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Disruption of micellar aggregates of ganglioside GM-1 by complexation with α-cyclodextrin

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Abstract

As previously observed for model glycolipids, micellar aggregates of the ganglioside GM-1 can be disrupted by the formation of inclusion complexes with α-cyclodextrin (α-CD). Evidence for such disaggregation was obtained from narrowing and shifting of ^{1}H- and ^{13}C-NMR signals, and decreasing of ^{13}C-NMR relaxation times (T_1) upon addition of α-CD to aqueous (D_2O) solutions of the ganglioside. As a result of the α-CD-induced disaggregation, GM-1 becomes permeable through 100 000 Da cut-off ultrafiltration membranes (which are virtually impermeable to normal GM-1 aggregates), and can be freed from phospholipid contaminants. β-Cyclodextrin (β-CD), which gives weaker complexes with GM-1, does not produce any significant disaggregation effects. Also, fully methylated β-cyclodextrin (Meβ-CD) and hydroxyethyl-β-cyclodextrin (HEβ-CD) were ineffective. A solid complex (which precipitates from solutions at α-CD/GM-1 molar ratios > 5) was obtained, and characterized by CP/MAS ^{13}C-NMR spectroscopy and by DSC.

Key words: α-Cyclodextrin; GM-1 ganglioside; Disaggregation; NMR; Ultrafiltration; Solid complex

1. Introduction

The monosialoganglioside GM-1 is a glycolipid component of the nervous system, with important biological functions and pharmacological properties (Svennerholm, 1963; Wiegand, 1985). The molecules of GM-1 consist of a hydrophilic head (the pentasaccharide galactosyl-*N*-acetylgalactosaminyl (*N*-acetylneuraminyl) galactose) linked to the hydrophobic chains of ceramide (Cer) (Fig. 1). Like most other amphiphilic molecules, in aqueous systems GM-1 is in the form of tight aggregates, with the Cer hydrophobic chains sequestered from the aqueous enviroment, and the carbohydrate moiety exposed to water (Corti et al., 1987). As a result of this aggregation, the actual size of GM-1 in water at concentrations

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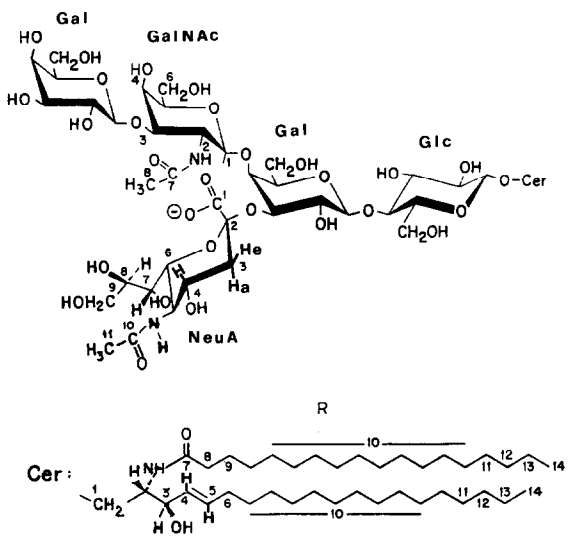

Fig. 1. Structure of GM-1 (Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; NeuA, neuraminic (*N*-acetylsialic) acid; Cer, ceramide).

higher than its, very low, critical micellar concentration (CMC) of approx. 10^{-8} (Formisano et al., 1979) largely exceeds that of its 'monomeric' molecules. The size and shape of GM-1 micelles influence the 'workability' of this glycolipid, and conceivably also its bioavaibility when administered as a drug. It was thus thought of interest to develop a method for controlling the aggregation state of GM-1 and possibly of other gangliosides. The inclusion propreties of cyclodextrins (Szeitli, 1988) could be exploited for this purpose. In fact, NMR studies on alkyl glycosides indicated that the micelles of these glicolipids can be disrupted by addition of α-cyclodextrin (α-CD) through the formation of inclusion complexes (Casu et al., 1990). In the present work, the NMR study was extended to aqueous systems containg GM-1 and α-CD, obtaining evidence that the cyclodextrin also forms inclusion complexes with GM-1. A solid complex of GM-1/α-CD was also obtained. Further evidence that cyclodextrins can disrupt micellar aggregates of GM-1 was obtained through ultrafiltration experiments, which rationalize preliminary findings (Casu et al., 1992) that purification of the ganglioside from phospholipids is achievable by ultrafiltration in the presence of α-CD.

