Changes in the physicochemical properties of transferrin upon chemical derivatization with ceramides

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Abstract: The interaction of native and derivatized transferrin with lecithin, cholesterol and their mixtures was studied using monomolecular layers of phospholipids. Moreover, the thermodynamic parameters corresponding to these interactions: the mixing excess free energy, interaction energy, and interaction parameter were also calculated.

The ability of these two proteins to insert into monolayers was studied working at constant area.

The formation of aggregates or secondary structures in solution was determined by the fluorescence and polarization values of ANS (anilinonaphthalene sulfonate), measured at different protein/ANS relationships.

Key words: Ceramides – liposomes – compression isotherms – surface activity – transferrin

Abbreviations: Transferrin (TF); derivatized transferrin (DTF); lecithin (PC); cholesterol (CHOL); ceramides (CER); ANS (anilinonaphthalene sulfonate); trinitro benzenesulfonate method (TNBS)

Introduction

Recently, transferrin has been applied to the treatment of cancer processes associated with doxorrubicin [1]. It seems that this protein can be used to target citostatics to certain membranes or organs.

The use of proteins as carriers for small molecules has long been established, but recently the introduction of liposomes as carriers has allowed the possibility to combine target and carrier in the same preparation [2, 3].

One approach to link a protein to the surface of a liposome is to derivatize the protein to render it more hydrophobic, followed by incubation with drug-loaded liposomes [4, 5]. This process seems easier to carry out than the direct linkage of the protein to the liposome, but can partially destroy or solubilize vesicles if the hydrophobicity of the resultant derivatized protein is too high.

Moreover, in some cases this hydrophobic derivatization can be so strong that it induces conformational changes in the protein so that active centers can be better exposed or hidden. In some cases derivatization can also promote the formation of some type of micelles or aggregates that could entrap by themselves small molecules without the presence of lipids. All these speculations require a physicochemical study to know how hydrophobic modifications change the interaction of proteins with lipids.

In the present paper, we describe the study of these interactions applied to transferrin, using monomolecular layers of phospholipids as membrane model. We have also determined the surface hydrophobicity of both native and derivatized protein by means of ANS-fluorescent marker.

Experimental

Chemicals

Transferrin was supplied by Behring. Lecithin (PC) Merck, purified as described by Singleton

[6] cholesterol (CHOL) and ceramides (CER) were from Sigma. Sodium cyanoborohydride was from Sigma and sodium periodate from Merck. Borate-buffer for derivatization of transferrin was prepared from 20 mM borate and 120 mM NaCl adjusted to pH: 8.4. Fluorescence studies were carried out with the protein dissolved in sodium acetate buffer, pH: 7.4 (TAC).

Methods

Derivatization of transferrin: The derivatization of transferrin was carried out as described by Heat [7]. Details are given in [8]. Briefly: Ceramides were oxidized at pH: 8.4 (borate buffer) with sodium periodate 60 mM, and after removal of the excess of reactant by dialysis overnight against the same buffer, were incubated with transferrin for 2 h at room temperature. The reaction mixture was treated with 2 M sodium cianoborohydride for 16 h at 4 °C. The excess of reducing reactant was removed overnight by dialysis against water. The level of derivatization process was determined by measuring the percentage of amino groups before and after reaction, by the trinitro benzenesulfonate method [9] (TNBS), and aminoacid analysis.

Physicochemical studies: Two sets of experiments were carried out to determine the surface activity of native and derivatized TF.

a) *Compression isotherms*: were performed on a Langmuir film balance equipped with a Wilhelmy platinum plate, as described by Verger et al. [10]. The output of pressure pickup (Beckmann Sartorius LM 600 microbalance) was calibrated by recording the well known isotherm of stearic acid, which is characterized by a sharp phase transition at 25 mN/m on pure water at 20 °C. The teflon trough (surface area 495 cm^2, volume 330 mL) was regularly cleaned with hot chronic acid; moreover, before each experiment it was washed with ethanol and rinsed with twice distilled water.

