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Cyclomaltooligosaccharide binding and solubilization of hydroxyfatty acid matrices in aqueous solution: calorimetric titration and ^{13}C NMR investigations of molecular recognition

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Abstract

Cyclomaltooligosaccharides (cyclodextrins, CDs) increase cutinase activity with both naturally occurring and synthetic cuticular substrates. Little is known about the interactions of CDs with cutin or cutin-like substrates such as 16-hydroxypalmitate (16-OH-P). We report herein investigations into the thermochemistry of β-CD, hydroxypropyl-β-CD (HP-β-CD) or α-CD interactions with palmitic acid (P), 16-OH-P and polyesters (synthetic cutin) derived therefrom under conditions coincident with maximal cutinase activity (pH 9, glycine/NaOH buffer) at 25 °C using isothermal titration calorimetry (ITC). The thermodynamic parameters for HP-β-CD•lipid inclusion complex formation and subsequent solubilization, which were studied in heterogeneous phase suspensions, displayed enthalpy–entropy compensation typical of processes driven by solvation phenomena ($\alpha = T\partial\Delta S/\Delta H = 1.03$, $T\Delta S_0 = 17.72$ kJ mol^{-1}; for 130 literature [α- and β-CD] values: $\alpha = 0.92$, $T\Delta S_0 = 15.11$ kJ mol^{-1}). In the 16-OH-P (Na^+) experiments ΔH and ΔS ($\Delta H = 42 \pm 8$ kJ mol^{-1}, $\Delta S = 206 \pm 24$ J mol^{-1} K^{-1}) values were large relative to those reported elsewhere for diverse CD•guest complexes ($\Delta H = -50$ to 0 kJ mol^{-1}, $\Delta S = -170$ to 30 J mol^{-1} K^{-1}) since ΔH resulted from the combined processes of binding and solubilization. ^{13}C NMR and ITC experiments indicated that HP-β-CD•lipid complexes had a 1:1 stoichiometry. A constant background lipid concentration-dependent endothermic process (ΔH^*) also observed

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Abbreviations: K, binding, formation or association constant; ΔG, Gibb's function = $\Delta H - T\Delta S$; ΔH, enthalpy change; ΔH^*, background lipid concentration-dependent endothermic process which might be associated with the binding of CDs to the solid lipid matrix without concomitant solubilization; ΔQ, heat changes from integration of calorimetric titration endo- or exotherms; ΔS, entropy change; α, slope of the $T\Delta S$ vs ΔH plot; $T\Delta S_0$, intercept (at $\Delta H = 0$) of the $T\Delta S$ vs ΔH plot; T, temperature in units of Kelvin (K) or Centigrade (°C); HP-β-CD, hydroxypropyl derivative of cyclomaltoheptaose; β-CD, cyclomaltoheptaose; α-CD, cyclomaltohexaose; synthetic cutin, $CO_2H(CH_2)_{14}CH_2O$–$[CO(CH_2)_{14}CH_2O]_n$–$CO(CH_2)_{14}CH_2OH$ (ave. $n \sim 3$); 16-OH-P, $CO_2H(CH_2)_{14}CH_2OH$, 16-hydroxyhexadecanoic acid, 16-hydroxy-palmitic acid; P, $CO_2H(CH_2)_{14}CH_3$, hexadecanoic acid, palmitic acid.

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using both P and 16-OH-P substrates ($\Delta H^* \sim 4.8 \pm 0.5\,kJ\,mol^{-1}$) as HP-$\beta$-CD was titrated into the heterogeneous lipid slurry. At a lower pH (6, 100 mM Na^+ phosphate buffer) neither a soluble HP-β-CD•16-OH-P complex was formed nor background ΔH^* observed. At pH 9 no substantial binding was evident when synthetic cutin ($\Delta Q = -240 \pm 61\ \mu J$, $\Delta Q_{control} = -231 \pm 31\ \mu J$) was used as a substrate; a similar result was obtained using β-CD. Titrations using α-CD did, however, display a weak interaction ($K = 119 \pm 53\ M^{-1}$, $\Delta H = 1.1 \pm 0.9\ kJ\ mol^{-1}$, $\Delta S = 43.4 \pm 3.7\ J\ mol^{-1}\ K^{-1}$) with the synthetic cuticular matrix. Thus, either CDs do not bind to the insoluble cutin matrix or they do but with a small ΔH. The fact that HP-β-CD binds the synthetic cutin monomer and weak binding was observed in the α-CD•synthetic cutin system tends to argue for the latter interpretation.

Keywords: Cyclomaltoheptaose; β-Cyclodextrin; Cyclomaltohexaose; α-Cyclodextrin; Hydroxypropylcyclomaltoheptaose; Hydroxypropyl-β-cyclodextrin; Hexadecanoic acid; Palmitic acid; 16-Hydroxyhexadecanoic acid; 16-Hydroxy-palmitic acid; Cutin; Hydroxy fatty acids; Binding; Enthalpy–entropy compensation; Thermodynamics; ΔH; ΔS; ΔG; Calorimetry; Isothermal titration calorimetry; ITC; NMR

1. Introduction

Large quantities of low-valued cutin, an extremely hydrophobic polyester of various hydroxy-fatty acid (HFA) derivatives [1], are generated from the processing of fruits and vegetables. The two largest sources of fruit and vegetable wastes which contain a significant fraction of cutin are associated with tomato (mainly 10,16-dihydroxyhexadecanoic acid or 10,16-dihydroxypalmitic acid) and apple processing (Dr. Harold L. Durkin, President, Market Development Professionals, 57 Mohawk Street, Stratford, CT 06497, personal communication). Wastes (peel and seed) from tomato processing alone (ca. $5 \times 10^6\,kg\,yr^{-1}$, wet weight) could be a renewable source for natural value-added chemicals for industrial purposes via partial depolymerization of the cuticular matrix (i.e., wax-like oligomers and HFAs).

Natural waxes are gaining popularity (S. Puleo, Vice President Research and Development, Koster Keunen, 1021 Echo Lake Road, PO Box 69, Watertown, CT 06795, pers. comm.) in the chemical industry since they can potentially replace certain synthetic equivalents. Waxes are also an important raw material for the cosmetic, pharmaceutical and food industries as well as for various consumer care products and coatings. Cutin monomers, or HFAs, could also be useful as a substitute for the products of saponified castor oil for utilization in lubricating oils, greases, nylon-11, corrosion inhibitors, metal coatings, cosmetics, soaps, plastics and sealants (Dr. Harold L. Durkin, personal communication). Certain HFAs, which are abundant in cutin, could be substitutes for the commercially well-established ricinoleic acid or its hydrogenated product, 12-hydroxystearic acid. Neither the technology nor economics of isolating these HFAs or their oligomers from processor wastes has been determined, but there is a possibility that they could be produced at a lower cost than current sources. For all these reasons our laboratory has been investigating processes for economically obtaining cutin fragments and HFAs from food-processing wastes.

One approach to acquiring cutin oligomers and HFAs, as well as gaining greater insight into cutin's structure [2], involves the utilization of bacterial esterases with 'cutinase' [3,4] activity to partially depolymerize the waste cuticle into organic solvent-soluble fractions. Recently, a synthetic cutin has been produced from 16-hydroxypalmitate [2] (16-hydroxyhexadecanoic acid, 16-OH-P) which acts as a cutinase substrate *only* in the presence of cyclomaltoheptaose (β-cyclodextrin, β-CD). In general, cyclodextrins (CDs) increase cutinase activity with naturally occurring cuticular substrates ([2] and unpublished results of Dr. Herve Gerard). One hypothesis for the apparent increase in enzyme cutinase activity is that the CDs bind to the matrix, increase the wettability of the polymer, and thereby increase the affinity of cutinase for this hydrophobic polyester substrate. Another, less likely alternative is related to the fact that cyclodextrins alone can perform various (carbon ester, phosphate ester and amide) hydrolyses [5]. These hypotheses seem reasonable because β-CD and hydroxypropyl derivatives (HP-β-CD) are known to bind primary/secondary aliphatic alcohols [6] and various lipids [7–12], including palmitic acid [10,13,14] (hexadecanoic acid, P), as well as vitamin A palmitate [15] and

chloramphenicol palmitate [16,17]. However, little is known about the interactions, if any, of CDs with cutin or cutin-like substances (HFAs with a terminal hydroxyl group) such as 16-OH-P or polyesters (synthetic cutin) derived therefrom.