2. Materials and methods

2.1. Materials

Both crude (second partition stage) and pure GM-1 (lot VV.157.T) were prepared at Crinos Research Laboratories. α-CD was supplied by Consortium Elektrochem. Ind. GmbH, Germany. β-CD, Meβ-CD and HE-β-CD were from Medimpex, Milan, Italy. All other chemicals and reagents were of analytical grade. Deionized double-distilled water was used throughout the studies.

2.2. NMR spectroscopy

^{1}H-NMR spectra were obtained on a Bruker AC 300 and AMX500 spectrometers using both a 5 mm broad band and a dual 5 mm probe, at 27°C. Samples of GM-1 and/or α-CD or GM-1/α-CD complex (10 mg/ml) were dissolved in either DMSO-d_6 or D_2O (99.7% D) after three exchanges with D_2O. Samples at different α-CD/GM-1 ratios ($R = 1$–30) were prepared by adding different amounts of a 6.43×10^{-3} M solution of α-CD (previously lyophilized three times with D_2O and redissolved in 0.5 ml D_2O) to 0.5 ml of a GM-1 solution (6.43×10^{-4} M). Spectra were obtained by coaddition of 516–1028 scans, using a 90° flip angle. Chemical shifts were referred to external trimethylsylilpropanesulphonic acid (TSP).

^{13}C-NMR spectra of GM-1 (4×10^{-3} M), alone or in the presence of α-CD, were obtained at 75 or 100 MHz, on a Bruker CXP 300 or AM400 spectrometer equipped with a 10 mm probe (about 15 000 scans). Chemical shifts were referred to external TSP. The ^{13}C spin-lattice relaxation times (T_1) were calculated by fitting the experimental values determined by the inversion-recovery method, using the calculation routines of the Bruker program (version DISMNR90) for the Aspect 3000 computer. The pulse sequence was D_1–180–τ–90–FID, were τ is the variable

delay (30) μs–3.5 s in 10 experiments) and D_1 denotes the relaxation delay (4 s).

2.3. Ultrafiltration

Experiments for finding optimal α-CD/GM-1 ratios were performed with 253 mg (0.16 mMol) GM-1 in 25 ml H_2O, added to 25 ml of solutions of α-CD in H_2O (containing 1–3.2–5 times, respectively, the molar concentration of the cyclodextrin with respect to the ganglioside). Solutions were ultrafiltered through Amicon Diaflo membranes (cut-off 100 000 Da; YM100, 43 mm diameter, Amicon Corp., MA., U.S.A.). 25 2-ml fractions were collected in the permeate, and analyzed (by TLC) for their GM-1 content. The same experimental conditions were used with β-CD, Me-β-CD, and HE-β-CD.

Experiments on the purification of GM-1 from phospholipid contaminants were performed with 444 mg of ganglioside-phospholipid mixture (56.7% = 0.161 mmol GM-1) in 25 ml H_2O, respectively, added to 25 ml solutions containing 1–3.2–4–6.4 molar excess of α-CD. Each solution was diafiltered across the above-described membranes against H_2O, collecting five 10-ml fractions for analysis. An additional experiment was carried out by diafiltering at constant volume at 60°C, with concentrations corresponding to the 6.4 α-CD/GM-1 molar ratio, collecting five 50-ml fractions.

2.4. TLC analysis of permeates

The content of GM-1 in ultrafiltration permeates and assessment of purity of the gangliosides in experiments starting from crude GM-1 were determined by thin-layer chromatography (TLC), by direct visual comparison of intensities of the spot with those of known amounts of reference substances. The analysis was conducted on HP-TLC plates (silica gel) using $CHCl_3$/methanol/0.3% aqueous $CaCl_2$ in a 60:35:8 ratio as elution solvent. For each sample, the TLC analysis was performed on two plates at the same time, for simultaneous determination of spots with two reagents (Ehrlich reagent and anisaldehyde, the latter being prepared by dissolving 1 ml anisaldehyde in glacial acetic acid, bringing the volume to 100 ml, and adding to the solution 2 ml of 86% (w/v) sulphuric acid). Detection limits were about 1 μg for GM-1 and 0.5 μg for α-CD.

2.5. Solid-state cross-polarization-magic angle spinning (CP-MAS) ^{13}C-NMR spectroscopy

The spectra were recorded with a Bruker CXP-300 spectrometer at 75 MHz. The cross-polarization was 1 ms, while the repetition time and the ^{1}H 90° pulse were 4 s and 4.75 μs, respectively. The chemical shifts were measured with respect to TMS, with benzene as secondary reference (128 ppm). The number of scans was 1000–3000 and the rotational speed 3.4 kHz.

