Use of 'soluble lipids' for biochemical processes: linoleic acid–cyclodextrin inclusion complexes in aqueous solutions

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The equilibria of linoleic acid (LA)–cyclodextrin (CD) complexes were studied to investigate the behaviour of 'soluble lipids' in solution as a function of factors that typically affect biochemical processes, such as pH, temperature and CD structure. The above complexes are formed with a stoichiometry of 1:2 in solution. The first CD molecule interacts with LA through hydrogen bonds when the pH is below the fatty acid pK; hydrophobic interactions may also play an important role at high pH. The second CD molecule makes only hydrophobic contact with the

LA hydrocarbon chain. The formation of hydrogen bonds is dependent on the inner diameter of the CD whereas the strength of the hydrophobic interactions between CD and LA can be related to the presence of hydrophobic groups in the CD. The first CD molecule interacts more strongly with LA at increased temperatures. The quantitative description of the LA–CD interaction allows absolute control of the effects produced by the lipid on biochemical processes.

INTRODUCTION

Recently, a series of new chemicals called 'soluble lipids' has appeared on the market. These consist of formulations of cyclodextrins (CDs) and hydrophobic substances such as fatty acids (FA) and steroids, and are marketed as solid complexes [1]. CDs are cyclic molecules formed from six to eight sugar residues, the conformation of which makes the inner channel more hydrophobic than the external surface of the CD molecule [2]. Their ability to interact with lipids and other hydrophobic molecules is based on the formation of so-called inclusion compounds, where the lipid is included in the hydrophobic torus, thus shifting the hydration water molecules of the internal groups in a thermodynamically favourable manner [2]. In this way, lipids can be dissolved in aqueous solutions, making them easier to handle, for example in serum-free culture media [3], or as substrate [4] or enzyme inhibitor [5–7]. The included lipid is also less prone to autoxidation [8].

The solid complexes partly dissociate when dissolved in aqueous solution, thus releasing a usually unknown amount of lipid into the medium. Although the use of these 'soluble lipids' can be based on empirical observations (for example, the effect on cell cultures of a dose of the complex under certain conditions of pH and temperature and in a given culture medium), characterization of the equilibrium between free and complexed lipid should allow quantification of the effects of the 'soluble lipid'.

In this work, we investigate linoleic acid (LA)–CD inclusion complexes as a model system of 'soluble lipids' and study the stoichiometry of the complexes in equilibrium. The equilibrium binding constants are determined in an attempt to characterize the system. In addition, the effects on equilibrium constants of factors that typically affect biochemical processes, such as pH, temperature and CD structure, are studied.

MATERIALS AND METHODS

LA was purchased from Cayman Chemical Co. (Paris, France). β -CD was obtained from Sigma (Madrid, Spain) and α -, γ - and methyl- β -CD (6–7.8 methyl residues per CD) were purchased

from Aldrich (Madrid, Spain). Diphenylhexatriene was a product of Fluka (Madrid, Spain), and tetrahydrofuran was from Merck (Darmstadt, Germany). All other chemicals used were of the highest purity available.

Fluorimetric determination of critical micellar concentration (c.m.c.)

The LA (c.m.c.) was determined as a function of pH and temperature by means of a fluorescence spectroscopy method described by Chattopadhyay and London [9] and adapted for

Figure 1 Dependence of relative fluorescence intensity of diphenylhexatriene at 430 nm (excitation wavelength 358 nm) on LA concentration

The FA c.m.c. was determined graphically as the intercept of the lines that define the increase in fluorescence in the pre- and post-micellar regions, as indicated. ●, pH 6.3 no CD; △, pH 8.0 no CD; ■, pH 6.3 and 1 mM β -CD; □, pH 8.0 and 1 mM β -CD.

Abbreviations used: FA, fatty acids; CD, cyclodextrin; LA, linoleic acid; c.m.c., critical micellar concentration; c.m.c.*, total FA c.m.c. in the presence of CD.

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Scheme 1 Equilibria between a long-chain FA and CD

FA by Serth et al. [10]. Samples of volume 2 ml contained 0.1 M potassium phosphate or potassium borate buffer, 0.88 μM diphenylhexatriene (supplied in 2 μl of tetrahydrofuran), 1% (v/v) ethanol and the required concentration of CD and FA. The samples were flushed with N_2 and incubated for 30 min at the desired temperature for equilibration, in the dark to prevent photoisomerization of the fluorescent probe.

Fluorescence intensity was measured at 430 nm (358 nm excitation wavelength) in a Kontron SFM-25 spectrofluorimeter equipped with thermostatically controlled cells. The relative fluorescence values were plotted against LA concentration, and the c.m.c. was determined as the intersection between the lines defining fluorescence intensity in the pre- and post-micellar regions (see Figure 1).