Lipid-films were spread on transferrin (TF) and derivatized transferrin (DTF) solutions at pH: 7.4, using a Hamilton microsyringe, and at least 10 min allowed for solvent evaporation. Films were compressed continuously at a rate of 4.2 cm/min; changes in the compression rate did not alter the shape of isotherms. All the isotherms were run at least three times in the direction of increasing pressure with freshly prepared films. The accuracy of the system under the conditions in which the bulk of the reported measurements were made was $\pm$ 0.5 mN/m for surface pressure. All measurements were carried out at 21 $\pm$ 1 °C.

b) *Penetration kinetics*: A small circular trough of 70 mL capacity was used to determine the pressure increases produced after injection of the protein under a phospholipid monolayer, spread at different surface pressures.

In this way it was possible to measure the penetration kinetics of the protein into the monolayer at constant area. Moreover, when the same experiments were carried out without monolayer, the pressure increases measured, allowed us to calculate the superficial excess of the solute Γ, applying the Gibbs equation.

Results and discussion

Derivatization of transferrin

Native TF was derivatized with ceramides using a weight relationship: (TF/CER: 20/6). This value has been previously determined to give the highest efficiency [8]. The level of substitution was quantified by the TNBS method, using TNBS-alanine as reference, and aminoacid analysis taking the lysine content as standard. By both methods the substitution values calculated ranged around 8%. As transferrin contains 59 amino groups per molecule, this level of substitution represents 4–5 residues of ceramides per transferrin molecule.

Physicochemical studies

a) Surface activity of native and derivatized transferrin: The presence of TF and DTF in an aqueous subphase modifies the surface tension of the water, giving a measurable change in the surface pressure of these solutions. The pressure increased very quickly after injection, but the process was left for 1 h to be sure that the system had reached equilibrium. Pressure increases were dependent on the concentration of the protein in the subphase till saturation was observed up to 30 nM (Fig. 1). The surface excess in absence of

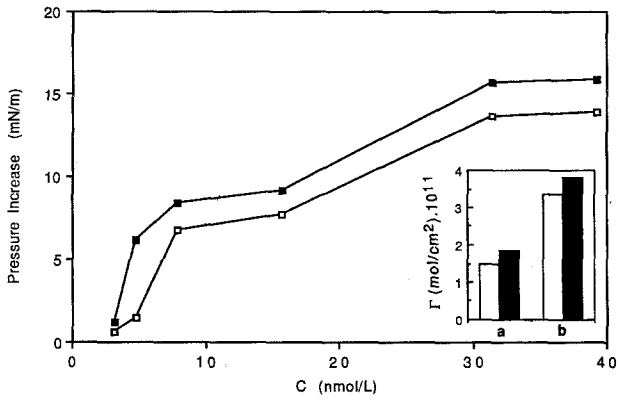

Fig. 1. Surface activity of native –□– and derivatized –■– transferrin as a function of the protein concentration in the subphase. Insert: Superficial excess (mol/cm$^2 \cdot 10^{11}$) of TF □ and DTF ■, at a) 7.86 and b) 39.29 nmol/l

a monolayer was calculated applying the Gibbs equation (1) and is given in the inset of Fig. 1.

$$\Gamma = -\frac{1}{RT}\frac{\Delta P}{\Delta \ln C}, \qquad (1)$$

where R is 8.3×10^{-7} mN/°K·mol, T is 294 °K, ΔP is the pressure increase, and C is the initial concentration of the protein in the subphase.

Differences between TF and DTF are not very strong, reflecting the small level of substitution of the protein.

b) Interaction of transferrin with mixed monolayers of PC/CHOL: As the most usual components of liposomes are PC/CHOL (1:1), we considered it interesting to study the interaction of this protein with a mixture of both lipids. Nevertheless, first of all the miscibility of PC and CHOL, was determined, being these lipids spread on PBS. Purified egg PC was chosen because it is the main component in liposomes formulations and biological membranes. The lack of homogeneity in the PC alkyl chains has a low influence in the miscibility with CHOL as has already been shown by Shah [11, 12].

