INTERACTION OF A SERUM APO-LIPOPROTEIN WITH ORDERED AND FLUID LIPID BILAYERS. CORRELATION BETWEEN LIPID AND PROTEIN STRUCTURE

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1. Introduction

Many aspects of lipid-protein interaction are poorly understood. For example, little is known about (1) the impact of the lipid structure on the conformation of membrane proteins and (2) the effects of membrane proteins on lipid structure. We have used lipid phase transitions to investigate these correlations for a lipid-binding apo-lipoprotein, apo-Lp-ala. This protein can be isolated in water-soluble form from the human plasma very low density lipoproteins [1]; it contains 79 amino acids of known sequence [2] with three tryptophane residues in the carboxyl-terminal half (positions 42, 54 and 65). In aqueous environment the protein has a disordered conformation [1,3].

So far the interaction of apo-Lp-ala with lipids has been studied using egg lecithin; this lipid induces a marked increase in the helix content from about 22% to 54% at lipid saturation [1,3] which is reached for a lipid: protein molar ratio $m^* \approx 54$. Segrest et al. [4] proposed that apo-Lp-ala can form an amphiphatic helix the ion pairs of which associate with the zwitterionic polar groups of lecithin.

In the present paper we have used different synthetic lipids (mainly lecithins) with characteristic transition temperatures T_t. Therefore it was possible to study the interaction of apo-Lp-ala with ordered ($T < T_t$) and fluid ($T > T_t$) lipid layers. The protein conformation was monitored by circular dichroism (CD) and intrinsic fluorescence measurements; the influence of apo-Lp-ala on the lipid structure was judged from phase transition curves and light scattering measurements.

2. Materials and methods

2.1. *Lipids*

Dimyristoyl-, dipalmitoyl-, and distearoyl-lecithin (C14-lec., C16-lec., C18-lec.) were obtained from Fluka: in aqueous dispersions these lipids have transition temperatures $T_t = 23°C$, 41°C and 58°C, respectively [5]. Dimyristoyl-phosphatidic acid (C14-PA) and -methyl phosphatidic acid (C14-MPA) were gifts of Dr Eibl [6]. Lipid dispersions were prepared by sonication for about 3 min at $T > T_t$. *N*-Bromosuccinimide (NBS) was obtained from Merck. Apo-Lp-ala 1 was prepared according to Brown et al. [1].

2.2. *Fluorescence spectra*

Fluorescence spectra were measured on a FICA fluorimeter corrected for instrumental spectral responses. When necessary the spectra were corrected for inner filter and light scattering effects due to the added lipid.

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2.3. *Lipid phase transitions*

Lipid phase transitions were monitored by 90°-light scattering (350 nm) or fluorescence measurements using ANS or NPN as probes [7].

2.4. *Circular dichroism*

Circular dichroism was measured on a Cary 60 equipped with a CD accessory. The mean residue ellipticity θ_{222} in units of deg. cm^2 dmole^{-1} was calculated from the ellipticity angle at 222 nm using a mean residue weight of 111 [3].

3. Results and discussion

3.1. *Interaction of apo-Lp-ala with ordered and fluid lipid bilayers studied by CD and intrinsic fluorescence measurements.*

CD interaction of apo-Lp-ala with synthetic lecithins induces a change from a typical random-coil spectrum (trough at 204 nm) to a spectrum with prominent negative troughs at 222 and 208 nm. This is the case for $T > T_t$ and $T < T_t$. Below T_t the spectra at lipid saturation resemble those of α-helical polypeptides [8,9]; they exhibit less fine structure at $T > T_t$. The maximal values of θ_{222} and of the lipid: protein molar ratio, m*, required for saturation are listed in table 1 for different lecithins at different temperatures. For a given temperature below T_t the different lecithins induce about the same maximal ellipticity; smaller values are observed for $T > T_t$.

Below T_t more lipid is required to induce maximal helicity. in both cases ($T \gtrless T_t$) m* increases with increasing chain length. As will be shown below the rather small value of m* for C14-lec. (m*≈ 20 at $T > T_t$) reflects the existence of a qualitatively different lipid-protein structure.

