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Thermodynamics of Hydrogen Bond and Hydrophobic Interactions in Cyclodextrin Complexes

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ABSTRACT Values of K, $\Delta G°$, $\Delta H°$, $\Delta S°$ and ΔC_p° for the binding reaction of small organic ligands forming 1:1 complexes with either α- or β-cyclodextrin were obtained by titration calorimetry from 15°C to 45°C. A hydrogen bond or hydrophobic interaction was introduced by adding a single functional group to the ligand. The thermodynamics of binding with and without the added group are compared to estimate the contribution of the hydrogen bond or hydrophobic interaction. A change in the environment of a functional group is required to influence the binding thermodynamics, but molecular size-dependent solute-solvent interactions have no effect. For phenolic O-H-O hydrogen bond formation, $\Delta H°$ varies from -2 to -1.4 kcal mol^{-1} from 15°C to 45°C, and ΔC_p° is increased by 18 cal K^{-1} mol^{-1}. The hydrophobic interaction has an opposite effect: in α-cyclodextrin, $\Delta C_p^\circ = -13.3$ cal K^{-1} mol^{-1} per ligand -CH_2-, identical to values found for the transfer of a -CH_2- group from water to a nonpolar environment. At room temperature, the hydrogen bond and the -CH_2- interaction each contribute about -600 cal mol^{-1} to the stability ($\Delta G°$) of the complex. With increased temperature, the hydrogen bond stability decreases (i.e., hydrogen bonds "melt"), but the stability of the hydrophobic interaction remains essentially constant.

INTRODUCTION

Electrostatic, hydrophobic, and hydrogen bonding interactions are the principal forces determining the stability of biological macromolecules. In this contribution, we give a thermodynamic profile of binding in a small molecule model system to characterize the temperature-dependent behavior of a hydrogen bond and of a hydrophobic-type interaction. We examine these interactions through a study of the formation of simple 1:1 complexes between small organic molecules and either α- or β-cyclodextrin (hexa- and hepta-amylose, respectively) as a common receptor. Because we introduce only the smallest possible chemical perturbation, that of a single functional group, and because of the relative simplicity of the reacting system, the hydrogen bond or hydrophobic interaction introduced into the complex is reasonably well defined. By comparing the values of the thermodynamic quantities in the presence and in the absence of this additional interaction, many of the common contributions to the thermodynamics in the individual binding reactions (e.g., effects of solvation/desolvation, changes in mole number, loss of translational and rotational entropy) may be largely eliminated. The remaining difference then provides an estimate of the contribution of the hydrogen bond and hydrophobic interaction per se to the thermodynamics of binding in these complexes. The requisite values of the thermodynamic quantities at different temperatures have been obtained by isothermal titration calorimetry. This technique yields the binding constant, K, and the enthalpy change, $\Delta H°$, from which the free energy change, $\Delta G°$, the entropy change, $\Delta S°$, and the change in heat capacity upon binding, ΔC_p°, are obtained. Finally, we consider the implications of our results for the thermal stability of biological macromolecules.

MATERIALS AND METHODS

Ultraviolet absorption spectra were measured with a Varian Cary model 210 spectrophotometer (Mulgrave, Australia), and fluorescence emission spectra were obtained with a Spectronic Instruments model AB II flourometer (Rochester, NY).

Thermal titrations were carried out in the OMEGA reaction cell of a MicroCal MC-2 calorimeter (Northampton, MA). A Keithley 150B amplifier (Cleveland, OH) was incorporated into the cell feedback circuit of the MC-2 to improve performance by reducing short-term noise. The instrument was calibrated electrically at each temperature. In an experiment, 15 7-μl portions of 15 mM ligand were injected into the 1.3-ml thermostatted cell containing 2.5 mM cyclodextrin and stirred at 400 rpm. Injections were for 20 s at 3-min intervals. The heat of diluting the ligand into buffer was obtained in an identical run and subtracted from the ligand + cyclodextrin titration. The cyclodextrins were obtained from Sigma (St. Louis, MO), and the other chemicals were from Aldrich (Milwaukee, WI). These materials were of the highest purity available and were used without further purification. The purity information and moisture content of these materials are given elsewhere (Rekharsky et al., 1995, and manuscript in preparation). All solutions were in 0.05 M Na phosphate buffer at pH = 6.9, where it is known from the pKs that the acids and amines are singly charged species. Using the same stock solution of ligand, a single titration was carried out at each temperature.