Moreover, mixed monolayers PC/CHOL of several molar compositions were spread on subphases containing TF or DTF, and the values of area/molecule calculated from the compresion isotherms. As reference, the same experiments were carried out with aqueous solutions of phosphate buffer (PBS). As an example, the compression isotherms of PC, CHOL, and PC/CHOL, spread on DTF are given in Fig. 2.

The presence of protein in the subphase has a small influence in the miscibility pattern of PC/CHOL. In general, isotherms were slightly expanded with reference to PBS subphases, especially for monolayers rich in cholesterol. Nevertheless, the presence of the protein has no effect on the collapse pressure, nor in the compressibility of the monolayers. This behavior is illustrated in Fig. 3.

The small differences described were quantified applying the following equations. The values of ΔG_m^{ex} have been calculated by applying Eq. (2). This equation has been obtained by following the

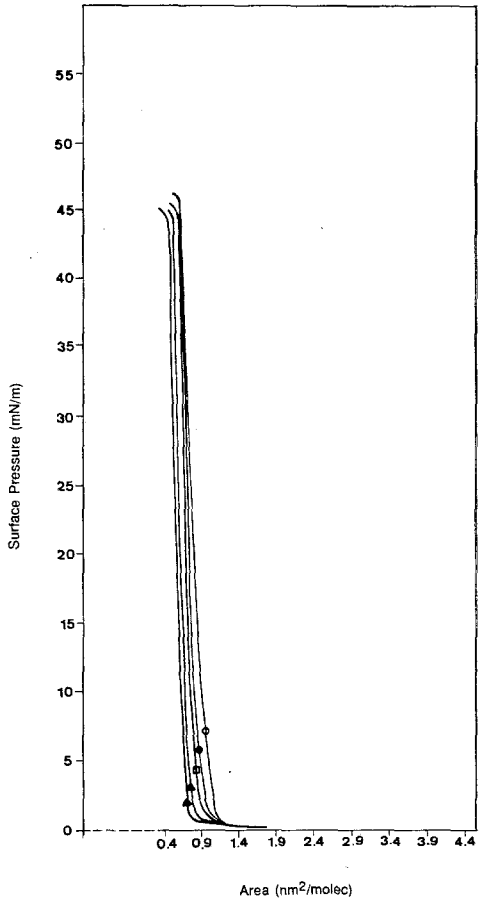

Fig. 2. Compression isotherms of PC, CHOL and mixed monolayers of PC/CHOL. PC –○–, CHOL –△–, PC/CHOL 0.8:0.2–●–, PC/CHOL 0.5:0.5 –□–, PC/CHOL 0.2:0.8 –▲–, spread on DTF subphase. Concentration of protein: $1.393 \cdot 10^{-11}$ M

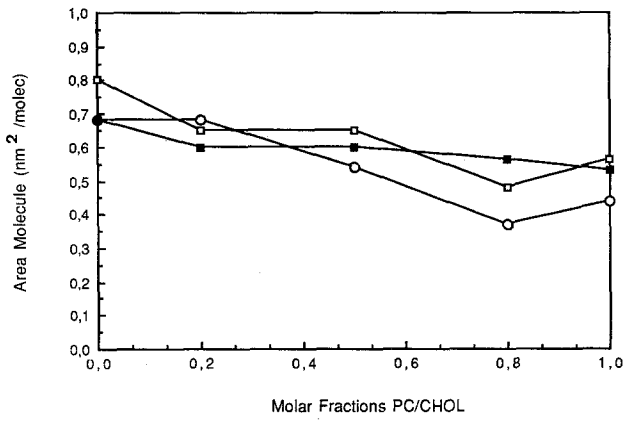

Fig. 3. Mean area molecule in mixed monolayers of PC/CHOL, measured at 20 mN/m. Subphases: PBS -○-, TF -□- and DTF -■-