A comparable increase in negative ellipticity is induced by several other lipids with different polar groups (e.g. C14-PA; C14-MPA, $\theta_{222}^{max} = -1.25 \times 10^{-4}$, m*≈20 at 45°C) and also by SDS, m*≈100. Only cephalin which forms relatively rigid bilayers has a smaller effect ($\theta_{222}^{max} = -0.8 \times 10^{-4}$ for C14-ceph. at 20°C). Therefore the induction of α-helicity in apo-Lp-ala seems to result mainly from interactions of the protein (or part of it) with the lipid hydrocarbon chains. Specific interactions with the lipid polar groups seem not to be important. This compares well with the fact that apo-Lp-ala assumes a highly helical conformation also in 2-chloroethanol [3].

3.1.1 Intrinsic fluorescence

The emission spectrum of apo-Lp-ala in water (λ_{exc} = 290 nm pH 8.5, 20°C) is similar to that of free tryptophane [10,11] with λ_{em}^{max} = 348 nm (fig.1a, curve 0) and a quantum yield Q ≈ 0.065 per tryptophane.

Increasing temperature reduces the quantum yield (cf. fig.3b) without markedly changing the spectral shape, as in the case of free tryptophane [12]. Titration of apo-Lp-ala with lecithin dispersions causes (1) a blue shift of λ_{em}^{max} to about 330 nm (cf.

Table 1
Titration of the circular dichroism of apo-Lp-ala with synthetic lecithins at $T \gtrless T_t$

	$\theta_{222}^{max} \times 10^{-4}$ [deg cm^2 dmole^{-1}]		m* = Lipid: protein molar ratio at saturation	
	$T < T_t$, (15°C)	$T > T_t$, (60°C)	$T < T_t$	$T > T_t$
C14-lec.	−1.5 ± 0.1	−0.9 ± 0.1	55 ± 5, 15°C	20 ± 5, 30°C
C16-lec.	−1.5 ± 0.1	−1.0 ± 0.1	240 ± 20, 20°C	200 ± 20, 55°C
C18-lec.	−1.5 ± 0.1	−0.9 ± 0.1	290 ± 20, 20°C	220 ± 20, 60°C

Initial protein concentration $c_p = 4 \times 10^{-6}$ M, 0.1 M K_2HPO_4, pH 8.5. The mean residue ellipticity θ_{222} shows an initial linear increase with increasing lipid content and reaches a saturation value θ_{222}^{max} for a lipid protein molar ratio $\gtrsim$ m*. In the absence of lipid $\theta_{222} = -0.4 \times 10^{-4}$ with little temperature dependence between 10 and 60°C. The given values of θ_{222}^{max} were measured under identical instrumental conditions at m = 650 in 0.1 M K_2HPO_4 and pH 8.5.

