 1989). The relative rate of reduction of DPPH by α-tocopherol in the ApoA-II/DMPC complexes as a function of temperature is also shown (●). Error bars represent standard deviation.

LCAT activity

The effect of α-tocopherol on the activity of LCAT was determined by using model HDLs containing 2 mol % cholesterol. The values for K_M and V_{Max}, which were determined from steady-state kinetic measurements (Pownall et al., 1985; Massey et al., 1985a) were the same for model HDLs having between 0 and 8 mol % α-tocopherol (Fig. 7).

Kinetics of association of ApoA-I with DMPC liposomes

Association of saturated phospholipids and apolipoproteins is a function of temperature and the addition of small

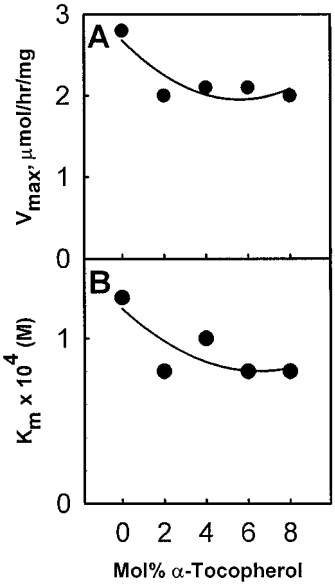

FIGURE 7 Effect of α-tocopherol on the LCAT activity against model HDLs. The model HDLs formed by the cholate dialysis procedure were composed of ApoA-I, DMPC, and cholesterol (1/100/2 M/M/M) and various amounts of α-tocopherol, which are expressed in terms of mol % of DMPC. The model HDLs contained a tracer of [^{3}H]cholesterol to quantify the formation of cholesteryl esters. V_{Max} and K_M were determined from steady-state kinetics of reaction velocity versus the DMPC concentration (Pownall et al., 1978, 1979).

uncharged lipids. Association of ApoA-I with DMPC liposomes exhibits a maximal rate at the transition temperature of the lipid (Pownall et al., 1978), and incorporation of cholesterol into the liposomes dramatically increases the maximal rate of association (Pownall et al., 1979). The effects of α-tocopherol and temperature on the rate of association of ApoA-I and DMPC liposomes were determined (Fig. 8). In the absence of α-tocopherol, the rate of ApoA-I–DMPC association was greatest at 24°C; however, the rate of association was much slower above and below this temperature. The rate of association of ApoA-I with DMPC liposomes decreased with increased amounts of α-tocopherol in the liposomes, even with as little as 1 mol %. At 24°C, the rate of association of ApoA-I with liposomes containing 6 mol % α-tocopherol was approximately four orders of magnitude slower than that observed with pure DMPC liposomes. In addition, the temperature at which the maximal rate of association was observed decreased as a function of added α-tocopherol. Incorporation of α-tocopherol into the DMPC liposomes produced two effects, which were a decrease in the rate of association and a reduction in the temperature of the maximal rate of association.

DISCUSSION

The modification of the physical properties of model HDLs by α-tocopherol is distinct from that of cholesterol. Although incorporation of α-tocopherol into model HDLs increases their size (Fig. 2), the magnitude of the effect is not nearly as large as that of added cholesterol, which at 24 mol % in DMPC combines with ApoA-I to form particles that are large enough (Stoke's radius >750 nm) to be totally excluded from Sepharose CL-4B (Massey et al., 1985b). The effect of cholesterol on the Stoke's radii of single-bilayer vesicles and model HDLs is thought to be due to the replacement of the truncated cone shape of phospholipid with the inverted cone shape of cholesterol (Israelachvili et al., 1980). The effect of α-tocopherol on particle size is smaller because its shape is very close to cylindrical.