Goodrich [13] and Pagano and Gershfeld [14] approaches. Numerical values were calculated according to the mathematical method of Simpson. The region below the lowest reproducible pressure was assumed to go to zero at the lift-off point. For this reason the two-phase liquid-gas and phase gaseous regions are not included in the integration.

$$\Delta G_m^{ex} = \int_{\Pi \to 0}^{\Pi} A_{12} d\Pi - N_1 \int_{\Pi \to 0}^{\Pi} A_1 d\Pi - N_2 \int_{\Pi \to 0}^{\Pi} A_2 d\Pi \quad (2)$$

A_{12} is the mean molar area in the mixed film, A_1 and A_2 are the molar areas of the two pure components, and N_1 and N_2 are the molar fractions of monolayers components 1 and 2.

The values of interaction parameter (α) at different pressures and the enthalpy corresponding to these interactions, ΔH, have been calculated by applying Eqs. (3) and (4) derived from Joos et al. [15–17].

$$\alpha = \frac{\Delta G_m^{ex}}{RT(x_1 x_2^2 + x_2 x_1^2)} \quad (3)$$

$$\Delta H = \frac{RT\alpha}{Z} \quad (4)$$

x_1 and x_2 are molar fractions (1 and 2 being the same components as defined for Eq. (2)). For the determination of the coordination number (Z), we followed the model of Quickenden and Tan [18], considering that in a closely packed monolayer (collapse) each molecule is surrounded by six neighbors. These values are given in Tables 1 and 2.

One can appreciate that the energies involved in the mixing process are small and of the same order as RT (1228.92 J/mol). Moreover, at molar fraction 0.5:0.5, as the packing factor is 4, when applying Eq. (4), ΔG_m^{ex} is equal to ΔH. Assuming that $\Delta G_m^{ex} = \Delta H_m^{ex} - T\Delta S_m^{ex}$ and as T is not null, ΔS_m^{ex} has to be zero when $\Delta G_m^{ex} = \Delta H_m^{ex}$. This means that at this molar composition the entropy of the system is zero, thus indicating that the mixture is highly ordered and stable.

c) Penetration studies: The penetration of TF and DTF on PC, CHOL, and PC/CHOL monolayers was carried out working at 5 and 20 mN/m. The composition of monolayers was chosen 1:1 to mimic the liposomes content and 0.2:0.8 because at this molar composition miscibility shows the highest deviation from ideality. The time-course is very slow and after 60 min there is no clear tendency towards saturation, as is shown in Fig. 4 for DTF.

The pressure increases determined after 60 min at 5 mN/m of initial surface pressure are represented in Fig. 5.

DTF gives higher pressure increases than TF for all the lipid compositions assayed. Moreover, the maximum penetration is observed with monolayers of PC/CHOL 0.2:0.8, the ones that gave the highest and negative deviation from ideality. The same behavior was found at 20 mN/m of initial pressure, but in this case differences between TF and DTF are almost nonexistent. Considering the lipid composition of the monolayer, one can say that the presence of cholesterol has a positive effect on the penetration; this fact was the opposite to that expected according to the well known rigidifying effect of CHOL on

Table 1. Excess free energy of mixing (ΔG_m^{ex}, J/mol) in mixed monolayers of PC/CHOL at different molar fractions

Molar fraction PC/CHOL	ΔG_m^{ex} at 20 mN/m (J/mol)		
	PBS	TF	DTF
0.8/0.2	409.31	− 599.50	− 732.52
0.5/0.5	− 607.94	− 123.40	− 141.45
0.2/0.8	− 1727.51	− 1952.64	− 209.46

Table 2. Interaction parameters and energies in PC/CHOL mixed monolayers, measured at different pressures