We report herein an investigation into the binding of HP-β-CD, β- and α-CD with 16-OH-P, P or synthetic cutin under conditions coincident with maximal cutinase activity (pH 9, glycine/NaOH buffer) at 25 °C using isothermal titration calorimetry (ITC). We have chosen ITC because it is one of the few direct methods available to determine if binding occurs in heterogeneous systems. ITC quantifies the change in the heat of the system and eliminates the need for partitioning techniques (e.g., equilibrium dialysis) or for ligands to have spectroscopic properties ($\Delta\delta$ for NMR, $\Delta\lambda$ for UV) which differ in the free and bound states [18,19]. The heat 'signal' [20] measured by ITC is an ubiquitous property of systems involving binding interactions and makes ancillary techniques for obtaining ΔH (T dependence of K [18,19,21] and assumes $\Delta C_p = 0$ [22]) unnecessary. In fact ITC is the only technique [20,23] that allows simultaneous determination of all binding parameters.

2. Results and discussion

HP-β-CD interactions with 16-OH-P, the synthetic cutin monomer, and P.—In order to broach questions about CD-cutin interactions, we investigated the binding of HP-β-CD with synthetic cutin's water-insoluble monomer, 16-OH-P, under conditions coincident with maximal cutinase activity (pH 9, glycine/NaOH buffer) at 25 °C using ITC. We chose this particular CD because it is more soluble than (ca. eight-fold), and retains most of the binding characteristics of the parent compound [24]. We have worked with 16-OH-P because it is the starting material for producing synthetic cutin and has most of its physical properties (high melting point, 91–99 °C, and hydrophobicity).

Table 1 presents a summary of our results from numerous studies at pH 9 as a function of differing [16-OH-P]:[HP-β-CD] ratios and pretreatment of the waxy monomer matrix (± dialysis against a great excess of buffer) to produce, presumably, the Na^+ salt. Thus, lipid–buffer suspensions of 16-OH-P were made and either dialyzed against a great excess of buffer ([+] designation in tables and figures) or used directly [−]. Many of the results presented herein express the titration treatments in terms of the ratio of [CD] to [lipid], or vice versa. This is done because, in ITC experiments, no single concentration of reactants is constant. The initial reactant concentration in the sample cell (lipid) becomes more dilute as titrant (CD) is added and the CD becomes more concentrated. The average of 5 [+] to 10 [−] sets of ITC experiments (Table 1: 16-OH-P [−]: $\Delta H = 19$ kJ mol^{-1}, $\Delta G = -18$ kJ mol^{-1}, $K = 1779$ L mol^{-1}, $\Delta S = 124$ J mol^{-1} K^{-1}; 16-OH-P [+]: $\Delta H = 42$ kJ mol^{-1}, $\Delta G = -20$ kJ mol^{-1}, $K = 3022$ L mol^{-1}, $\Delta S = 206$ J mol^{-1} K^{-1}) show that pretreatment by dialysis resulted in about a two-fold greater ΔH, probably due to solubility differences. There was no obvious trend in the variation (10–20%) of ΔH, ΔG and ΔS as a function of lipid concentration and the variation observed seems to reflect the error induced by deconvolving K and ΔH from data of highly different qualitative features which result from varying reactant concentrations. At a lower pH (6, 100 mM Na^+ phosphate buffer; 16-OH-P [+] with HP-β-CD) ITC and NMR experiments indicated that no CD complex was formed inasmuch as ΔQs were not significantly different than for controls and no complexed lipid was found to be in solution (^{13}C NMR, data not shown).

Fig. 1 shows typical derivative curves, normalized to their respective ΔHs, for two calorimetric titrations of widely different lipid concentrations ([16-OH-P]:[HP-β-CD] = 0.8 and 8, upper and lower curves, respectively) as a function of increasing [HP-β-CD]. The major difference between these two fits is apparent stoichiometry (0.8 [+], $n = 0.84 \pm 0.02$; 8 [−], $n = 0.03 \pm 0.001$). Under heterogeneous conditions n is a parameter related more to fractional binding and solubilization than true stoichiometry. However if the 16-OH-P matrix *fully* dissolves (i.e., completely complexed by the CD) n would represent the true stoichiometric ratio of lipid:CD at equilibrium. The lipid samples from the experiments depicted in Fig. 1 were not completely solubilized therefore n represents the molar fraction of the 16-OH-P matrix, which was bound and solubilized by the CD assuming $n = 1$, the most common [25] CD•host stoichiometry. Fig. 2 shows the ^{13}C NMR spectrum (inset: titration results) of a solution of HP-β-CD which had been presaturated ([16-OH-P]:[HP-β-CD] ~60) with 16-OH-P. Integration of the anomeric region (representing 7 Cs)

Table 1
Thermodynamic parameters derived from isothermal titration calorimetry concerning the binding, and subsequent solubilization, of 16-hydroxypalmitate (16-OH-P) by hydroxypropyl-β-cyclodextrin (HP-β-CD) at 25 °C in glycine buffer (pH ca. 9)

[16-OH-P]:[HP-β-CD][a]	ΔH/kJ mole^{-1}		ΔS/J mole^{-1} K^{-1}		ΔG/kJ mole^{-1}	
	+[b]	−	+	−	+	−
0.1	33 s[c]	12 s	181	107	−21	−20
ε[d]	2	1	2	1	0.5	0.4
0.2	48 s	22 ns	227	134	−20	−18
ε	2	2	1	1	0.3	0.4
0.3	44 s	—	213	—	−20	—
ε	3	—	2	—	1	—
0.4	50 s	—	229	—	−18	—
ε	3	—	2	—	0.5	—
0.6	—	2.3 ns	—	133	—	−17
ε	—	4	—	2	—	1
0.8	34 ns	16 ns	181	113	−20	−18
ε	1	1	1	1	0.4	0.3
1	—	24 ns	—	135	—	−16
ε	—	2	—	1	—	0.3
2	—	19 ns	—	127	—	−19
ε	—	1	—	1	—	0.3
3	—	13 ns	—	107	—	−19
ε	—	1	—	2	—	1
4	—	16 ns	—	117	—	−19
ε	—	1	—	1	—	0.3
6	—	19 n	—	123	—	−18
ε	—	2	—	2	—	1
8	—	22 ns	—	140	—	−20
ε	—	1	—	2	—	1
Average	41.8	18.6	206.3	123.8	−19.7	−18.3
S.D.	7.9	4.2	23.9	12.1	1.0	1.2

[a] [HP-β-CD]$_{final}$~9.6 mM.
[b] Pretreatment of the starting material: + dialyzed, − not dialyzed.
[c] s = solubilized; ns = not completely solubilized.
[d] ε = asymptotic standard error.

and the 16-OH-P resonances (representing the 15 non-carbonyl Cs, marked by stars) indicated that the ratio of CD:lipid was ca. 1:1 ([16-OH-P]:[HP-β-CD] = 0.96). The NMR-determined apparent stoichiometry was validated by ITC experiments (five samples, Table 1, 's') which indicated that $n = 1.02 \pm 0.03$. A similar finding was noted for HP-β-CD•palmitate complexes (P [−]: $n = 1.3 \pm 0.1$; $\pm\varepsilon$; only the lowest [P]:[CD], 0.1, was fully solubilized).