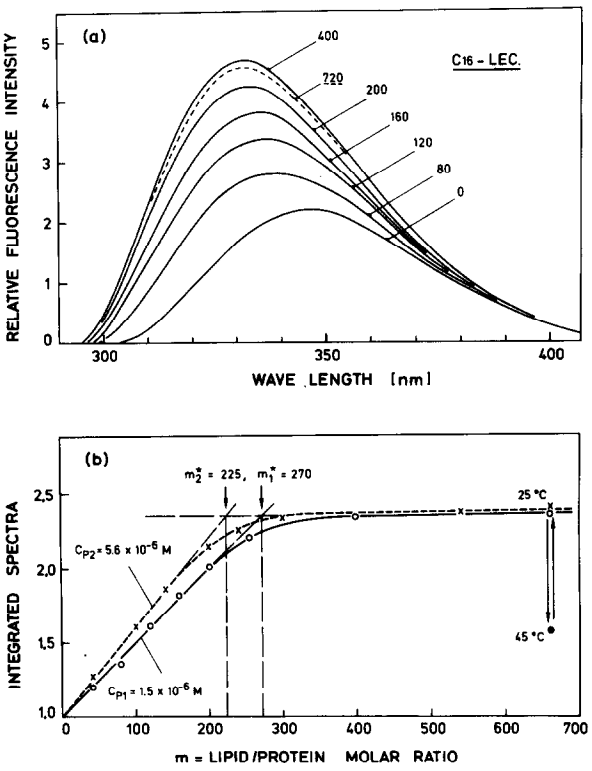

Fig.1. Effect of C16-lec. on the intrinsic fluorescence of apo-Lp-ala at 25°C ($T < T_t$), pH 8.5, 0.1 M K_2HPO_4. Exc. at 290 nm. a) Spectral changes with increasing lipid: protein molar ratio m. Initial protein concentration 1.5×10^{-6} M. The spectra are corrected for instrumental wavelength responses and light scattering, however, not for dilution. For m = 400 the dilution factor would be 1.12. b) Relative increase in quantum yield with increasing lipid content for two different protein concentrations c_{p1} and c_{p2}. Corrected for dilution. Heating of the sample with m = 660 to 45°C causes a reversible decrease in quantum yield.

[3]) and (2) a pronounced *increase* in quantum yield Q. After an initial linear region Q reaches a plateau for $m \gtrsim m^*$ (cf. fig.1b). Similar results were obtained with the other lecithins for $T \gtrsim T_t$ (cf. table 2) The spectral changes in fig. 1a can be simulated by lowering the dielectric constant ϵ, of the solvent for apo-Lp-ala by the addition of dioxane. A twofold increase in Q is observed for $\epsilon \approx 50$ suggesting that after interaction with lecithins one or more of the tryptophane residues are still in a relatively polar environment. Similar experiments with C14-PA, C14-MPA and SDS indicate that the increase in Q shows a marked dependence on the structure of the lipid polar groups. Whereas all of these lipids induce a blue-shift of λ_{em}^{max} to about 330 nm the quantum yield increases only by factors 1.04, 1.22 and 1.33 for SDS, C14-PA and C14-MPA, respectively, compared to the twofold increase for the lecithins. This supports the assumption that the tryptophane-containing part of apo-Lp-ala is located in the region of the lipid polar groups.

3.2. *Binding constants*

If the protein does not destroy the lipid bilayer we may assume in the simplest case that independent 'binding sites' exist on (or within) the lipid layers. Then we may define a binding constant K_B (= $1/K_D$) and a number of lipid molecules, m_0^*, providing one binding site. These parameters can, in principle, be estimated from fluorescence (or CD) titration curves as shown in fig.1b. The initial slope of these curves and the value of m* exhibit a small, but definite dependenc on the protein concentration c_p. One can easily show that in the above model the value of m* is given by

Table 2
Titration of the intrinsic fluorescence of apo-Lp-ala with synthetic lecithins at $T \gtrsim T_t$

	Increase in quantum yield Q_{max}/Qo		λ_{em}^{max} [nm] at lipid saturation	m* = Lipid: protein molar ratio at saturation	
	$T < T_t$, (15°C)	$T > T_t$ (60°C)	$T \gtrsim T_t$	$T < T_t$	$T > T_t$
C14-lec.	2.15 ± 0.2	2.5 ± 0.2	332 ± 2	60 ± 10, 15°C	20 ± 5, 30°C
C16-lec.	2.20 ± 0.2	2.2 ± 0.2	332 ± 2	280 ± 20, 30°C	160 ± 20, 45°C
C18-lec	2.30 ± 0.2	1.8 ± 0.2	332 ± 2	320 ± 20, 20°C	–

Exc.: 290 nm, 2×10^{-6} M protein, 0.1 M K_2HPO_4, 0.5 M NaCl, pH 8.5. Q_{max} and Q_o denote the quantum yields in the absence of lipid and at lipid saturation, respectively. The value of Q_o at 60°C is about one third of that at 15°C. m* is defined in fig.1b.

$m^* = m_0^* (1 + K_D/c_p)$. Therefore m* is expected to decrease with increasing c_p (unless $K_D \ll c_p$) and the actually observed values represent upper limits for m_0^*. The values of K_D and m_0^* can be estimated from two titrations with different protein concentrations yielding m_1^* and m_2^*. Using the results of fig.1b we arrive at $K_B \approx 2.4 \times 10^6$ moles/litre (ΔG = 8.8 kcal/mole) and $m_0^* = 220$ for C16-lec. at 25°C ($T < T_t$). Similar experiments with C18-lec. at 30°C yield $K_B \approx 1.7 \times 10^6$ (ΔG = 8.6 kcal/mole) and $m_0^* = 280$. The values for C14-lec. at 30°C ($T > T_t$) are, however, markedly different ($K_B = 0.15 \times 10^6$ and $m_0^* = 10$) and as will be shown below the assumption of independent binding sites on intact lipid layers is certainly not valid in this case.