	(a) PC/CHOL: 0.8/0.2					
Π	α			ΔH (J/mol)		
(mN/m)	PBS	TF	DTF	PBS	TF	DTF
10	0.23	− 0.31	− 1.28	142.96	− 383.72	− 784.88
20	1.05	− 1.53	− 1.85	639.54	− 936.74	− 1130.48
	(b) PC/CHOL: 0.5/0.5					
Π	α			ΔH (J/mol)		
(mN/m)	PBS	TF	DTF	PBS	TF	DTF
10	− 0.64	− 0.11	− 0.12	− 391.25	− 132.42	− 72.23
20	− 0.99	− 0.20	− 0.23	− 607.94	− 123.39	− 141.45
	(c) PC/CHOL: 0.2/0.8					
Π	α			ΔH (J/mol)		
(mN/m)	PBS	TF	DTF	PBS	TF	DTF
10	− 2.34	− 2.82	− 0.27	− 1431.44	− 1726.76	− 165.53
20	− 4.41	− 4.99	− 0.54	− 1799.50	− 3050.98	− 327.29

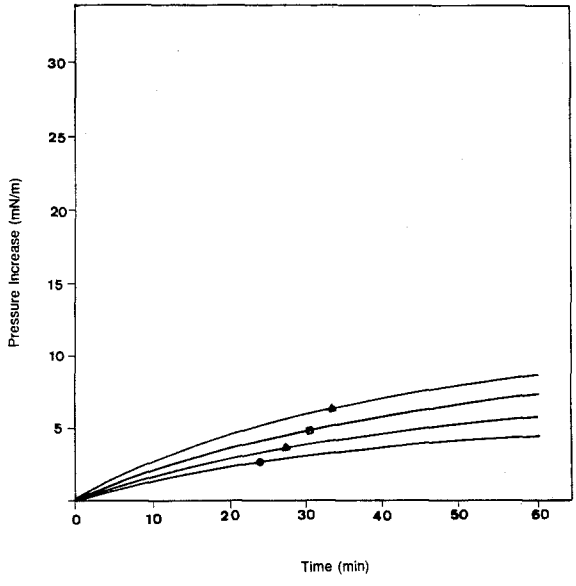

Fig. 4. Penetration kinetics of derivatized transferrin ($5.5.10^{-10}$ mols) into PC ⊖, CHOL △, PC/CHOL 0.5:0.5 ⊟ and PC/CHOL 0.2:0.8 ▲, spread at initial surface pressure of 5 mN/m

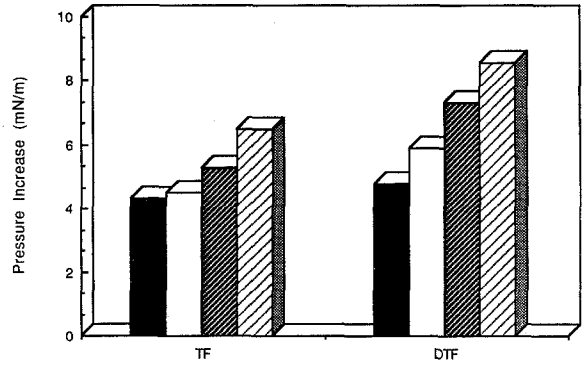

Fig. 5. Surface pressure increases induced by injection of TF and DTF under a PC ■, CHOL □, PC/CHOL 0.5:0.5 ▨ and PC/CHOL 0.2:0.8 ▨. Initial surface pressure 5 mN/m

unsaturated lecithins. One can hypothesize that the presence of the protein and its insertion can form a three-component monolayer with different properties.

d) Interaction of ANS with transferrin and derivatized transferrin: Solutions of native and derivatized transferrin were titrated with ANS. Moreover, as reference and to control quenching problems the same concentrations of ANS were diluted in TAC, and measured. In the three series of experiments the fluorescence intensity at 480 nm, the λ_{max} and polarization were determined.