The data were analyzed as described by Wiseman et al. (1989), using software supplied with the calorimeter (Yang, 1993). Only those ligands giving an excellent fit to the 1:1 binding model for the reaction

$$\text{Cyclodextrin (aq)} + \text{Ligand (aq)} = \text{Cyclodextrin} \cdot \text{Ligand (aq)} \quad (1)$$

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are reported upon in this paper. In the range of K = 10 to 1000, varying n, K, and $\Delta H°$, the values of n obtained from the fit were always close to unity. Thus, we set n = 1 and fit the titration data to the two variables K and $\Delta H°$. These fits were robust in the sense that removing up to half the data from either end of the set yielded the same values of K and $\Delta H°$. For the range K = 100 to 1000, which encompasses most of the work described herein, two standard deviations of the mean obtained from six experiments over a threefold cyclodextrin concentration range and a 10-fold cyclodextrin-to-ligand mole ratio was ~1% for K and 70 cal mol^{-1} for $\Delta H°$ at 25°C (1 cal = 4.184 J). Therefore, at 25°C, differences between two values of K exceeding 1.5% and $\Delta H°$ exceeding 100 cal mol^{-1} may be regarded as significant. For values of K = 100 to 1000, the uncertainties returned by the Microcal Origin software approximate the true sample standard deviations. In our Tables, twice these uncertainities are reported. They reflect the greater scatter of the experimental points at higher temperatures. The equilibrium constant for reaction 1 is

$$K = a(\text{cyclodextrin} \cdot \text{ligand})/\{a(\text{cyclodextrin})a(\text{ligand})\}, \quad (2)$$

where a is the activity of the species denoted. The hypothetical ideal solution of unit molality was chosen as the standard state for solutes. Because species concentrations were dilute and reaction 1 is charge symmetrical, so that the activity coefficients of the charged species will tend to cancel, we shall assume nonideality corrections to be negligible. The values of $\Delta G°$ and $\Delta S°$ were calculated from the relationships, $\Delta G° = -RT \ln K$ and $\Delta S° = (\Delta H° - \Delta G°)/T$. ΔC_p° was obtained from linear regression of $\Delta H°$ upon T.

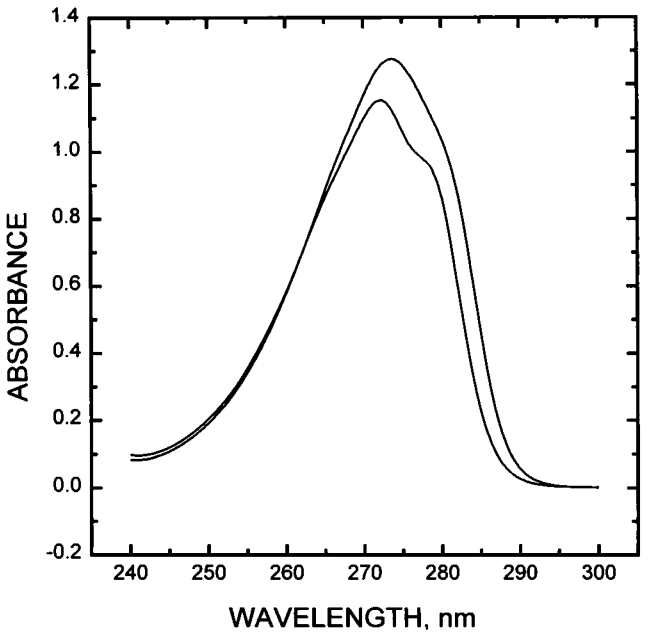

FIGURE 1 Lower curve, Ultraviolet absorption spectrum of tyramine (4-hydroxyphenethylamine); upper curve, tyramine in the presence of β-cyclodextrin. The red-shifted spectra from both absorption and fluorescence emission (not shown) are indicative of the formation of a phenolic hydrogen bond. Concentrations: tyramine, 0.8 mM; β-cyclodextrin, 10 mM; in 50 mM sodium phosphate at pH 6.9, 25°C.