Determination of equilibrium constants

Equilibrium constants between free and complexed FA were determined by using a model involving the sequential binding of two CD molecules to one FA molecule (Scheme 1). The mass balance of the FA and CD in an aqueous solution may be represented by eqns. (1) and (2):

$$[\text{FA}]_t = [\text{FA}]_f + [\text{FA-CD}] + [\text{FA-CD}_2] \quad (1)$$

$$[\text{CD}]_t = [\text{CD}]_f + [\text{FA-CD}] + [\text{FA-CD}_2] \quad (2)$$

where subscripts f and t stand for free and total respectively. K_1 and K_2 are the equilibrium constants, which are defined as follows:

$$K_1 = \frac{[\text{FA-CD}]}{[\text{FA}]_f [\text{CD}]_f} \quad (3)$$

$$K_2 = \frac{[\text{FA-CD}_2]}{[\text{FA-CD}]_f [\text{CD}]_f} \quad (4)$$

Reorganizing eqns. (3) and (4) we have:

$$[\text{FA-CD}] = K_1 [\text{FA}]_f [\text{CD}]_f \quad (5)$$

$$[\text{FA-CD}_2] = K_1 K_2 [\text{FA}]_f [\text{CD}]_f^2 \quad (6)$$

Substituting eqns. (5) and (6) into (1), then:

$$[\text{FA}]_t = [\text{FA}]_f + K_1 [\text{FA}]_f [\text{CD}]_f + K_1 K_2 [\text{FA}]_f [\text{CD}]_f^2 \quad (7)$$

$[\text{CD}]_f$ can be deduced from eqn. (2) following substitution of eqns. (5) and (6) and rearrangement:

$$[\text{CD}]_f = \frac{-(1 + K_1 [\text{FA}]_f) + \sqrt{\{(1 + K_1 [\text{FA}]_f)^2 + 4K_1 K_2 [\text{FA}]_f [\text{CD}]_t\}}}{2K_1 K_2 [\text{FA}]_f} \quad (8)$$

$[\text{CD}]_t$ and $[\text{FA}]_t$ are known variables whereas $[\text{FA}]_f$ and the equilibrium constants are unknown. It has been shown by speed-of-sound measurements [11] that the apparent c.m.c. of an amphiphile such decyltrimethylammonium bromide in the presence of β -CD (c.m.c.*) is actually the sum of the c.m.c.s of the pure amphiphile (c.m.c._o) plus the concentration of inclusion complexes. Analogously, in the present case, $[\text{FA}]_t$ and $[\text{FA}]_f$ can

be replaced by c.m.c.* (the total FA c.m.c. in the presence of CD) and c.m.c._o (the total FA c.m.c. in the absence of CD) respectively, and thus the unknown equilibrium constants K_1 and K_2 can be estimated by non-linear regression of apparent c.m.c.* versus total CD concentration using the following equations:

$$\text{c.m.c.}^* = \text{c.m.c.}_o + K_1 \text{c.m.c.}_o X + K_1 K_2 \text{c.m.c.}_o X^2 \quad (9)$$

where

$$X = \frac{-(1 + K_1 \text{c.m.c.}_o) + \sqrt{\{(1 + K_1 \text{c.m.c.}_o)^2 + 4K_1 K_2 \text{c.m.c.}_o [\text{CD}]_t\}}}{2K_1 K_2 \text{c.m.c.}_o} \quad (10)$$

Non-linear regression fitting was performed by using a Marquardt algorithm implemented in the SigmaPlot v5.1 computer program (Jandel Scientific).

RESULTS AND DISCUSSION

FA forms aggregates above a certain critical concentration. As shown in Figure 1, the appearance of these aggregates at increased concentrations of LA can be detected by measuring the fluorescence of a probe such as diphenylhexatriene, the quantum yield of which increases when surrounded by an apolar environment such as that of the aggregate core [10]. In the presence of CDs, the aggregation behaviour of the FA changes to higher c.m.c.* values. Figure 1 shows such an effect of β -CD in different conditions.

Effect of β -CD concentration on c.m.c. of FA

On increasing β -CD concentrations a concomitant increase in the apparent c.m.c.* of FA was observed (Figure 2). From this graph a 1:1 stoichiometry for the inclusion compound, LA- β -CD, can be directly ruled out as this would have yielded a linear dependence, such as has been described for the interaction of a series of guest molecules with β -CD [2,11]. Analysis of dehydrated complexes of β -CD with FA has revealed stoichiometric ratios

Figure 2 Effect of β -CD concentration on the LA c.m.c.