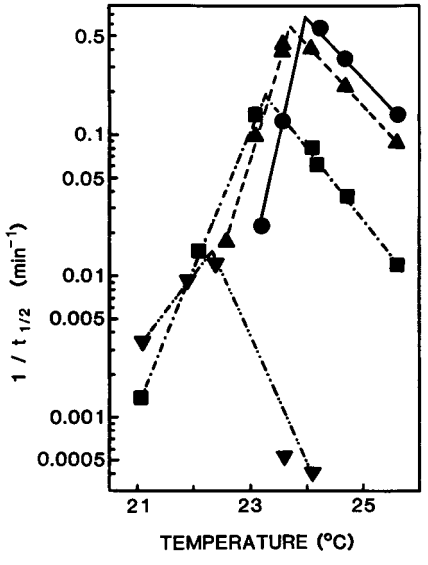

FIGURE 8 Kinetics of association of ApoA-I with DMPC/α-tocopherol liposomes followed by changes in turbidity as a function of mol % α-tocopherol and temperature. Liposomes (0.5 mg of phospholipid) and ApoA-I (0.2 mg) were preincubated at the appropriate temperature and mixed to give a final volume of 3 ml. The rate constant $k_{1/2} = 1/t_{1/2}$ represents the time required for 50% reduction in light scattering at 325 nm. The data shown are for 0 mol % (●), 1 mol % (▲), 3 mol % (■), and 6 mol % (▼) α-tocopherol.

The temperature dependence of the polarization of HC and DPH fluorescence reveals differences in the phase behavior of model HDLs containing α-tocopherol and the effects of α-tocopherol on molecular mobility within the particles. At $T < T_C$, α-tocopherol had only a small effect on the fluorescence polarization; at $T > T_C$, however, a large, concentration-dependent decrease in polarization was observed (Fig. 4, *A* and *B*). This was observed for both DPH, which probes molecular mobility within the hydrocarbon region of the model HDLs, and HC, which monitors mobility within the interfacial region (Fig. 4 *B*). These data suggest that above the transition temperature, α-tocopherol decreases lateral and rotational mobility in model HDLs and that this effect extends to both the interfacial and the hydrocarbon regions of the particles. Although incorporation of cholesterol into model HDLs raises its transition temperature (Massey et al., 1985b), our data revealed no effect of α-tocopherol on the T_C of DMPC. This suggests that α-tocopherol does not greatly modify the lipid-lipid interactions between DMPC molecules.

The fluorescence spectral properties of Prodan, which are highly sensitive to solvent polarity (Massey et al., 1985c, Weber and Farris, 1979), make it a good probe of polarity and its heterogeneity in model lipid systems. Whereas a large Stoke's shift is indicative of a polar environment, the breadth of the fluorescence spectrum is a reliable indicator of the heterogeneity of the environment around Prodan. Unlike the effects of cholesterol, addition of α-tocopherol had only a small effect on the fluorescence properties of Prodan. The maximal value in $\nu_{1/2}$ occurred at the same temperature for all concentrations studied and varied only slightly among the different model HDLs (Fig. 5 *C*). The temperature dependence of the fluorescence maximum was only slightly different when the α-tocopherol content was increased from 0 to 24 mol % α-tocopherol (Fig. 5 *B*). The difference is due to α-tocopherol's broadening the phase transition (Fig. 5 *A*). The effects of cholesterol on Prodan fluorescence in model HDLs composed of ApoA-I and DMPC are distinctly different: at 18 mol % cholesterol a wavelength maximum characteristic of gel-phase lipid is observed even at $T > T_C$ (Massey et al., 1985c). This result suggested that cholesterol decreases the water penetrability into liquid-crystalline bilayers (Massey et al., 1985c; Straume and Litman, 1987) but that α-tocopherol does not.

In model HDLs containing ApoA-I, DMPC, and [^{3}H]cholesterol, addition of up to 8 mol % α-tocopherol had no effect on cholesterol esterification by LCAT (Fig. 7). Based on the small differences in DPH fluorescence polarization,

0 to 6 mol % α-tocopherol induced little change in lipid fluidity of model HDL substrates at the temperature of the assay (37°C) (Fig. 4 *A*). Thus, even though it contains an alcohol functional group, α-tocopherol is neither a competing substrate nor a competitive inhibitor of LCAT. Moreover, α-tocopherol does not modify the physical properties of the model HDLs enough to affect LCAT activity by means of other mechanisms.