RESULTS

Spectroscopic demonstration of hydrogen bond formation

The ultraviolet absorption spectrum of 4-hydroxyphenethylamine (tyramine) is shifted 2 nm to longer wavelengths in the presence of β-cyclodextrin (Fig. 1). The fluorescence spectrum of tyramine (not shown), excited at 285 nm, is a broad skewed envelope with a peak at 308 nm and a tail approaching baseline at ~370 nm. This spectrum is shifted 1 nm to longer wavelength in the presence of β-cyclodextrin. At the concentrations used, only ~40% of the tyramine interacts with β-cyclodextrin; thus the magnitude of the wavelength shift due to the complex may be about twice as great. In contrast, 4-methoxyphenethylamine, which binds with comparable affinity, K = 93 (Rekharsky et al., 1995), and which cannot form a hydrogen-bond at the 4 position, shows no discernible difference in the emission spectrum in the presence and in the absence of β-cyclodextrin.

Similar shifts to longer wavelengths in the fluorescence emission spectra in the presence of β-cyclodextrin were found with two OH- aromatic substituted derivatives of hydrocinnamate (3-phenylpropionate). 3-(4-Hydroxyphenyl)propionate was red-shifted 1 nm to a peak at 305 nm, and 3-(2-hydroxyphenyl)propionate was red-shifted 1.6 nm to a peak at 301 nm (data not shown). The combination of red-shifted absorption and fluorescence emission spectra are indicative of hydrogen bond formation of aromatic alcohols and tyrosine (Ross et al., 1992). The behavior we have observed is identical to that for a tyrosine hydroxyl group forming a hydrogen bond in a hydrophobic protein environment (Khrapunov and Dragan, 1989). We interpret our spectroscopic results to indicate hydrogen bond formation between β-cyclodextrin and the aromatic hydroxyl groups of these ligands.

Thermodynamic results of a hydrogen bond interaction

The thermodynamic quantities from 15°C to 45°C for the binding to β-cyclodextrin of the reference molecules phenethylamine and hydrocinnamate and their phenolic hydroxyl substituted derivatives, which are capable of forming an additional hydrogen bond, are reported in Table 1. The presence of the hydrogen bond, depending upon the temperature, is characterized by a 1.4 to 2.0 kcal mol^{-1} more exothermic values of $\Delta H°$ and more negative values of $\Delta S°$ than the reference ligand. Although the enthalpy change always makes a stabilizing contribution, the presence of the hydrogen bond may result in either a net stabilizing effect, as in 3-(4-hydroxyphenyl) propionate, or have reduced stability, as in 3-(2-hydroxyphenyl) propionate. In the latter case, the destabilization relative to hydrocinnamate arises from an enhanced unfavorable (negative) entropic contribution.

Like most association reactions in water, the heat capacity change of these binding reactions is always negative. However, the effect of the hydrogen bond is to make ΔC_p° less negative than for the binding reaction of the parent compound lacking the hydrogen bond. Thus, the hydrogen bond makes a positive contribution to ΔC_p°. The characteristic behavior of the enthalpy change as a function of