Other NMR data (Table 2) indicate that as the ratio of [16-OH-P]:[HP-β-CD] increased from 0 to 0.4 (representing the four soluble [+] samples, Table 1) the change in chemical shift ($\Delta\delta$) gradually increased and saturated (50–90%) at 0.3–0.4. The $\Delta\delta s$ for the most downfield anomeric resonance showed a maximal change ($\Delta\delta_{max}$) of 0.56 ppm, while those of the other anomeric resonance were significantly greater ($\Delta\delta_{max} = 0.93$ ppm). Lesser chemical shift perturbations were noted for the other major CD resonances not associated with the hydroxypropyl substituents ($\Delta\delta_{max} = 0.31$ ppm). 16-OH-P chemical shifts did not alter as all the lipid present was complexed. These NMR data indicate that 16-OH-P resides in the CD cavity inasmuch as the anomeric Cs, which are situated in the middle [26] of the CD's torus, experienced the largest perturbations in chemical shift.

As a comparison to the 16-OH-P data (Table 1) we performed several experiments ([P]:[HP-β-CD]:

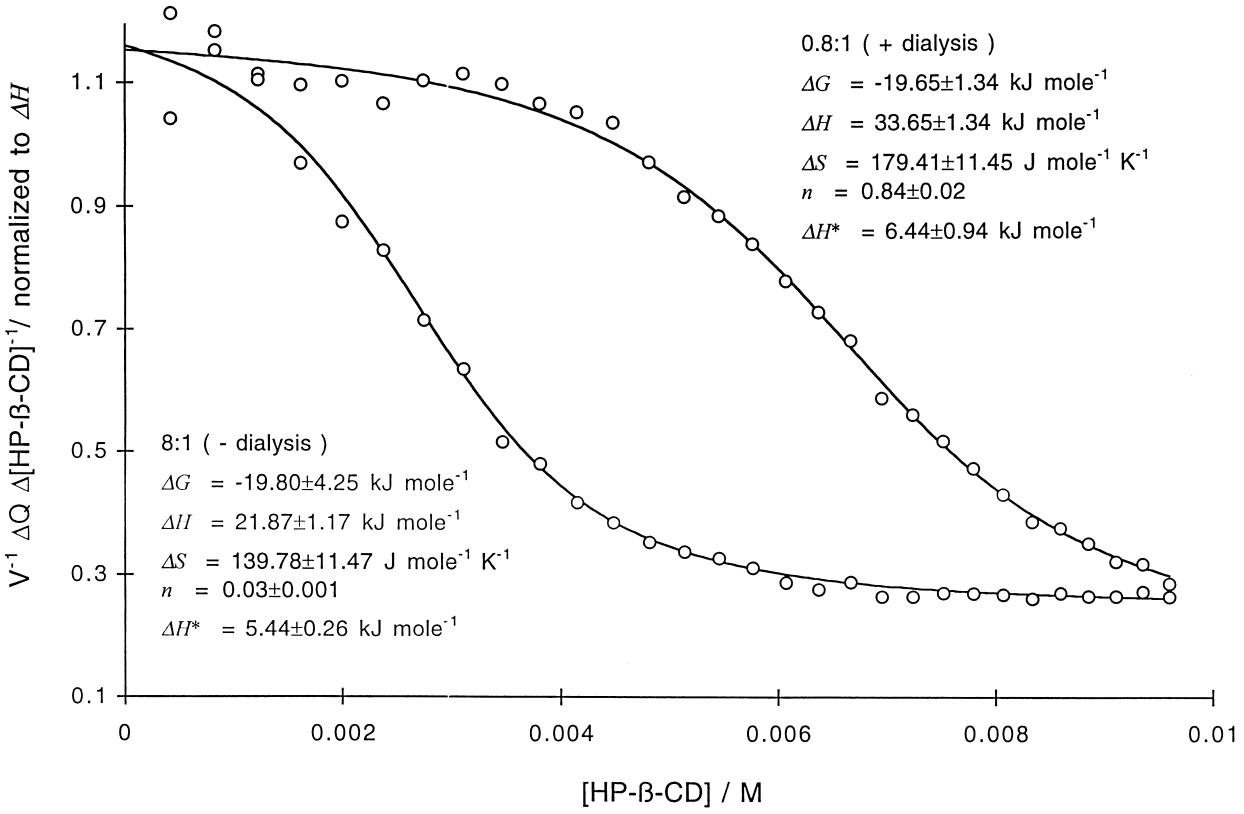

Fig. 1. Plot of ΔQ, in the derivative form, as a function of [HP-β-CD]; $[CD]_{final}$ 9.6 mM. In the top derivative fit [16-OH-palmitic acid]:$[HP\text{-}\beta\text{-}CD]_{final}$ was ca. 0.8, whereupon 16-OH-P was predialyzed [+] against a great molar excess of glycine/NaOH buffer at pH 9. In the lower derivative curve the final molar ratio of reactants was ca. 8 and not predialyzed [−]. Data represent the mean of two ITC experiments performed at 25 °C.

0.1, 0.2, 0.6, 1, 2, 6 and 8) with palmitic acid (Table 3, mp ~63–64 °C). We assumed that HP-β-CD would bind, and solubilize, P since randomly methylated-β-CD [10,13,14] has been used to this end with C_{14} fatty acids and P, under different conditions, for the purpose of studying fatty acid metabolism by *Mycobacteria* spp. We observed an outcome similar to 16-OH-P with regard to binding affinity (P [−]:ΔG −18 kJ mol^{-1}) under the same conditions but with a somewhat smaller ΔH (P [−]:ΔH = 13 kJ mol^{-1}, ΔS = 103 J mol^{-1} K^{-1}). However, during the course of our investigations we noticed a large background endothermic process (Fig. 3) for both 16-OH-P and P as HP-β-CD was titrated into the lipid-glycine/NaOH buffer slurry; this phenomenon was accounted for in our curve-fitting model by an additional parameter, ΔH^*. Fig. 3 displays both raw data (inset) and the resultant curve fit to the ΔH^*-modified equation (Experimental) after subtracting out the heat of dilution/mixing of HP-β-CD ($\Delta Q_{control} = -231 \pm 41$ μJ) for P and 16-OH-P (final concentration of lipid:CD ~8). The most significant, and surprising, difference in the behavior of these two lipid–buffer systems is shown in Fig. 3 ([lipid]:[HP-β-CD] levels ~8). At this elevated level of lipid (ca. 21 mg mL^{-1}), the shape of the 16-OH-P [+] titration curve was mainly modulated by the normal isothermal (endothermic) process, $\Delta H_{binding} + \Delta H_{solution}$. However, the P [−] (ca. 20 mg mL^{-1}) data were completely dominated (Fig. 3, inset: marked P) by ΔH^* (4933 ± 296 J mol^{-1}) inasmuch as the individual calorimetric titration peaks were all virtually identical. Similar results were noted for [P]:[HP-β-CD] ~6 (ΔH^* = 5641 ± 624 J mol^{-1}). Thus, ΔH^* appears not to be directly related to $\Delta H_{solution}$ since ΔH^* was equivalent in either sample (Fig. 3) and very little of the P•CD complex was solubilized (^{13}C NMR, data not shown).