According to the fluorescence intensity values the ability of ANS molecules to insert into hydrophobic zones in DTF was better than in TF at all the molar relationships ANS/Protein assayed (Fig. 6). This result was to be expected from the differences in the surface activity of both samples. Moreover, the higher hydrophobicity of DTF was also evident from the blue shift experienced by the λ_{max}, compared with native transferrin (Table 3). Nevertheless, when increasing the relationship ANS/protein, at a constant protein concentration there is a small red shift in both cases. The fact was not an artifact because the values did not change after dilution. We think that, at this point, the saturation of the protein with ANS renders the protein more hydrophilic; in this situation the ANS molecules have a slightly lower hydrophobic environment. Double reciprocal analysis of the data generated a linear plot in each case, consistent with a single type of ANS binding site for TF and DTF (not shown). The K_d values for the ANS-induced fluorescence changes were: $8.44\ 10^{-4}$ for TF and $2.64\ 10^{-4}$ for DTF.

As far as the polarization fluorescence is concerned we found that ANS motion is more restricted in DTF than in TF. All these values remained constant when reading the same preparations at 100 times dilution, thus we can discard the presence of artifacts.

At low contents of ANS (4.5 μM), the polarization values in DTF are 40% higher than in TF. Nevertheless, when increasing the ANS/protein relationship polarization increases for TF, but remains almost constant for DTF. This behavior suggests that DTF in solution has a favored secondary structure that is able to admit ANS molecules without any detectable change in it. On the contrary, it seems that TF has a less rigid structure and as ANS molecules become incorporated into the protein there is a tendency to stabilize and rigidify this secondary structure.

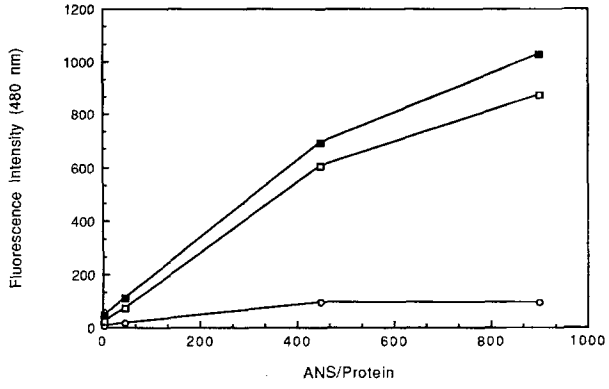

Fig. 6. ANS fluorescence titrations of TF -□- and DTF -■-; background fluorescence -○-. The protein concentration was $11 \cdot 10^{-6}$ M in both cases. ANS fluorescence was monitored at 480 nm

Table 3. Maximum wavelength values of ANS, in acetate buffer, TF and DTF solutions

ANS/protein	λ_{max}		
	TAC	TF	DTF
4.50	515.7	499.2	488.1
45.00	515.3	502.2	490.4
450.00	518.6	506.4	500.1
900.00	513.3	500.0	496.9

Conclusions

The results obtained in all these experiences show that upon derivatization transferrin becomes more hydrophobic. The interaction of this protein in native and derivatized forms with PC/CHOL mixtures is highly dependent on the composition of the mixtures, being maximum at PC/CHOL 0.2:0.8. This composition is the one that shows higher deviation from ideality (compression). Nevertheless, this composition is not valid to prepare liposomes as the maximum content of cholesterol is 0.5 of molar fraction. Moreover, experiments carried out compressing extension monolayers, spread on protein solutions, indicate that the incorporation of protein to the surface estimated by area increases is very small. These low values can be attributed to a lower sensibility in the area measurements compared with pressure determinations in our system.

ANS fluorescence titrations show the same differences in hydrophobicity as reported in surface studies, indicating that this small fluorophore can be associated with the protein surface, thus giving fluorescence yields in an ANS concentration-dependent way.

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