Symbols represent the experimental data. Conditions were pH 6.3 and 25 °C. Equilibrium constants were obtained by non-linear regression fitting using eqns. (9) and (10), developed for a sequential binding scheme. A similar expression was deduced for a concerted binding scheme such as $2\text{CD} + \text{FA} \rightleftharpoons \text{FA/CD}_2$. —, Fitting using eqns. (9) and (10); ----, fitting using the equation deduced for concerted binding. Error bars are $\pm$ S.E.M. of three determinations.

Figure 3 Effect of pH and β -CD concentration on the apparent c.m.c.* of LA

The buffers used were 0.1 M sodium acetate for pH 4.0, 0.1 M sodium phosphate for pH 6.3–8.0 and 0.1 M sodium borate for pH 9.0–10.0. The temperature was 25 °C.

between 1:2 and 1:2.5 when the chain length ranges from 15 to 18 carbon atoms [12]. In dilute solutions such as those used in the experiments described here, no complexes higher than 1:2 would normally be expected, although, in a crystalline state, up to three CD molecules can be accommodated along the completely extended LA molecule. Figure 2 shows fitting of the experimental data to eqn. (9) and to another equation (not shown) deduced similarly from a concerted scheme where the species 1:1 does not exist. The sequential binding scheme was considered to give the best description of the dependence of c.m.c. on CD concentration. The equilibrium constants thus obtained characterize the formation of 1:1 and 1:2 complexes according to Scheme 1.

Effect of pH on equilibrium constants

The equilibrium constants for LA- β -CD interaction were determined as above in the pH range 4.0–10. Figure 3 shows a

Figure 4 Effect of pH on the equilibrium constants of β -CD-LA

The buffers used were as in Figure 3. Error bars are those given by non-linear regression fitting. ●, K_1 ; ■, K_2 .

Table 1 Effect of temperature on the equilibrium constants of β -CD-LA complexation

The pH of the buffer was adjusted at the indicated temperature. pH 7.6 was achieved with 0.1 M sodium phosphate buffer and pH 9.0 with sodium borate. Results $\pm$ S.E.M. as given by non-linear regression fitting.

Temperature (°C)	pH 7.6		pH 9.0	
	K_1 (mM $^{-1}$)	K_2 (mM $^{-1}$)	K_1 (mM $^{-1}$)	K_2 (mM $^{-1}$)
10	9.5 ± 1.9	1.4 ± 0.4	1.1 ± 0.4	3.3 ± 0.7
25	10.5 ± 3.0	1.8 ± 0.5	1.5 ± 0.1	1.7 ± 0.5
37	12.8 ± 3.2	1.0 ± 0.3	1.8 ± 0.4	0.9 ± 0.3

three-dimensional graph of changes in c.m.c.* with variations in pH and β -CD concentration. When no CD was present, c.m.c.* values were in good agreement with those reported in the literature [13]. The c.m.c.* increased uniformly on increasing pH at low β -CD concentration but, on increasing the concentration of β -CD, a maximum of c.m.c.* appeared around pH 7.6. Figure 4 shows the effect of pH on the equilibrium constants, determined using data from Figure 3. A strong dependence of K_1 on pH can be seen, passing from a stable value of about 11 000 M $^{-1}$ to another stable value of about 1000 M $^{-1}$ in just 1.5 pH units, as happens during the titration of a weak ionizable group. As β -CD does not possess any ionizable groups it is presumably the carboxy group of the FA that is being titrated. In fact, the pH at which the mean value of K_1 is obtained is very close to an LA pK_a of 7.9, as determined in 95% ethanol [14].

A likely explanation for the dependence of K_1 on pH is that the protonated FA carboxyl group forms a hydrogen bond with hydrophilic groups of CD at pH values below the pK_a value, as with other ionized weak electrolytes [15]. Indeed, hydrogen-bonding is one of the most important types of interaction on the stabilization of inclusion complexes of guest molecules with CDs [2,16]. Based on NMR studies, it has been proposed that the carboxy group of FA forms hydrogen bonds with the -OH at position 6 in the sugar residues, although this does not result in significant changes in the pK_a of FA [17].

Figure 4 also shows the effect of pH on K_2 . It can be seen that there is almost no effect in the pH range studied, indicating that no ionizable group takes part in the interaction of the second CD molecule with the 1:1 complex; instead, only the hydrocarbon chain of the FA is probably involved by means of hydrophobic interactions, the other principal way in which inclusion complexes are stabilized [2,16].