TABLE 1 Thermodynamics of a hydrogen bond interaction with β-cyclodextrin

Ligand	T (°C)	K	$\Delta G°$ (cal mol^{-1})	$\Delta H°$ (cal mol^{-1})	$\Delta S°$ (cal mol^{-1} K^{-1})	ΔC_p° (cal mol^{-1} K^{-1})
Phenethylamine$^+$*	25.0	24 ± 2	−1890 ± 50	−1540 ± 90	1.2 ± 0.3	
	35.0	26 ± 2	−1990 ± 40	−2110 ± 90	−0.4 ± 0.3	−58 ± 1
	45.0	26 ± 1	−2060 ± 30	−2710 ± 70	−2.1 ± 0.2	
4-Hydroxyphenethylamine$^+$ (tyramine$^+$)	14.5	83 ± 1	−2520 ± 10	−2940 ± 20	−1.5 ± 0.1	
	25.0	70 ± 2	−2520 ± 10	−3310 ± 40	−2.7 ± 0.1	
	35.0	60 ± 2	−2510 ± 20	−3640 ± 60	−3.7 ± 0.2	−36 ± 3
	45.0	49 ± 1	−2460 ± 10	−4050 ± 40	−5.0 ± 0.2	
3-Phenylpropionate$^-$ (hydrocinnamate$^-$)	14.5	132 ± 5	−2790 ± 20	−1140 ± 20	5.7 ± 0.1	
	25.0	149 ± 4	−2970 ± 20	−1750 ± 20	4.1 ± 0.1	
	35.0	144 ± 4	−3040 ± 20	−2360 ± 40	2.2 ± 0.1	−61 ± 1
	45.0	137 ± 4	−3110 ± 20	−2990 ± 40	0.4 ± 0.1	
3-(4-Hydroxyphenyl)propionate$^-$	14.5	355 ± 2	−3360 ± 10	−3000 ± 10	1.2 ± 0.1	
	25.0	297 ± 4	−3370 ± 10	−3400 ± 20	−0.1 ± 0.1	
	35.0	243 ± 4	−3360 ± 10	−3930 ± 30	−1.8 ± 0.1	−45 ± 4
	45.0	202 ± 3	−3360 ± 10	−4340 ± 30	−3.1 ± 0.1	
3-(2-Hydroxyphenyl)propionate$^-$	14.5	93 ± 4	−2590 ± 30	−3260 ± 70	−2.3 ± 0.2	
	25.0	81 ± 2	−2600 ± 20	−3620 ± 50	−3.4 ± 0.2	
	35.0	67 ± 3	−2580 ± 30	−4200 ± 110	−5.3 ± 0.4	−44 ± 6
	45.0	58 ± 2	−2570 ± 20	−4540 ± 100	−6.2 ± 0.3	

Uncertainties are two standard deviations returned by fitting programs.
*No data were obtained at 14.5°C because of insolubility.

temperature for the ligand with the additional phenolic hydrogen bond (*filled symbols*) and the parent compound (*open symbols*) is shown in Fig. 2.

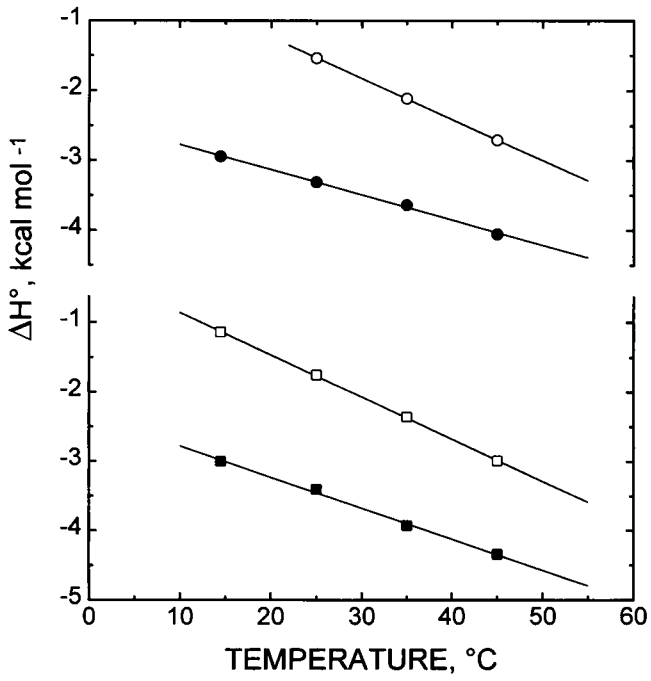

FIGURE 2 Effect of a hydrogen bond upon $\Delta H°$ versus T behavior of ligand reactions with β-cyclodextrin. Top: ○, Phenethylamine; ●, 4-hydroxyphenethylamine. Bottom: □, 3-phenylpropionate; ■, 3(4-hydroxyphenyl)propionate. Best-fit regression lines have a reduced negative slope as a result of hydrogen bond formation.