We have normalized ΔH^* to ΔH so that 16-OH-P ([−] and [+]) results can be shown on the same scale (Fig. 4). As the [CD]:[16-OH-P] ratio rose to about 2, $\Delta H^* \div \Delta H$ was observed to verge upon 0 (−0.009 ± 0.026, $\Delta H^* = -0.51 \pm 0.83$ kJ mol^{-1}). At a high [16-OH-P] ΔH^* approached 25–35% of

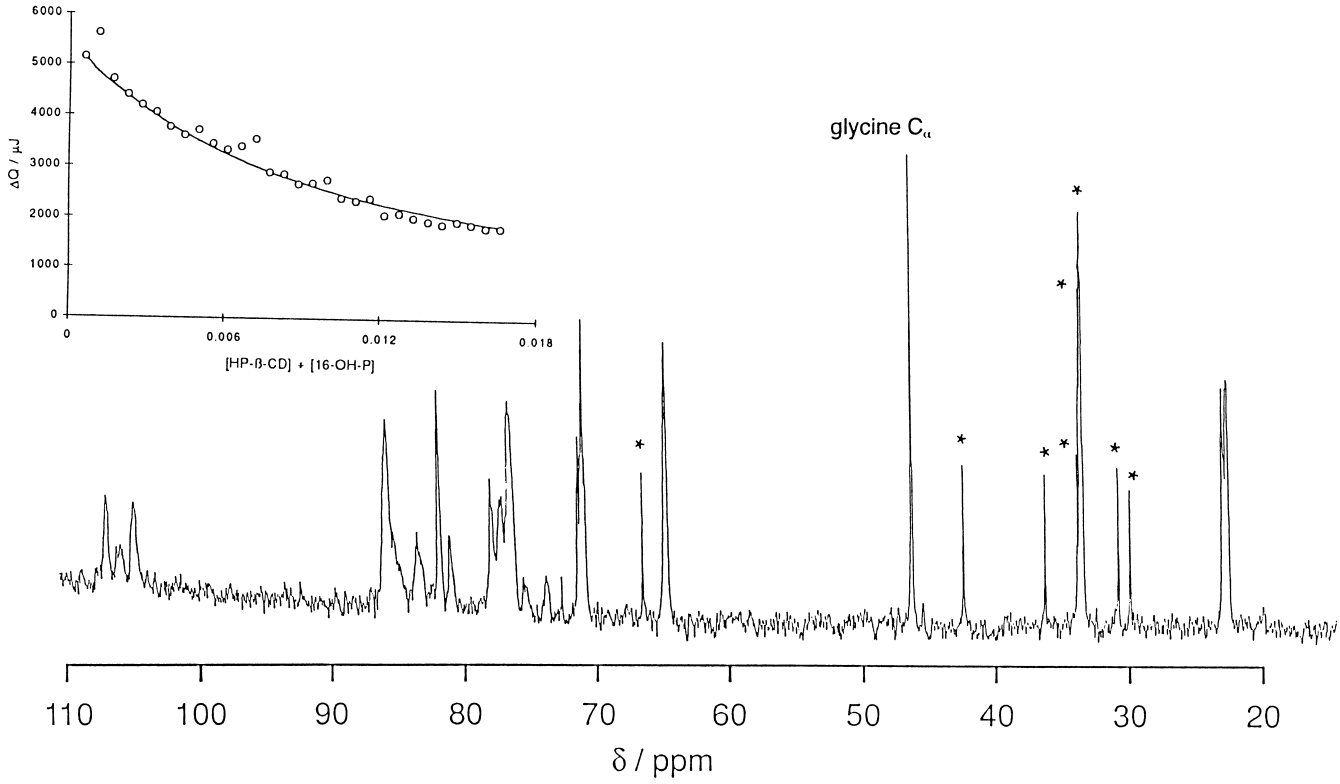

Fig. 2. ^{13}C NMR (ca. 100 MHz) spectrum of a ca. 1:1 molar complex of HP-β-CD•16-OH-palmitic acid [−] that resulted (after removal of solids) from the titration (inset) of 40 mM CD into the buffer/lipid (pH 9). Stars mark the 16-OH-P resonances. Chemical shifts relative to the α C of glycine (ca. 46 ppm). Inset: plot of ΔQ as a function of [CD]:[lipid]; $[CD]_{final}$ 9.6 mM, [16-OH-P]:$[HP\text{-}\beta\text{-}CD]_{final}$ ca. 60.

ΔH ($27 \pm 5\%$, $\Delta H^* = 4.66 \pm 0.47$ kJ mol^{-1}). This secondary process, evidenced by ΔH^*, seems to be related to the formation of another, perhaps insoluble, HP-β-CD•lipid complex (Fig. 4, inset) since 16-OH-P [−] data (circles) show a typical saturation curve, or adsorption isotherm [27], as a function of the unsolubilized lipid matrix concentration, $\{1-n\}\times$[16-OH-P]. ΔH^* data for P [−] show a similar lipid saturation response (diamonds). All these data indicate that there was a weak interaction between the titrant, HP-β-CD, and the lipid matrix which cannot be accounted for by titrating HP-β-CD into the buffer alone (e.g., the control titration).

Knowing the stoichiometry ($n = 1$) and assuming that n, in an incompletely solubilized lipid–CD system, is related to fractional molar binding and subsequent solubilization of the waxy matrix by the CD, we have estimated the concentration in the solution phase of the HP-β-CD•16-OH-P complex in the ITC cells at the end of each titration series (Fig. 5; $n \times [16-OH-P]_{final}$). Generally, the predialyzed [+] lipid sample was far more soluble, by these criteria, than its undialyzed [−] counterpart. Thus, as the 16-OH-P [+] concentration (Na^+ salt) in the ITC cells was increased by 1 unit in concentration the soluble complex increased by 0.84. Contrariwise, the unpretreated lipid•CD complex gradually increased to about 2.4 mM and leveled off. The inset graph in Fig. 5 shows the relationship of n for both treatments with respect to [16-OH-P]:[CD] ratio and clearly demonstrates that as [16-OH-P]:[CD] approaches 0.5, n approaches unity. These data support our assertion that n is related to fractional solubilization (e.g., $n \times [16\text{-OH-P}]_{final} \sim [16\text{-OH-P}]_{solution} \sim [HP\text{-}\beta\text{-CD}\bullet 16\text{-OH-P}]$).

Interaction of synthetic cutin with β-, *HP*-β- *and* α-*CD using ITC*.—Based upon the promising results (1:1 stoichiometry and large ΔH) of experiments on the synthetic cutin monomer, we investigated the binding of HP-β-CD, β- and α-CD with synthetic cutin in glycine/NaOH buffer (pH 9) at 25 °C using ITC. Upon extensive testing HP-β-CD titrations were observed to display no substantial heats of binding when synthetic cutin ($\Delta Q = -240 \pm 61\ \mu J$; $\Delta Q_{control} = -231 \pm 31\ \mu J$) was used as a substrate; a similar result was found

Table 2
Changes in ^{13}C chemical shift ($\Delta\delta$) for HP-β-CD as a function of 16-hydroxypalmitate (16-OH-P) concentration

[16-OH-P]:[HP-β-CD]	Anomeric 1		Anomeric 2		Ave. $\Delta\delta \pm$ S.D.	Other[a]	
	δ/ppm[b]	$\Delta\delta$/ppm	δ/ppm	$\Delta\delta$/ppm		δ/ppm	$\Delta\delta$/ppm
0	105.86	0	103.41	0	0 ± 0	81.16	0.00
0.1	105.89	0.04	103.68	0.27	0.16 ± 0.17	81.26	0.10
0.2	105.99	0.14	103.81	0.40	0.27 ± 0.19	81.37	0.21
0.3	106.17	0.31	103.88	0.47	0.39 ± 0.11	81.44	0.28
0.4	106.11	0.26	104.06	0.65	0.46 ± 0.28	81.41	0.26
60[c]	106.42	0.56	104.34	0.93	0.75 ± 0.26	81.46	0.31

[a] Average of the five major resonances between 77 and 85 ppm.
[b] All chemical shifts are relative to the α C of glycine.
[c] This treatment was not predialysed against glycine/NaOH buffer.

when titrating with β-CD in both synthetic and apple cutin buffer suspensions. However, a weak interaction (Fig. 6: $K = 119$ L mol^{-1}, $\Delta H = 1.11$ kJ mol^{-1}, $\Delta S = 43.44$ J mol^{-1} K^{-1}, $n = 0.28$) was observed when α-CD (100 mM) was titrated into a synthetic cutin (ca. 8 mg in 760 μL) suspension. In Fig. 6 the signal-to-noise is poor because ΔH is at the very limit of detection (e.g., $\Delta Q - \Delta Q_{control} \leq$ca. 100 μJ) whereupon both K (119 L mol^{-1}) and ΔH ($\pm$1 kJ mol^{-1}) are small and ITC quantification of binding becomes problematic. Assuming a true 1:1 stoichiometry ($n = 1$) and that the average degree of polymerization (DP) of the substrate was ca. 5 (by size-exclusion HPLC and TLC analyses, data not shown), our value of n indicated that only 28% (on a molar basis) of the total synthetic cutin bound, or interacted with, α-CD. Thus, either β-CDs do not bind to the insoluble cutin matrix or they do but with a vanishingly small ΔH and K. That HP-β-CD does bind with the synthetic cutin monomer heterogeneously and extremely weak binding was observed in the α-CD•synthetic cutin system tends to argue for the latter interpretation.