3.3. *Accessibility of the tryptophane residues*

The tryptophane residues of apo-Lp-ala in water are readily accessible to fluorescence quenching by NO_3^- and oxidation by NBS: (1) the quenching constant for NO_3^- is 18 $mole^{-1}$ at 20°C and pH 8 compared to 20 $mole^{-1}$ for free tryptophane [13]; (2) the addition of 2 molecules of NBS per tryptophane at pH 8 completely abolishes the tryptophane fluorescence. In contrast, the tryptophane residues are largely protected in the presence of excess lecithin ($m \gg m^*$). For C16-lec. at 20°C and pH 8 the quenching constant for NO_3^- is only 7.2 $Mole^{-1}$ and treatment with NBS decreases the quantum yield to only 70% of the initial value. This protection is not observed for lipids with smaller polar groups, for example C14-MPA or SDS, although these lipids induce about the same α-helix content as the lecithins; in these cases oxidation occurs with practically the same efficiency as for free apo-Lp-ala, without marked effect on the helix content.

3.4. *Effect of apo-Lp-ala on the lipid structure*

The influence of apo-Lp-ala on the lipid structure can be judged by its effect on the lipid phase transition (temperature width and amplitude of the transition); changes in the vesicle size can be monitored by 90°-light scattering.

For C16-lec. and C18-lec. the protein practically does not affect the transition curves and the light scattering, indicating that the lipid structure is not markedly disturbed. In contrast, for C14-lec. at 30°C ($T > T_t$) apo-Lp-ala causes a marked decrease in light scattering (cf. fig.2a). This suggests that apo-Lp-ala breaks down the original liposomes into smaller 'complexes'. Interestingly, this process does not take place below T_t. For example at 10°C apo-Lp-ala decreases the scattering amplitude by not more than 5–10% (fig.2a). When the temperature is raised, however, one observes a large irreversible decrease in scattering amplitude at about T_t = 23°C (Fig.2b). Sub-

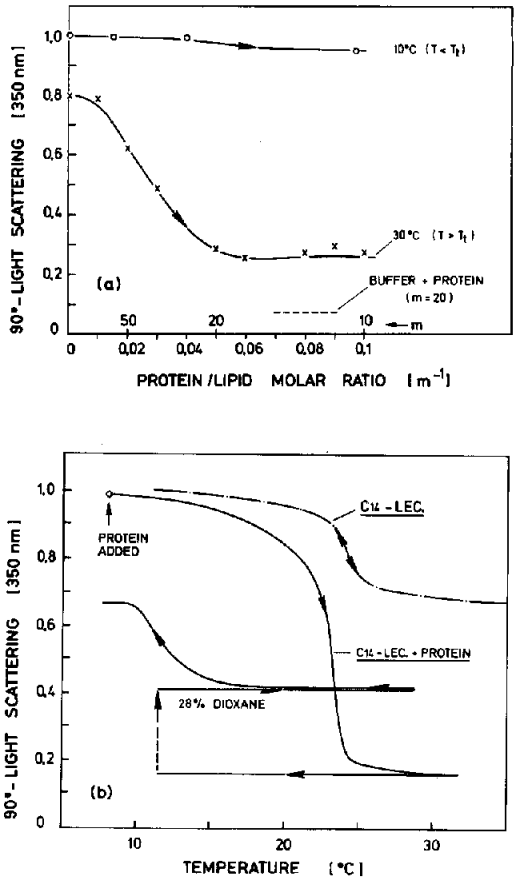

Fig. 2. Effect of apo-Lp-ala on the 90°-light scattering of C14-lec. dispersions. 0.5×10^{-4} M lipid, 0.1 M K_2HPO_4, 0.5 M NaCl, pH 8. a) At 30°C ($T > T_t$) apo-Lp-ala causes a large decrease in the scattering amplitude. At 10°C the protein has practically no effect. Intensities are normalized to the value at 10°C and corrected for dilution. b) Apo-Lp-ala was added to a dispersion of C14-lec. at 10°C to yield a lipid: protein molar ratio m=20. Subsequent heating leads to a large irreversible decrease in scattering amplitude at about 23°C and to a disappearance of the lipid phase transition. Addition of dioxane (28% v/v) at 12°C increases the light scattering and re-establishes the transition at about 12°C. Upper curve: light-scattering indication of the phase transition of pure C14-lec.