The effect of the gel-to-liquid crystalline phase transition on the microenvironment of α-tocopherol in ApoA-II/DMPC complexes was examined by the fluorescence properties of the chromanol ring and the chemical reactivity of α-tocopherol with DPPH (Bellemare and Fragata, 1980). According to both methods, the microenvironment for the chromanol ring is different in the gel and liquid-crystalline phases and the chromanol ring is located at the lipid-water interface (Ekiel et al., 1988). Using environmentally sensitive fluorescence lipid probes and the intrinsic spectral properties of tyrosine residues, we previously demonstrated that the lipid-water interface of DMPC and the lipid-protein interface of ApoA-II/DMPC both change polarity during a phase transition (Massey et al., 1985c; Pownall et al., 1978). Similarly, the physical state of the phospholipid in the complex affects the microenvironment and chemical reactivity of the chromanol α-tocopherol.

DMPC in model HDLs consists of a "boundary" lipid, which is directly associated with protein and does not undergo a gel-to-liquid crystalline phase transition, and a "bulk" lipid, which is not in contact with protein and has properties similar to those of DMPC in liposomes. Previous physical studies (Massey et al., 1985b; Nakagaki et al., 1985; Silvius et al., 1984) suggest a model in which cholesterol is excluded from the boundary lipid because of poor packing of the rigid sterol moiety among the lipids and the irregular protein surface. Phosphatidylcholine (PC), which has a great deal of flexibility in its acyl chains, can be readily accommodated by the protein surface. Like PC, α-tocopherol is more flexible than cholesterol (Ekiel et al., 1988). The concentration-dependent increase in fluorescence polarization and decreased acyl chain mobility suggest that within a fluid-lipid matrix, α-tocopherol is distributed throughout both the bulk and boundary lipid. The lack of effect of α-tocopherol on polarization below T_C suggests that α-tocopherol behaves as an impurity that is "frozen out" of the DMPC molecules that undergo a phase transition into the boundary region. This would be consistent with the effect of α-tocopherol in saturated PC bilayers, where it is preferentially excluded from the gel-phase lipids (Villalain et al., 1986; Ortiz et al., 1987; Massey et al., 1982).

Other studies have suggested that the spontaneous association of apolipoproteins with DMPC is a function of surface permeability, that association occurs by means of the insertion of ApoA-I into preexisting defects in the lipid surface, and that the rate of association is a direct function of the number of defects in the lipid surface. According to this model, the defects form at the boundary between two different lipid phases. In pure DMPC at its transition temperature, defects occur between gel and liquid-crystalline phase lipids (Pownall et al., 1978; Reijngould and Phillips, 1984); in the presence of cholesterol, defects form between domains of pure DMPC and those that are cholesterol rich (Pownall et al., 1979, 1987; Estep et al., 1978; Genz et al., 1986). The number of defects is maximal at the gel-liquid crystalline transition of the lipid (Pownall et al., 1978; Reijngould and Phillips, 1984) and is increased by the addition of up to 12 mol % cholesterol. Consequently, the rate of association is highest at the transition temperature of DMPC that contains 12 mol % cholesterol (Pownall et al., 1979). The effects of α-tocopherol on the properties of DMPC and the kinetics of association of ApoA-I and DMPC are distinct from those of cholesterol. As with DMPC and cholesterol-DMPC mixtures, the maximal rate of association with ApoA-I occurred at the lipid transition temperature. In contrast to cholesterol, the addition of α-tocopherol lowered rather than raised the transition temperature of DMPC (Massey et al., 1982). Thus, as the amount of α-tocopherol increases the temperature at which the maximal rate of association occurs decreases. In addition, α-tocopherol inhibits the association of ApoA-I and DMPC in a dose-dependent manner (Figs. 1 and 2 *A*).