Table 3
Thermodynamic parameters, derived from ITC experiments, concerning the binding of palmitic acid (P) to hydroxypropyl-β-cyclodextrin (HP-β-CD) at 25 °C in (ca. pH 9) glycine buffer

[P][a]				
[HP-β-CD]	ΔH/kJ mole^{-1}		ΔS/J mole^{-1} K^{1}	ΔG/kJ mole^{-1}
0.1	16	s[b]	117	−19
ε[c]	1		1	0.4
0.2	13	ns	110	−20
ε	1		2	1
0.6	14	ns	103	−17
ε	1		2	1
1	8	ns	85	−17
ε	1		4	1
2	14	ns	99	−16
ε	3		3	1
Average	13		103	−18
S.D.	3		12	2

[a] [HP-β-CD]$\sim$9.6 mM.
[b] s = solubilized; ns = not completely solubilized.
[c] ε = asymptotic standard error.

Comparison of HP-β-CD•16-OH-P [+/−],•P [−] and •synthetic cutin [−] thermodynamic parameters to literature values [28] for various α- and β-CD•guest adducts: enthalpy–entropy compensation.—Enthalpy–entropy compensation [29] is observed as an induced shift in ΔH resulting in a compensatory perturbation in ΔS. Regardless of how $\Delta H - \Delta S$ pairs are created (pH changes, alteration in host/guest species, etc.), all methods should produce only one linear relationship for each type of chemical interaction (e.g., CD•guest complexation being just one such class). Such an enthalpy–entropy compensatory process is believed to be driven by, according to Lumry and Rajender [29], either temperature-independent heat-capacity changes ($\Delta C_p \neq 0$ [22] and is constant with T) and/or alterations in the concentrations of at least two 'phenomenologically significant' types of H_2O, whereupon one population is associated with the bulk solution and the other is actively involved [19] in the process under investigation. In relatively simple processes, such as CD•guest adduct formation, where perturbations in the solvation of both host and guest species play a role in determining complex stability [19], plots of $T\Delta S - \Delta H$ pairs should result in a straight line with a slope, α, equal to about one [28]. Recently, Inoue and co-workers [28] have argued that an α approaching unity indicates that CD inclusion complexation induces substantial conformational changes involving the global reorganization of the original hydrogen-bond network within the CD molecule. A related

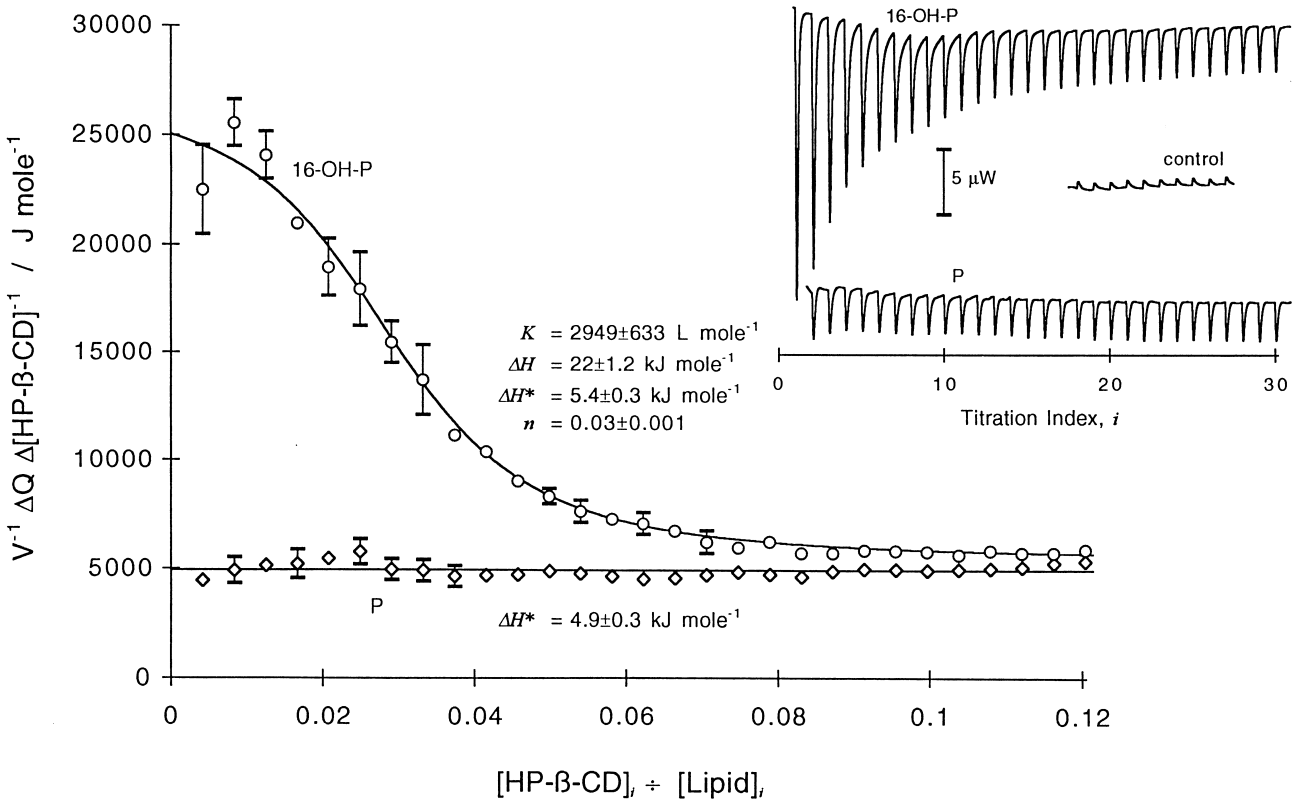

Fig. 3. Plot of ΔQ, in the derivative form, as a function of the molar ratio of [HP-β-CD]:[lipid]. In both curves [lipid]:[HP-β-CD]$_{final}$ was ca. 8. Circles, lipid = 16-OH-palmitic acid; diamonds, lipid = palmitic acid. Neither treatment was predialyzed against buffer [–]. Data represent the mean of two ITC experiments performed at 25 °C. Inset: raw data obtained for 30 automatic injections, each of 8 μL with 40 mM HP-β-CD, into the sample cell containing the lipid/buffer (pH 9) suspension or buffer alone (control).

parameter, $T\Delta S_0$ (intercept of the $T\Delta S$ vs ΔH scatter plot), is believed to be a measure of the CD's relative desolvation upon complex formation [28].