2.6. Thermal analysis

DSC scanning was performed with a Perkin Elmer DSC-4 apparatus. Samples (2–5 mg) were encapsulated in aluminium pans and programmed heating at a rate of 4°C/min in a dynamic nitrogen environment from 50 to 350°C was performed. The instrument was calibrated with indium.

3. Results and discussion

3.1. Studies in solution

3.1.1. NMR spectra

As previously reported for alkylglycosides (Casu et al., 1990), the NMR parameters sensitively reflect the interaction of cyclodextrins with amphiphilic molecules in aqueous solution. The ^{1}H-NMR spectrum of GM-1 in deuterium oxide consists of very broad signals, only the strongest ones (those associated with the CH_3 and CH_2 groups of Cer and of the acetamido CH_3 groups of GalNAc residues) being discernible even at a magnetic field as high as 500 MHz (Fig. 2).

As also illustrated in Fig. 2, in the presence of α-CD these signals become sharper, and shift to different extents. Whereas signals of the Cer moiety shift downfield upon addition of α-CD, the CH_3 signal of GalNAc is barely affected, and the

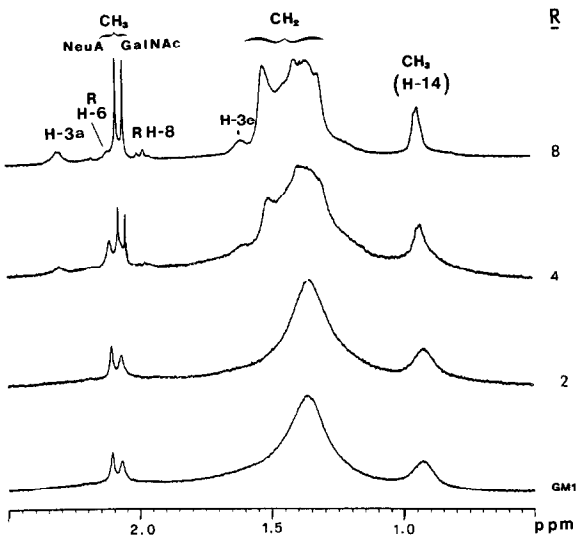

Fig. 2. ^{1}H-NMR spectra (500 MHz, D_2O) of the alkyl moieties of GM-1 alone and in the presence of α-CD at different α-CD/GM-1 molar ratios (R).

CH_3 signal of NeuA moves upfield. At α-CD/GM-1 molar ratios ≥ 4, other GM-1 signals (attributable to H-3_a and H-3_b of NeuA and to H-8 of the Cer moiety) show up in the spectrum (signal assignments were made by comparison with those of GM-1 in dimethyl sulphoxide (Harris and Thornton, 1978; Sonnino et al., 1988).

The highest signal resolution and largest shifts were observed for $R = 8$. For higher α-CD concentrations, the solution became turbid, with incipient precipitation of a solid complex (see later). For $R \geq 4$, the CH_3 signal of the Cer moiety (at ≈ 1 ppm) clearly shows two components. Values for α-CD-induced signal sharpening and shifts are given in Table 1 for the CH_3 protons.

The largest CD-induced spread of signals was observed for the -CH_2- protons in the 1.2–1.6

Table 1
Half-width change (Hz) of the CH_3 signal of GM-1 with increasing molar ratios α-CD/GM-1 (R)

	R							
	0	1	2	3	4	7	10	20
Hz	56.4	56.4	28.2	21.2	17.6	15.9	14.1	14.1
ΔHz	–	0	28.2	35.2	38.2	40.5	42.3	42.3

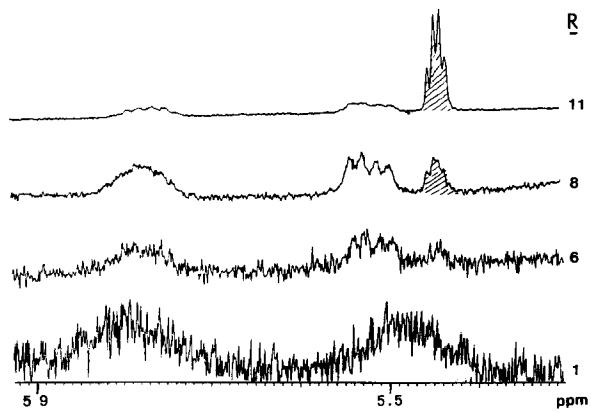

Fig. 3. ^{1}H-NMR spectra (500 MHz, D_2O) in the region of H-4 and H-5 of the Cer moiety, at different α-CD/GM-1 molar ratios (R).

ppm region, with a 'new', strong component at 1.55 ppm for $R = 8$. Most of the 'carbohydrate region' of the ^{1}H-NMR spectra of GM-1 solutions containing added αCD is dominated by the strong resonance of the cyclodextrin.