Thermodynamics of a CH_3- group interaction with β-cyclodextrin

The thermodynamic quantities from 15°C to 45°C for the binding of the reference ligands hydrocinnamate and cyclohexanol and methyl-substituted derivatives of these compounds, which form an additional CH_3- group interaction in the interior of the β-cyclodextrin cavity, are reported in Table 2. These associations are stabilized by both favorable enthalpic and entropic contributions. The heat capacity changes, ΔC_p°, are markedly negative quantities. The additional CH_3- group interaction stabilizes the complex by $\Delta\Delta G° \approx -500$ cal mol^{-1}; this arises almost entirely from an enhanced enthalpic contribution. The CH_3- group interaction in β-cyclodextrin results in a more negative heat capacity change, $\Delta\Delta C_p^\circ = -8$ cal K^{-1} mol^{-1}.

Thermodynamics of a -CH_2- group interaction with α-cyclodextrin

The thermodynamic parameters from 15°C to 45°C for the binding of α-cyclodextrin to the series of normal amines from pentylamine to octylamine are reported in Table 3. These data were obtained to examine the cumulative effect of introducing additional aliphatic methylene groups into the interior cavity of α-cyclodextrin. Hexanoate and heptanoate yield nearly the same -CH_2- group effect in all of the thermodynamic quantities as hexyl- and heptylamine (Table 3). As -CH_2- groups are added, the stability of these associations increase because of a more negative $\Delta H°$. With each additional -CH_2- interaction, the heat capacity change, ΔC_p°, becomes more negative, on the average, by ~13.3 cal

TABLE 2 Thermodynamics of an added CH_3- group interaction with β-cyclodextrin

Ligand	T (°C)	K	$\Delta G°$ (cal mol^{-1})	$\Delta H°$ (cal mol^{-1})	$\Delta S°$ (cal mol^{-1} K^{-1})	$\Delta C_p^°$ (cal mol^{-1} K^{-1})
3-Phenylpropionate$^-$						
(hydrocinnamate$^-$)	14.5	132 ± 5	−2790 ± 20	−1140 ± 20	5.7 ± 0.1	
	25.0	149 ± 4	−2970 ± 20	−1750 ± 20	4.1 ± 0.1	−61 ± 1
	35.0	144 ± 4	−3040 ± 20	−2360 ± 40	2.2 ± 0.1	
	45.0	137 ± 4	−3110 ± 20	−2990 ± 40	0.4 ± 0.1	
3-Phenylbutyrate$^-$	14.5	395 ± 12	−3420 ± 20	−1460 ± 20	6.8 ± 0.1	
	25.0	387 ± 6	−3530 ± 10	−2190 ± 10	4.5 ± 0.1	
	35.0	364 ± 4	−3610 ± 10	−2860 ± 10	2.5 ± 0.1	−69 ± 1
	45.0	323 ± 10	−3650 ± 20	−3560 ± 20	0.3 ± 0.1	
Cyclohexanol	14.5	844 ± 40	−3850 ± 30	−680 ± 10	11.0 ± 0.1	
	25.0	689 ± 11	−3870 ± 10	−1550 ± 10	7.8 ± 0.1	
	35.0	593 ± 11	−3910 ± 10	−2340 ± 10	5.1 ± 0.1	−79 ± 2
	45.0	515 ± 12	−3950 ± 20	−3100 ± 20	2.7 ± 0.1	
cis-4-Methylcyclohexanol	14.5	1710 ± 80	−4260 ± 30	−1340 ± 10	10.1 ± 0.1	
	25.0	1470 ± 50	−4320 ± 20	−2300 ± 20	6.8 ± 0.1	
	35.0	1330 ± 50	−4410 ± 30	−3120 ± 30	4.2 ± 0.1	−87 ± 2
	45.0	1080 ± 40	−4420 ± 20	−4010 ± 20	1.3 ± 0.1	

Uncertainties are two standard deviations as returned by fitting programs.

K^{-1} mol^{-1} (Table 3). The $\Delta H°$ values as a function of temperature for the interaction of the normal amines with α-cyclodextrin tend to converge toward a common value at −15°C, and the interaction of hydrocinnamates with β-cyclodextrin in the presence and absence of the added -CH_2- group show the same trend (see Fig. 3).