Fig. 7 shows $T\Delta S$ plotted against ΔH for our system (least-squares analysis {solid line}: $\alpha = 1.03$, $T\Delta S_0 = 17.72$ kJ mol^{-1}) as well as numerous values observed or cited by Inoue and co-workers [28] (least-squares analysis {dashed line}: $\alpha = 0.92$, $T\Delta S_0 = 15.11$ kJ mol^{-1}, r^2 0.76). The linear-regression analysis of all the data in Fig. 7 gives an α of unity and intercept, $T\Delta S_0$, of 16.76 kJ mol^{-1} (r^2 0.9). The thermodynamic parameters associated with the heterogeneous phase lipid•CD adduct formation, reported herein, show a remarkably tight grouping about the line of best fit (r^2 0.99). Clearly our values of ΔH (12 to 50 kJ mol^{-1}, excluding α-CD•synthetic cutin) and $T\Delta S$ (ca. 21 to 68 kJ mol^{-1}) were significantly larger than those reported elsewhere for diverse CD•guest complexes (ΔH ca. −50 to 0 kJ mol^{-1}, $T\Delta S$ ca. −40 to 20 kJ mol^{-1}). Most of the CD•guest studies reported in the literature [25,28] involve homogeneous phase binding reactions whereas, in our studies, we have measured solid-phase binding and subsequent movement of the CD•lipid complex into the solution phase. Since our enthalpy–entropy compensation analysis was close to homogeneous phase calculations we believe the magnitude of our thermodynamic findings reflect both binding and solubilization of the CD•lipid complex. Thermodynamically, any chemical process may be considered as the summation of 'part processes' [29]. Thus, for our system, these parts should be made up of a 'chemical', 'solvation' as well as a solid-to-solution phase part process. This additional process resulted in $T\Delta S - \Delta H$ data being significantly shifted in magnitude yet displaying normal CD adduct enthalpy–entropy compensation behavior (α near unity and large $T\Delta S_0$).

Based upon our $T\Delta S - \Delta H$ calculations (α 1.03, $T\Delta S_0$ 17.72 kJ mol^{-1}) and assuming that ΔH^* (4836 ± 531 J mol^{-1}: average of 5 16-OH-P [–] and 2 P [–] ITC experiments) at near saturation (Fig. 4, inset) represents HP-β-CD intercolation into the solid phase, we can estimate the solid-phase

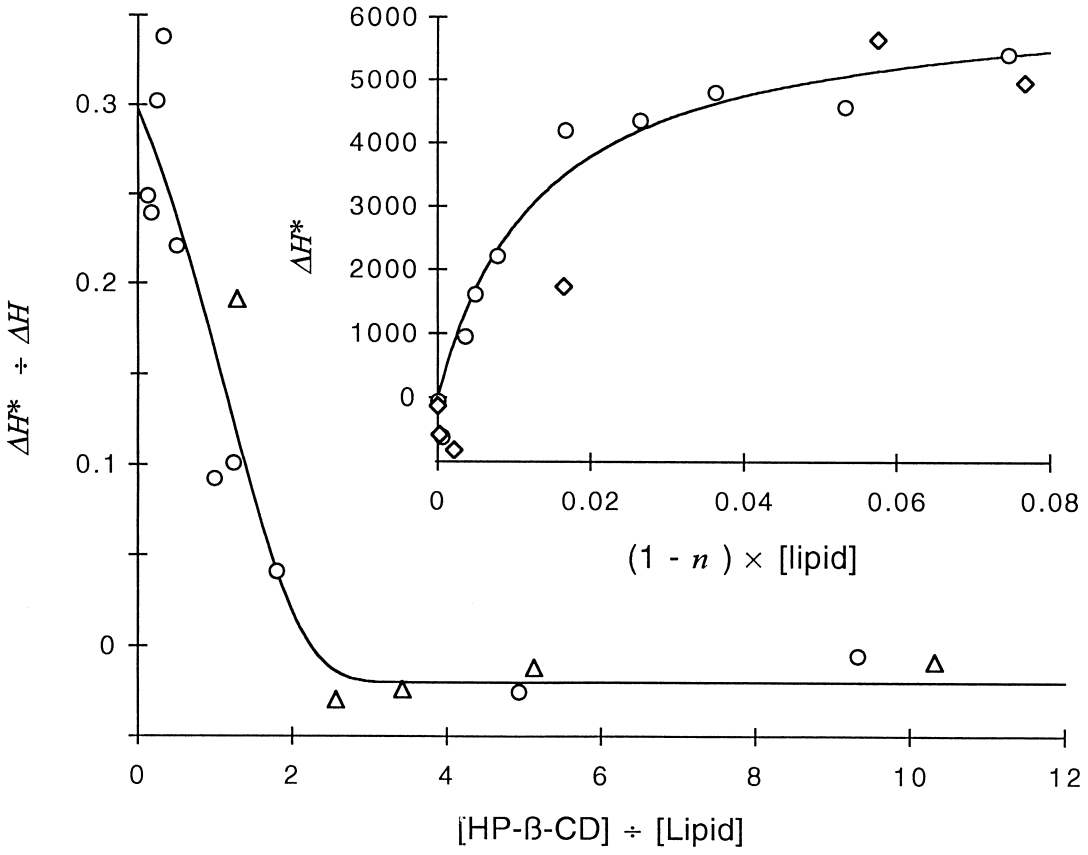

Fig. 4. Normalized ΔH^* dependency on ITC reactant concentration. Circles: 16-OH-palmitic acid [−]; triangles: 16-OH-palmitic acid [+]. Inset: dependency of ΔH^* on the fraction of unsolubilized lipid matrix, $(1-n)\times$[16-OH-P]. Diamonds: palmitic acid [−].

binding thermodynamic parameters ($\Delta S^* = 76$ J mol^{-1} K^{-1}, K^*1358 L mol^{-1}) associated with the 16-OH-P•HP-β-CD system. These values ($T\Delta S^* = 22.7$ kJ mol^{-1}, $\Delta H^* = 4.8$ kJ mol^{-1}; Fig. 7), when taken in the context of the enthalpy–entropy compensation plot of all data, then approach the upper end of the homogeneous phase [28] data pairs. Similarly, the α-CD•synthetic cutin (solid-phase binding only; Fig. 7, arrow) $T\Delta S - \Delta H$ pair resides at the higher end of the literature [28] CD•host enthalpy–entropy compensation scatter plot. These observations argue that solid-phase HFA-based CD interactions involve only chemical and solvation part processes with relatively small ΔHs of binding (1 to 5 kJ mol^{-1}). If this hypothesis is true then the solubilization of the HP-β-CD•16-OH-P or •P complexes involves a considerable endothermic component ($\Delta H_{solution} = 14$ to 37 kJ mol^{-1}).

3. Conclusions

The HFA 16-OH-P, which is the monomer for synthetic cutin, is bound by (Tables 1 and 2, Figs. 1 and 3) HP-β-CD and subsequently solubilized (Figs. 2 and 4). Both 16-OH-P and P show a secondary or background (Fig. 3) process, possibly solid-phase binding or CD intercalation within the lipid matrix without comcomitant solubilization, which is no more than 30% (Fig. 5) of the primary process (solid-phase binding and solubilization) and is lipid-concentration dependent (Fig. 5, inset). Lastly, α-CD weakly binds to synthetic cutin (Fig. 6) with a small ΔH. Based upon the enthalpy–entropy compensation behavior of various literature [28] α- and β-CD•guest complexes, we hypothesize that the major contribution to the P• and 16-OH-P•CD ΔHs, reported herein (Fig. 7), is solubilization. All these results argue that CDs do bind to solid lipid matrices, such as synthetic cutin and its monomer, but with a small ΔH (1 to 5 kJ mol^{-1}).