Apart from the high-field region (0–2.5 ppm) shown in Fig. 2, GM-1 signals can be observed in another 'window', between 5.3 and 5.9 ppm (Fig. 3). This region is typical for the olefinic protons H-4 and H-5 of the Cer moiety (Sonnino et al., 1988). Whereas the spectrum of GM-1 alone in this region is featureless, upon addition of α-CD the resonances of H-4 and H-5 become already evident $R = 1$, showing fine structure for higher R values. The H-4 signals move downfield, and those of H-5 upfield, with increasing concentrations of α-CD.

As illustrated in Fig. 4, the ^{13}C-NMR spectra also show sharpening and relative shifts of GM-1 signals upon addition of α-CD (signal assignments are according to Nerz-Stormes and Thornton, 1978 and Sillerud and Yu, 1983). Some of the signals (especially C-8 and C-13) are represented by more than one component.

Data for spin-lattice relaxation time (T_1) measured at 75 MHz for C-14, C-13, and C-10 of GM-1 alone and in the presence of α-CD ($R = 2$) (Table 2) show that the cyclodextrin induces a decrease in T_1 values of these carbons of the glycolipid. However, as is also shown in Table 2,

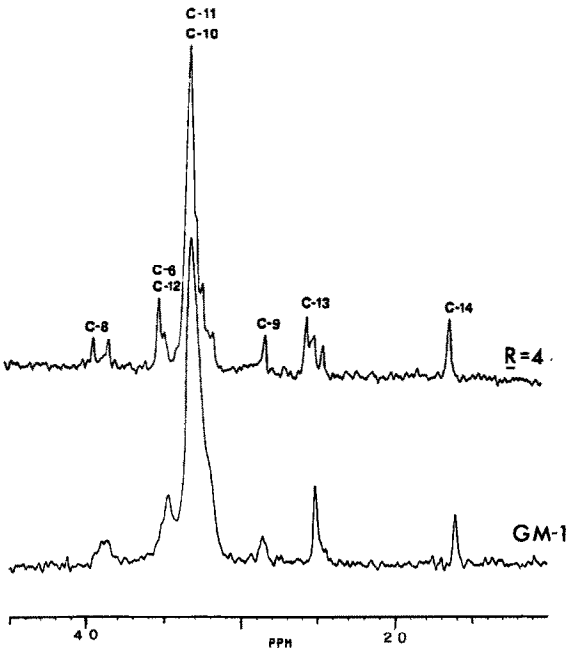

Fig. 4. ^{13}C-NMR spectra (100 MHz, D_2O) of the alkyl moieties of GM-1, alone and at a α-CD/GM-1 molar ratio (R) = 4.

such a decrease is consistently lower than observed (Casu et al., 1990) for similar carbons of C8-alkylglycoside in the presence of α-CD at the same molar ratio.

By analogy with alkyl glycoside/α-CD systems (Casu et al., 1990), the cyclodextrin-induced modifications in the NMR parameters of GM-1 are indicative of penetration of the alkyl chains of Cer into the cavities of α-CD. The observation of more than one ^{13}C signal for some carbons of GM-1 similarly suggests the presence of more than one form of complex in slow dynamic equilibrium. Large shifts of signals of Cer protons and carbons close to the carbohydrate moiety suggest that α-CD also induces a change in the conformation of the GM-1 molecule with respect to that of the ganglioside in its usual micellar state.