The inhibition could be either kinetically or thermodynamically controlled. Several studies have shown that the composition and properties of model HDLs formed by spontaneous association of ApoA-I and DMPC are identical to those formed by combining ApoA-I and lipids with a detergent followed by removal of the detergent (Massey et al., 1985b; Matz and Jonas, 1982; Pownall et al., 1982). Detergent removal methods "catalyze" the assembly of lipids and proteins under conditions in which spontaneous assembly is slow or does not occur, for example, above or below the transition temperature or in the presence of high cholesterol concentrations. As the cholate dialysis procedure leads to quantitative incorporation of ApoA-I, DMPC, and α-tocopherol into homogeneous model HDLs, their formation in the absence of a detergent must be kinetically controlled. The decreased rate of association of ApoA-I with DMPC observed with increasing α-tocopherol is probably due to a decrease in the number of defects in the lipid surface. Defects in lipid bilayers permit entrapped small molecules to escape. Previous experiments (Halks-Miller et al., 1985) have shown that addition of α-tocopherol to phospholipid liposomes decreases the rate at which a trapped dye escapes, suggesting that α-tocopherol decreases the size and/or number of surface defects. Our data are consistent with this observation and, according to the model in which the rate association of ApoA-I with DMPC is a function of the number of surface defects, the decrease in the rate of association seen with increasing α-tocopherol would have been predicted.

It is interesting to compare the structures of α-tocopherol and cholesterol and ask why one would increase the number of defects and the other would not. According to the physicochemical studies, cholesterol induces phase separation in lipid bilayers, whereas α-tocopherol does not (Lucy, 1978;

Lai et al., 1985; Wasall et al., 1986; Villalain et al., 1986; Ortiz et al., 1987; Massey et al., 1982; Ekiel et al., 1988). Both cholesterol and α-tocopherol contain a hydroxyl group that anchors that part of the molecule at the lipid-water interface. The remainder of the molecule is inserted into the hydrocarbon region of the lipid. Cholesterol is a flat, rigid molecule with a shape that is roughly that of a truncated cone in which the smaller end resides at the lipid-water interface (Israelachvili et al., 1980). Cholesterol does not pack into the DMPC-ApoA-I matrix efficiently because of its shape and its inability to flex into a more accommodating shape. As a consequence of the poor packing, cholesterol creates defects in the surface. On the other hand, α-tocopherol is nearly cylindrical and contains a long, flexible phytyl chain that can be rotated or bent to pack closely with the phospholipid acyl chains. As the difference in the shapes of cholesterol and α-tocopherol are small, it is likely that the high degree of flexibility within the phytyl chain permits α-tocopherol to insert into defects in the DMPC bilayer, reducing the number and size of defects. As the rate association of ApoA-I with DMPC is a function of the number of defects, incorporation of α-tocopherol into the bilayer is associated with a lower rate of DMPC-ApoA-I association.

Cholesterol alters physiological activities directly by a molecular interaction or indirectly by altering membrane fluidity (Gimpl et al., 1997) and polarity (Massey et al., 1985c). In the presence of high HDL levels, HDL and not LDL is the major plasma carrier of α-tocopherol (Traber et al., 1992; Clevidence and Lehman, 1989). However, even in subjects using dietary supplements containing α-tocopherol, plasma α-tocopherol concentrations are much lower than those of cholesterol and vary from 5.4 to 24.0 μg/ml. These respective levels correspond to $\sim$0.5 and 3 mol % α-tocopherol in HDLs. According to our data, these amounts of α-tocopherol in a cell membrane or plasma lipoprotein are too low to alter any activity that is a function of fluidity or of the polarity of the lipid surface. However, according to our kinetic data (Fig. 8), these α-tocopherol levels could be an important determinant of the rates of association of apolipoproteins with HDLs, so that the plasma concentration of unbound ApoA-I may be increased in response to high plasma α-tocopherol levels.

Although α-tocopherol is antioxidant in a variety of tissues and fluids, current evidence suggests that α-tocopherol exerts its other physiologic effects in cells, not plasma lipoproteins (Devaraj and Jialal, 1998). Within cell membranes, α-tocopherol could form α-tocopherol-rich domains (Sánchez-Migallón et al., 1996) and co-localize with other membrane-bound molecules that regulate cellular activities. These activities include inhibition of platelet aggregation (Freedman et al., 1996), inhibition of protein kinase C (Devaraj and Jialal, 1998; Freedman et al., 1996), and modulation of cytosolic phospholipase A_2 activity (Tran et al., 1996). Our data provide several possible mechanisms by which α-tocopherol could alter lipid structure within a microenvironment. These mechanisms include filling in defects within lipids or between lipids and proteins and reducing the local fluidity. However, many of these effects are small or require large amounts of α-tocopherol. Therefore, it is likely that many of the effects of α-tocopherol occur through specific interactions with cellular proteins.

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