4. Experimental

General.—β-CD was donated by the Cerestar USA (1100 Indianapolis Blvd., Hammond, IN 46320-1094, USA). Hydroxypropyl-β-CD (HP-β-CD,

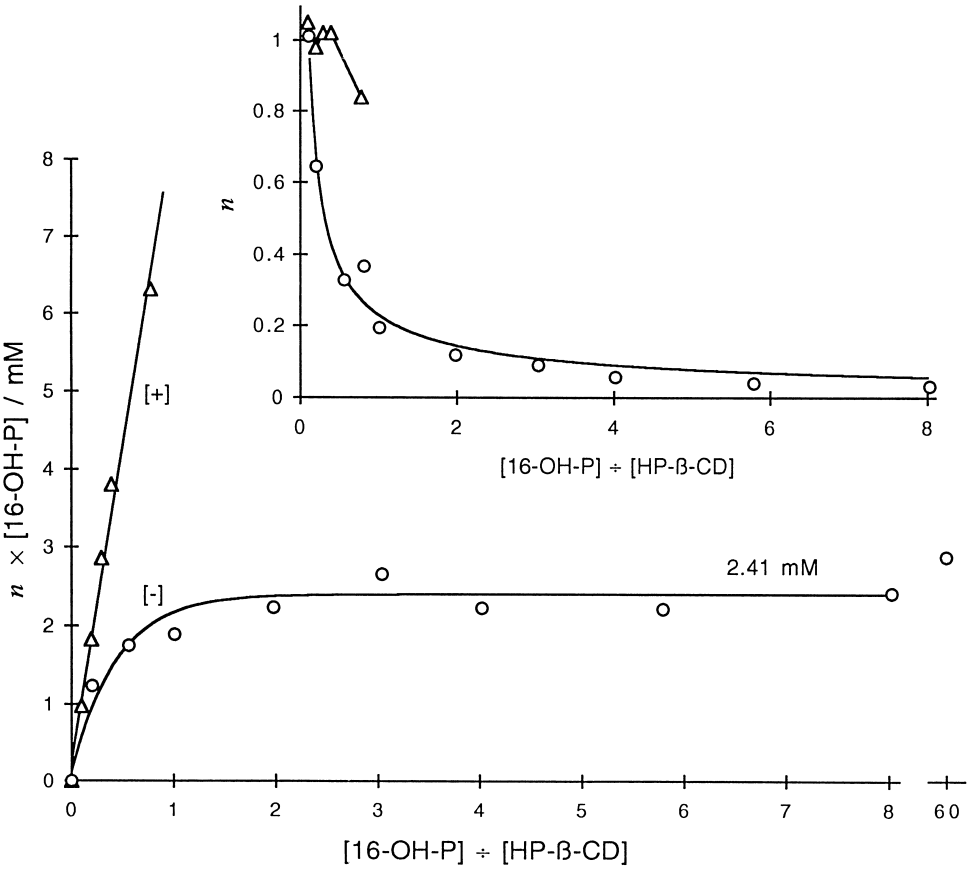

Fig. 5. Estimated 16-OH-palmitic acid•HP-β-CD complex concentration in solution phase as a function of ITC reactant concentration. Circles: 16-OH-palmitic acid [−]; triangles: 16-OH-palmitic acid [+]. Inset: plot of calculated n from curve fitting derivative ΔQ data as a function of reactant concentration.

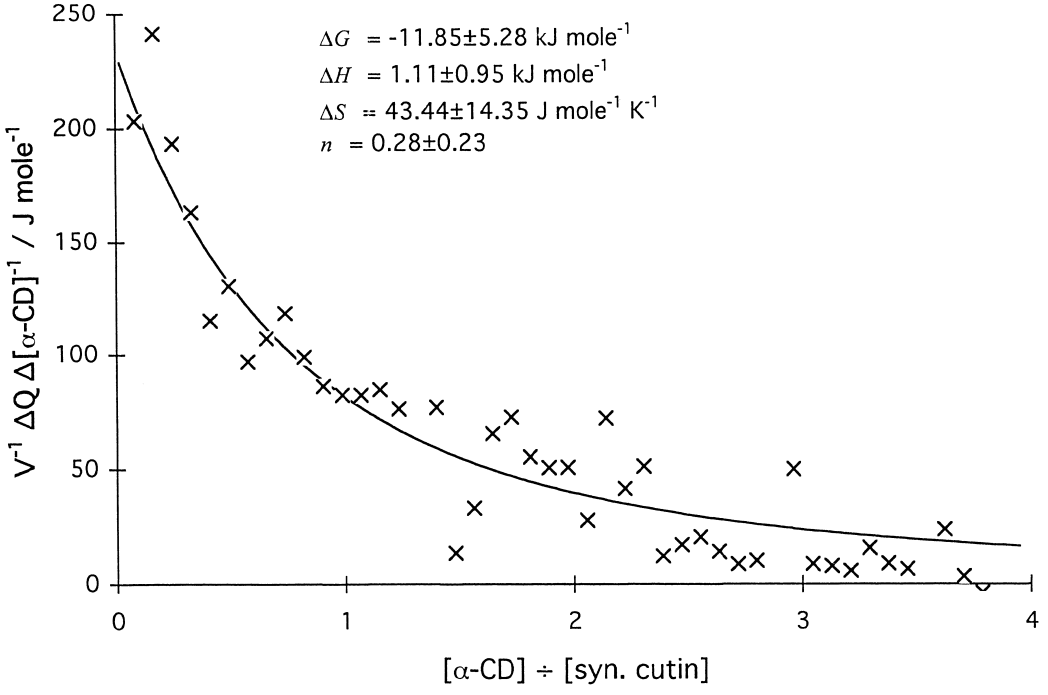

Fig. 6. Plot of ΔQ, in the derivative form, as a function of the molar ratio of [α-CD]:[synthetic cutin]. The synthetic cutin was not predialyzed against glycine/NaOH (pH 9) buffer [−].