3.1.2. Ultrafiltration

In order to investigate whether the α-CD-induced micellar disaggregation of GM-1 suggested by the NMR studies affected the ultrafiltration properties of the gangliosides, and could be exploited for its purification from some of its common contaminants, two series of ultrafiltration experiments though a 100000 Da cut-off membrane were performed as described in section 2. In the first series of experiments – aimed at optimizing α-CD/GM-1 molar ratios for the best recovery of GM-1 in the permeate – 0.16 mM GM-1 in H_2O was added to an equal volume of H_2O solutions containing: (A) an equimolar concentration of α-CD; (B) and (C) 3.2 and 5 molar excess, respectively, of the cyclodextrin. A blank experiment was carried out using a corresponding solution of GM-1 alone. No detectable amounts of ganglioside were found (by TLC analysis) in the permeates from the experiments with GM-1 alone. In contrast, significant amounts of GM-1 were already present in the first permeates from experiments performed in the presence of α-CD. The amounts of GM-1 in the permeates rapidly increased with increasing ultrafiltration times (such an increase being greater for higher α-CD/GM-1 ratios), and levelled off at about one-tenth of the total solution volume (data not shown), probably as a result of formation of a solid complex (see later), revealed by increasing turbidity of the retentate solutions.

Table 2
Influence of α-CD on the 75 MHz ^{13}C-NMR spin lattice relaxation times (T_1, s) at 23°C in D_2O of the alkyl moiety of GM-1 and n-octyl-β-D-glucopyranoside (T_1^0 in the absence of α-CD; T_1, in the presence of α-CD)

		-CH_3(C-14)	β-C-2(C-13)	-$(CH_2)_n$(C-10)
GM-1	T_1^0	1.47 ± 0.015	0.87 ± 0.027	0.45 ± 0.005
GM-1/α-CD 1:2	T_1	1.17 ± 0.038	0.81 ± 0.028	0.32 ± 0.01
	T_1^0/T_1	1.26	1.07	1.42
Glucose C_8	T_1^0/T_1	2.3 [a]	3.0 [a]	5.6 [a]

[a] From Casu et al. (1990).

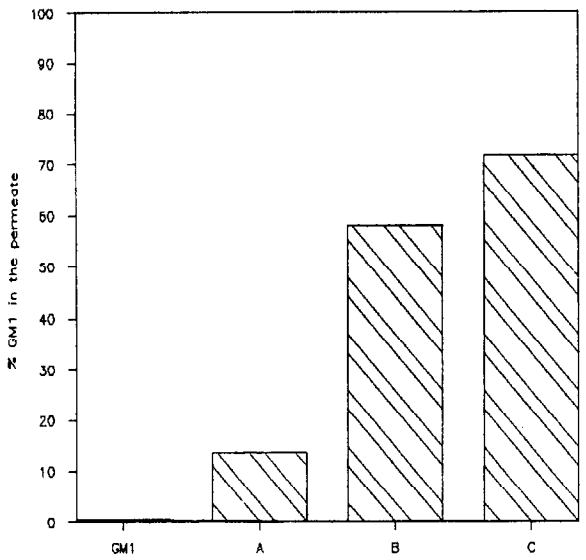

Fig. 5. Recovery of GM-1 in the permeate, as a function of the α-CD/GM-1 molar ratio (R) (GM1) GM-1 alone; (A) $R = 1$; (B) $R = 3.2$; (C)$R = 5$.

As illustrated in Fig. 5, up to 72% of the ganglioside was recovered in the permeate of the experiment with a 5 molar excess of α-CD. Other cyclodextrins (β-CD, Me-β-CD, and β-HECD) did not induce any significant passage of GM-1 through the membrane.

The second set of experiments, performed to evaluate the feasibility of purification of GM-1 from its usual phospholipid contaminants, was performed mixing a solution of crude ganglioside (consisting of 56.7% of GM-1, the complement to 100% being mainly phospholipids) with equal volumes of solutions containing: (D) an equimolar amount (0.16 mM) of α-CD, and (E), (F) and (G), a 3.2, 4, and 6.4 molar excess, respectively, of α-CD.

The percent recoveries of GM-1 in the permeates from these experiments are reported in Table 3, together with that from an experiment (H) conducted with a 6.4 molar excess cyclodextrin, at 60°C and constant volume to avoid precipitation of solid glycolipid-cyclodextrin complexes. These data show that up to 25% recovery of GM-1 (pure by TLC analysis) is achievable by ultrafiltration at room temperature of the crude mixture in the presence of α-CD. At 60°C and working under conditions of constant volume, more than 90% recovery of GM-1 was obtained.

Table 3
Efficiency of ultrafiltration for the purification of GM-1 from phospholipids

Experiment	α-CD/GM-1 molar ratio	%(w/w) of GM-1 recovered in the permeate
D	1.0	15
E	3.2	19
F	4.0	22
G	6.4	25
H	6.4	93

3.2. Solid-state studies

As previously mentioned, under the experimental conditions described in section 2, a precipitate was formed from GM-1 solutions containing added α-CD, for $R \geq 8$. ^{1}H-NMR analysis of the precipitate dissolved in dimethyl sulphoxide-d_6 indicated a composition of 1 GM-1 : 4α-CD.