Effect of temperature on the equilibrium constants

The effect of temperature on the equilibrium constants was studied for the LA- β -CD interaction at two different pH values, one below (pH 7.6) and the other above (pH 9.0) the pK_a of the free LA. To prevent the results being affected by changes in the buffer pK_a with temperature, the pH of the buffer was adjusted at the indicated temperature. Table 1 summarizes the results of this experiment. Although inclusion complexes usually dissociate when temperature is increased [2,16], in our experiment K_1 increased when the temperature increased both at pH 7.6 and 9.0. As K_1 depends on the interaction of the FA carboxy group with CD, this result might be interpreted as a higher degree of interaction at higher temperatures. Hydrogen bonds are usually weakened by heating, although in this case the contrary seems to be true. Instead of a rather unlikely strengthening of the hydrogen

Table 2 Effect of CD structure on the equilibrium constants of CD-LA complexation, at 25 °C and in 0.1 M sodium phosphate buffer, pH 6.3

The values for inner diameter are taken from ref. [2] except for the value in parentheses which is taken from [20]. Results $\pm$ S.E.M. as given by non-linear regression fitting.

CD type	Inner diameter (Å)	K_1 (mM ⁻¹)	K_2 (mM ⁻¹)
α -CD	4.7–5.2	14.3 ± 0.7	2.4 ± 0.2
β -CD	6.0–6.4 (7.4)	10.2 ± 1.4	2.6 ± 0.9
γ -CD	7.5–8.3	7.8 ± 0.2	1.0 ± 0.1
Methyl- β -CD	7.5	13.5 ± 0.4	9.1 ± 0.4

bonds, an increase in the pK_a of LA with temperature might alternatively explain this result, since the concentration of protonated species able to form hydrogen bonds would increase. Indeed, the midpoint titration of oleic acid was shown to occur at about 0.25 pH unit higher at 40 °C than at 6 °C [18].

K_2 varied irregularly with respect to pH. At pH 7.6 it showed a slight maximum at 25 °C whereas at pH 9.0 its value decreased monotonically as temperature increased. The behaviour at pH 7.6 seems to be rather complex as the change in K_2 was not monotonic with temperature. If we consider the presence of the *cis,cis*-1,4 double-bond system in LA, with a freely rotating methylene group in position 11, it is possible that a conformational change in the molecule takes place over the temperature range 10–37 °C, probably influenced by the transitory aggregation mode at those pH values [18]. However, at pH 9.0, where the aggregation mode is stable as negatively charged micelles, K_2 decreases uniformly. A weakening of the hydrophobic interactions between the FA hydrocarbon chain and the CD channel can be expected at increased temperatures, since a higher mobility of the FA tail produces a decrease in the hydrophobic effect and therefore a decrease in K_2 .

Effect of the CD structure on the equilibrium constants

The complexation equilibrium constants between LA and CDs were determined with different CDs in an attempt to characterize at a molecular level the interaction between the lipid and the host CD. Table 2 shows the values of K_1 and K_2 for the complexation of different CDs with the FA, as well as some structural data for the CDs.

Considering the species α -, β - and γ -CD it can be observed that K_1 increases as the inner diameter of the CD becomes smaller. Probably, the shorter average distance between the FA carboxy group and the CD hydroxy groups increases the chance of hydrogen-bond formation. In contrast, there was almost no effect on K_2 . In fact, the factor that dominates the interaction of the second CD molecule, the hydrophobic effect, is probably not

affected by the diameter of the macrocycle since the hydrophobicity of the sugar residues will not have changed. Moreover, the smallest CD tested is too large to provide a tight fit with the hydrocarbon chain of FA.

A qualitatively different result is obtained when methyl- β -CD is used instead of β -CD, the K_1 value being higher for the methylated CD. Methylation of β -CD occurs principally at position 2 of the sugar residues [19] situated on one side of the torus at the edge and orientated inward [2,16], thus increasing the hydrophobicity of the channel. The increase in K_1 could be due to the favoured hydrophobic interactions with one side of the CD molecule (that bearing the methyl groups), whereas on the other side the hydrogen-bonding with the 6-OH remains almost unchanged.

On the other hand, K_2 underwent a large increase with respect to the other CDs. The dramatic changes in the hydrophobicity of the CD torus provoked by the methylation of the internal -OH groups are sufficient to explain the behaviour of K_2 . Indeed, methyl- β -CD is preferred over β -CD in commercial preparations of CD-encapsulated FA [1].

In conclusion, through this work we have learnt (1) that characterization of 'soluble lipids' in solution allows absolute quantification of the effects produced by the lipid on biochemical processes and (2) that the effect of the lipid on such processes can be assigned to individual molecules.

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