sequent temperature scans show a complete absence of the lipid phase transition. Apparently the protein breaks down the lipid layers into smaller 'complexes' as soon as the lipid enters the fluid state and the lipid is reorganized in such a way that phase transitions are no longer possible. Electron micrographs of apo-Lp-ala incubated with C14-lec. at $T > T_t$ show, in fact, small particles with a diameter of about 80–100 Å compared to the original liposomes with a diameter of about 500–2000 Å.

As indicated in fig.2b the protein can be 're-extracted' from these complexes and the lipid phase transition re-established by the addition of 28% (v/v) dioxane. The new value of $T_t \approx 12°C$ is that of C14-lec. in 28% dioxane in the absence of apo-Lp-ala. As one would expect from these experiments the phase transition of C14-lec. is markedly affected even by small additions of apo-Lp-ala: the transition curves (measured using ANS) become broader, the amplitude [7] decreases, and for higher protein contents ($m < 20$) the transition disappears.

3.5. *Effect of lipid phase transitions on apo-Lp-ala*

Reversible effects of lipid phase transitions on the protein can be expected only for those lipids whose bilayer structure is maintained. As shown in fig.3a for C18-lec. and $m > m_o^*$ both the negative ellipticity θ_{222} and the tryptophane fluorescence of apo-Lp-ala exhibit abrupt changes at the ordered-fluid phase transition. Similar results were obtained with C16-lec. In addition the ordered → fluid phase transition impairs the fine structure of the CD spectrum.

These observations indicate that in the fluid lipid state ($T > T_t$) the protein has a somewhat lower helix content and its tryptophane-containing part is more exposed to the water. Fig.3b shows the effect of increasing amounts of C16-lec. on the temperature characteristics of the tryptophane fluorescence.

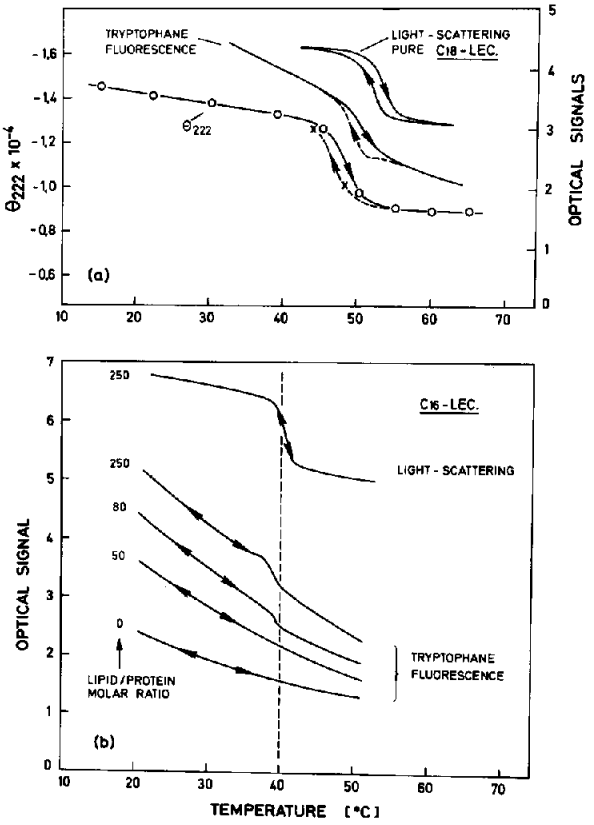

Fig.3. Effect of the lipid phase transition on the negative ellipticity θ_{222} and on the tryptophane fluorescence at 350 nm (exc. 290 nm) of apo-Lp-ala. a) Temperature scans in the presence of 10^{-3} M C18-lec.; Lipid: protein ratio m = 350; pH 8.5, 0.1 M K_2HPO_4. Upper curve: 90°-light scattering change of a pure C18-lec. dispersion. b) Temperature dependence of the tryptophane fluorescence for increasing contents of C16-lec.; 1.2×10^{-6} M protein, pH 8.5, 0.1 M K_2HPO_4. Upper curve: temperature scan of the 90°-light scattering for m = 250.