The solid state CP/MAS ^{13}C-NMR spectrum of the precipitate (Fig. 6) demonstrates signals of both the glycolipid and the cyclodextrin compo-

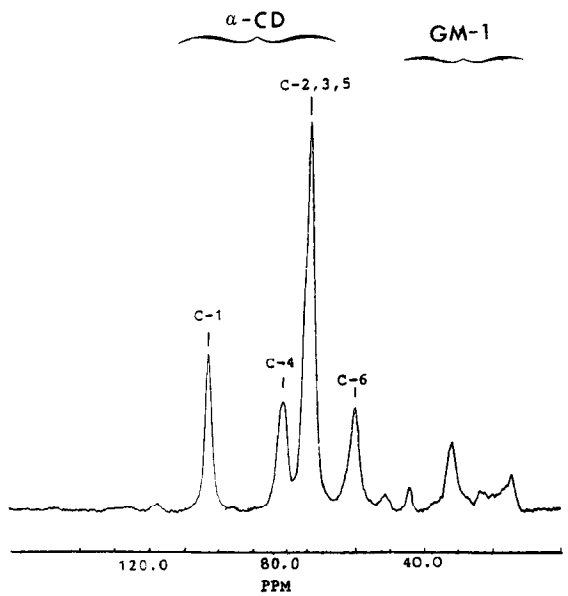

Fig. 6. CP/MAS ^{13}C-NMR spectrum of the solid GM-1/α-CD complex.

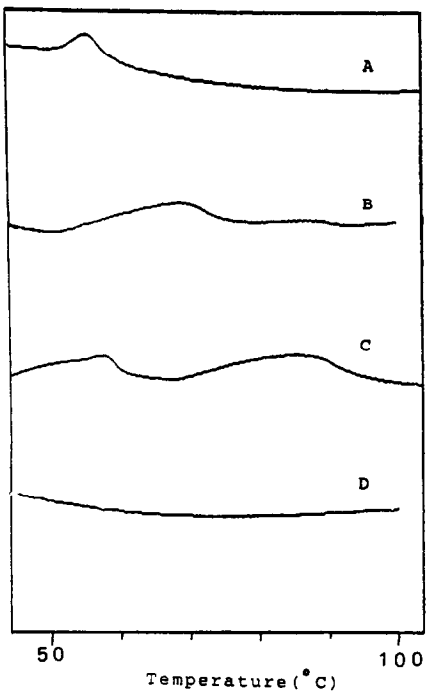

Fig. 7. DSC thermograms of solid GM-1/α-CD systems.(A) GM-1; (B) α-CD; (C) 1:4 (M/M) GM-1/α-CD physical mixture; (D) GM-1/α-CD inclusion complex.

nents. Signals C-1 and C-4 of α-CD (referred to signal C-6) are displaced (~ 100 Hz) with respect to values measured for α-CD alone. (signal assignments are according to Inoue et al., 1987). The precipitate was also characterized by differential scanning calorimetry (DSC). As shown in Fig. 7, the DSC thermogram of the precipitate (d) did not show the transitions observed for GM-1 alone (a), α-CD (b), or a 1:4 physical mixture of GM-1 and α-CD (c). Taken together, the ^{13}C-NMR and DSC data strongly suggest that the precipitate is a complex between GM-1 and α-CD.

4. Conclusions

As indicated by the sharpening of the ^{1}H- and ^{13}C-NMR signals and the decreasing ^{13}C T_1 values, α-CD has a strong influence on the state of aggregation of GM-1. The increased permeability of the ganglioside in the presence of the cyclodextrin directly confirms disruption of the ganglioside aggregates.

In more detail, the α-CD-induced ^{1}H- and ^{13}C-NMR shifts indicate that the Cer chains of GM-1 are imbedded in the microenvironment of the cydodextrin cavity, as expected for formation of inclusion complexes. As a result of this interaction, the original GM-1 aggregates are largely disrupted. This possibility of controlling the aggregation state of GM-1 made it possible to purify the glycolipid from large amounts of phospholipid contaminants, by ultrafiltration in the presence of α-CD.

The solid complex formed between GM-1 and α-CD under defined experimental conditions may be a useful pharmaceutical form to modify the biovailability of the glycolipid.

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