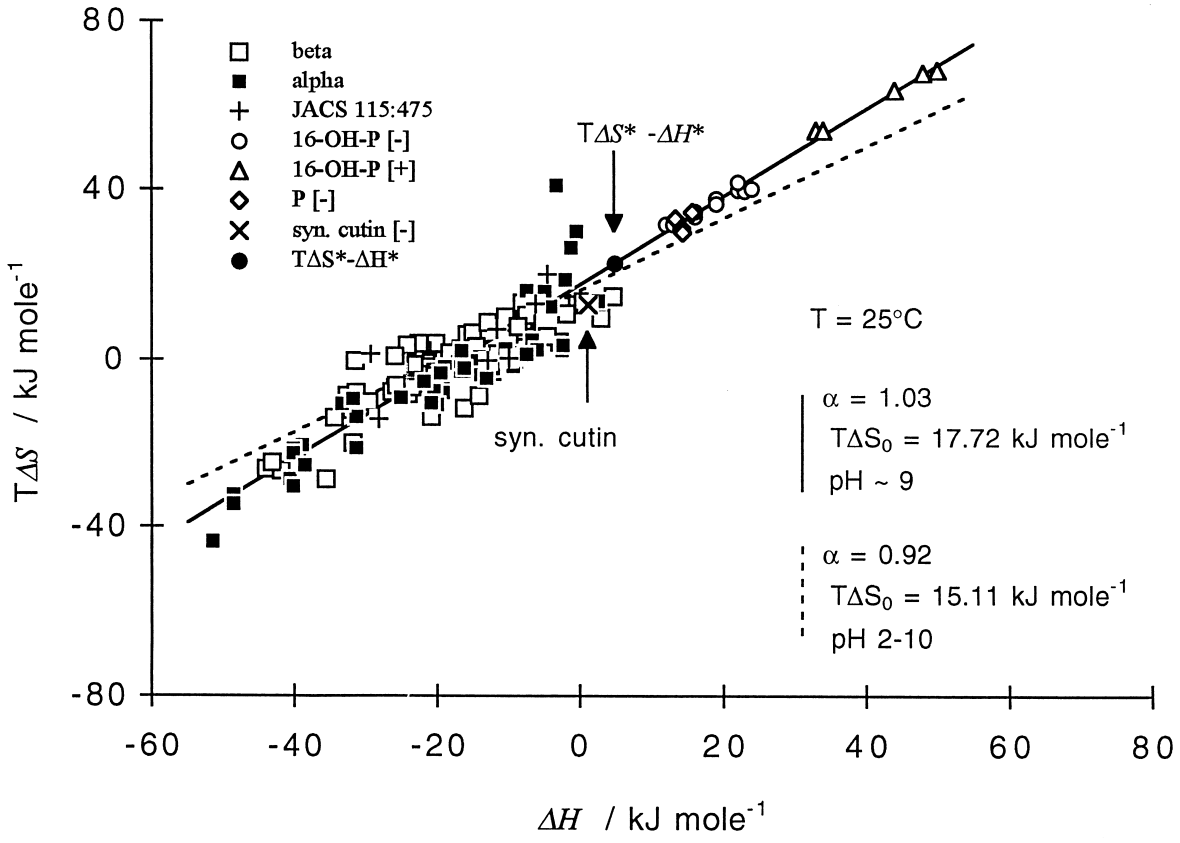

Fig. 7. Enthalpy–entropy compensation plot for various CD•guest complexes from the literature as well as all CD•lipid data reported herein. Symbols: literature [28] (β-CD), open squares; literature [28] (α-CD), closed squares; Inoue and co-workers [28], +; HP-β-CD•16-OH-palmitic acid [−dialysis], circles; HP-β-CD•16-OH-palmitic acid [+ dialysis], triangles; HP-β-CD•palmitic acid [−dialysis], open diamonds; α-CD•synthetic cutin [−dialysis], ×; $T\Delta S^* - \Delta H^*$ data (average of 7 ΔH^*s), closed circles. α and $T\Delta S_0$ from least squares analysis: solid line, CD•lipid data; dashed line, all data from [28].

MW 1500) was purchased from Aldrich Chemical Company (Cat. # 33,260-7). Palmitic acid (P, hexadecanoic acid), 16-hydroxypalmitic acid (16-OH-P, 16-hydroxyhexadecanoic acid) and α-CD were purchased from Sigma Chemical Company. The linear polyesters of 16-OH-P (average dp ~5 by size-exclusion HPLC and TLC analysis) were synthesized as described previously [2].

^{13}C NMR spectroscopy.—All studies were performed on a Varian Unity Plus NMR spectrometer operated at 9.4 T (100.56 MHz for ^{13}C). Typical spectrometer conditions were: 60 K data points, 25 kHz spectral width, 12.6 μs pulse width and 2.7 s recycle time; 20,000 transients were collected. Chemical shifts were assigned relative to glycine's α C (ca. 46 ppm). Samples were prepared for NMR analysis by combining suspensions (typically ca. 2 mL) from ITC experiments, spinning out any solids, drying under N_2, and dissolving in ca. 0.7 mL of 99.9 atom % D_2O.

Calorimetry.—These studies were performed on a Calorimetry Sciences Corporation (CSC, PO Box 799, Provo, UT 84603-0799) 4200 ITC. This device was checked for precision and accuracy by chemical calibration (25 °C): 2×10 titrations with 10 μL of 1 mM HCl into 250 mM TRIS buffer; observed ΔH: -44 ± 4 kJ mol^{-1}; theory [30]: -47 kJ mol^{-1}.

All experiments were performed in either glycine/NaOH (ca. pH 9: 50 mM glycine, 8.8 mM NaOH and 3 mM NaN_3) or phosphate (ca. pH 6: 1 vol of 0.2 M NaH_2PO_3 + 1.1 vol of 0.2 M Na_2HPO_3 and diluted to 4.2 vol with H_2O, 3 mM NaN_3) buffer. In these studies lipid–buffer suspensions of P, 16-OH-P or synthetic cutin (5–10 mL of 12–30 mg mL^{-1}) were made and either dialyzed against 5 L of buffer ([+] designation in tables and figures) or, as was usually the case, used directly [−]. All the buffer/lipid slurries were sonicated to induce the breaking up of large particles. The lipid suspensions were dispensed into the ITC sample cell (760 μL buffer in the reference cell) in 10–760 μL aliquots (pipetted while vigorously stirring), depending on the concentration desired, with the balance of the volume (760 μL total) made up

with buffer alone. All experiments with HP-β-CD as the titrant (40 mM in buffer) involved 30 titrations of 8 μL each (final concentration ca. 9.6 mM). α-CD experiments (8.1 mg of synthetic cutin [–]) involved titration (48 injections: 5 μL each of 100 mM) in glycine/NaOH buffer to a total final vol of 1 mL. Raw data were collected at a rate of one point every 4.5 s (1800 s between injections).

Raw data (dQ/dt, in units of μW, versus time) from titrations were integrated

$$\int_{t_0}^{t_f}\left(\frac{dQ}{d}\right)dt - \Delta Q_{\text{control}} = \Delta Q,$$

and the resultant corrected areas (ΔQ) iteratively fit [31] to an equation [20] describing the derivative ($V^{-1}\Delta Q\Delta[\text{CD}]^{-1}$; V = volume of cell at each ith injection; $\Delta[\text{CD}] = [\text{CD}]_i - [\text{CD}]_{i-1}$) of heat evolved (positive peaks, exothermic) or absorbed (negative peaks, endothermic) with respect to titrant ([CD]) and lipid ([L]) concentration after each injection whereupon

$$\frac{1}{V}\frac{\Delta Q}{\Delta[\text{CD}]} = \frac{1}{2}\left(1 + \frac{K[\text{L}]n - [\text{CD}]K - 1}{K\sqrt{\frac{\{1+[\text{CD}]K+[\text{L}]nK\}^2}{K^2} - 4n[\text{CD}][\text{L}]}}\right) \times \Delta H + \Delta H^*$$

This equation is the most common mathematical model for binding experiments [20,23]: K is the binding constant (assumed to be the same for all sites, n), ΔH is the enthalpy change in the system due to binding (as well as any other event subsequent to this), and n is the stoichiometric coefficient (Wiseman et al. [20] derive this equation for a 1:1 stoichiometry, $n = 1$). ΔH^* is an additional term we have added to correct for any constant background process not accounted for by subtracting out $\Delta Q_{\text{control}}$. ΔQs usually resulted from the average of 2, or more, independent titrations. Knowing [20] K and ΔH from iteratively solving the above relationship, ΔG and ΔS were obtained from

$$\Delta G = -\text{RT}\ \log_e K = \Delta H - \text{T}\Delta S$$
$$\Delta S = \text{R}\ \log_e K + \Delta H/\text{T}$$

We have tested our computational technique, a spreadsheet Gauss–Newton (GN) algorithm developed in our lab [31], against the Levenberg–Marquart (LM; [20,32,33]) method on the same data (titrations of 2′-CMP into RNaseA at 38 °C) using [20] identical mathematical models except that ours incorporated the ΔH^* term (discussed earlier in detail). The LM curve fitting procedure yielded: $\Delta H = -57$ kJ mol^{-1}, $\Delta S = -93$ J mol^{-1} K^{-1}, $n = 1.05$ [20]; the GN [32] procedure gave: $\Delta H = -56 \pm 0.3$ kJ mol^{-1}, $\Delta H^* = -0.4 \pm 0.2$ kJ mol^{-1}, $\Delta S = -87 \pm 3$ J mol^{-1} K^{-1}, $n = 1.11 \pm 0.003$ ($\pm\varepsilon$, asymptotic standard error); the GN procedure with ΔH^* fixed to 0 gave: $\Delta H = -56 \pm 0.5$ kJ mol^{-1}, $\Delta S = -88 \pm 4$ J mol^{-1} K^{-1}, $n = 1.11 \pm 0.005$. All results were in good agreement and argue that our ΔH^*-corrected derivative GN method gives reasonable thermodynamic parameters for ITC data.

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