4. Conclusions

The interaction of apo-Lp-ala with synthetic lecithins depends in a complex way on the physical state of the lipids ($T \gtrless T_t$), on the hydrocarbon chain length and on the pretreatment of the samples. Judged by the maximally inducible spectroscopic changes the protein can interact readily with ordered and fluid lipid structures. However, in the fluid state ($T > T_t$) less lipid molecules, m_o^*, are required per protein to induce the maximal effects: m_o^* ($T > T_t$) < m_o^* ($T < T_t$), Furthermore the resulting structures depend on the balance between bilayer stability (chain length, $T \gtrless T_t$) and the 'structural demand' of the protein.

1) With the long-chain lecithins C16-lec. and C18-lec the protein does not significantly alter the bilayer structure (no effect on the lipid phase transition);

$m_o^* \approx 300$ for $T < T_t$ and $m_o^* \approx 200$ $T > T_t$, $K_B \approx 2.0 \times 10^6$ litres/mole at 30°C.

2) In contrast with C14-lec. bilayers in the fluid state ($T > T_t$) the protein causes a profound and irreversible structural reorganization (formation of small lipid-protein 'complexes', disappearance of the lipid phase transition). These changes do, however, not take place below T_t. $m_o^* \approx 10$ for $T > T_t$, and $m_o^* \approx 50$ for $T < T_t$. Therefore, when mixtures of C14-lec. and apo-Lp-ala are prepared below T_t and heated to $T > T_t$ these changes occur irreversibly at $T \approx T_t$, when the lipid enters the fluid state.

An irreversible increase in interaction at the order→fluid transition may be observed also with C16- or C18-lec. under certain conditions. This is the case when lipid-protein mixtures are prepared below T_t and $m < m^*$ ($T < T_t$). Now, because m^* ($T > T_t$) $< m^*$ ($T > T_t$) the interaction increases when the samples are heated above T_t.

The situation is simpler in the presence of excess C16-lec. or C18-lec. ($m \gg m_o^*$). Then the phase transition leads to reversible changes in the spectral parameters of apo-Lp-ala. In view of the fact that lipid phase transitions can be induced also at constant temperature by alterations in pH, ionic strength and by divalent cations [6] this suggests a new possibility for the regulation of the conformation (and perhaps function) of certain membrane proteins.

As to the resulting protein conformation there is good evidence that the tryptophane residues are located in the lipid–water interface: they can readily be oxidized by NBS when the lipid polar groups are small (C14-MPA, PA, SDS), whereas they are partly protected in the case of lecithin. Quenching experiments with NO_3^- and differences in quantum yield support this view. In contrast, the induction of α-helicity is practically independent of the lipid polar groups and must result mainly from interactions of (part of) the protein with the lipid hydrocarbon chains. Specific interactions with the zwitterionic polar groups of lecithin [4] cannot be essential for helix formation. We propose that helix-formation takes place in the amino-half of the protein within the lipid hydrocarbon moiety whereas the carboxyl-half remains more or less exposed to the water. Any external functions of the protein would be expected to reside in the latter portion of the protein. Since the exact conformation depends on several factors as shown above, it is not possible to design a generally-valid model for the lipid-attached protein. The major structural correlation are: 1) interaction of apo-Lp-ala with lipids causes a marked increase in helix content; 2) the exact conformation of the carboxyl-half of the protein depends on the structure of the lipid polar groups; 3) when bilayer stability is small (C14-lec., $T > T_t$) it is the protein which determines the resulting structure; 4) when bilayer stability is high (C16-lec., C18-lec.) one observes reversible changes of the spectra parameters of the protein at the lipid phase transition.

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