Temperature dependence of *K* and overview of calorimetric results

The temperature dependence of the equilibrium binding constant, K, expressed by the integrated van't Hoff equation, is best handled by the equation of Clarke and Glew (1966):

$$R \ln K = -\Delta G°(\theta)/\theta + \Delta H°(\theta)[1/\theta - 1/T] - \Delta C_p^°[(1 - \theta/T) - \ln (T/\theta)], \quad (3)$$

which has the desirable property that the thermodynamic quantities are independent of one another. The arbitrary reference temperature, θ, is chosen to be 298.15°K. Equation 3 has been truncated at the term linear in $\Delta C_p^°$, because no temperature dependence of $\Delta C_p^°$ was detectable in our

TABLE 3 Thermodynamics of an aliphatic -CH_2- group interaction with α-cyclodextrin

Ligand	T (°C)	K	$\Delta G°$ (cal mol^{-1})	$\Delta H°$ (cal mol^{-1})	$\Delta S°$ (cal mol^{-1} K^{-1})	$\Delta C_p^°$ (cal mol^{-1} K^{-1})
n-Pentylamine$^+$	14.5	111 ± 3	−2690 ± 20	−2580 ± 40	0.4 ± 0.1	
	25.0	93 ± 2	−2690 ± 10	−3250 ± 40	−1.9 ± 0.2	
	35.0	82 ± 3	−2700 ± 20	−3830 ± 90	−3.7 ± 0.3	−65 ± 5
	45.0	60 ± 2	−2600 ± 20	−4590 ± 100	−6.3 ± 0.3	
n-Hexylamine$^+$	14.5	484 ± 7	−3530 ± 10	−3320 ± 20	0.7 ± 0.1	
	25.0	383 ± 5	−3520 ± 10	−4200 ± 20	−2.3 ± 0.1	
	35.0	301 ± 8	−3500 ± 20	−5000 ± 60	−4.9 ± 0.2	−78 ± 4
	45.0	227 ± 7	−3430 ± 20	−5710 ± 80	−7.2 ± 0.3	
n-Heptylamine$^+$	14.5	1370 ± 20	−4130 ± 10	−3710 ± 20	1.5 ± 0.1	
	25.0	1070 ± 38	−4130 ± 20	−4750 ± 50	−2.1 ± 0.2	
	35.0	790 ± 26	−4090 ± 20	−5650 ± 70	−5.1 ± 0.2	−92 ± 4
	45.0	592 ± 19	−4040 ± 20	−6530 ± 80	−7.8 ± 0.3	
n-Octylamine$^+$	14.5	3060 ± 198	−4590 ± 40	−4190 ± 60	1.4 ± 0.2	
	25.0	2330 ± 240	−4590 ± 70	−5270 ± 130	−2.3 ± 0.5	
	35.0	1570 ± 120	−4510 ± 50	−6480 ± 150	−6.4 ± 0.5	−108 ± 6
	45.0	1160 ± 58	−4460 ± 30	−7430 ± 130	−9.3 ± 0.4	
n-Hexanoate$^-$	14.5	358 ± 6	−3360 ± 10	−2620 ± 20	2.6 ± 0.1	
	25.0	291 ± 10	−3360 ± 20	−3460 ± 50	−0.3 ± 0.2	
	35.0	249 ± 8	−3380 ± 20	−4070 ± 60	−2.3 ± 0.2	−74 ± 2
	45.0	204 ± 2	−3360 ± 10	−4900 ± 50	−4.8 ± 0.2	
n-Heptanoate$^-$	14.5	1040 ± 20	−3970 ± 10	−3340 ± 20	2.2 ± 0.1	
	25.0	815 ± 18	−3970 ± 10	−4230 ± 30	−0.9 ± 0.1	
	35.0	657 ± 14	−3970 ± 10	−5000 ± 40	−3.3 ± 0.1	−84 ± 2
	45.0	502 ± 10	−3930 ± 10	−5910 ± 50	−6.2 ± 0.2	

Uncertainties are two standard deviations as returned by fitting programs.

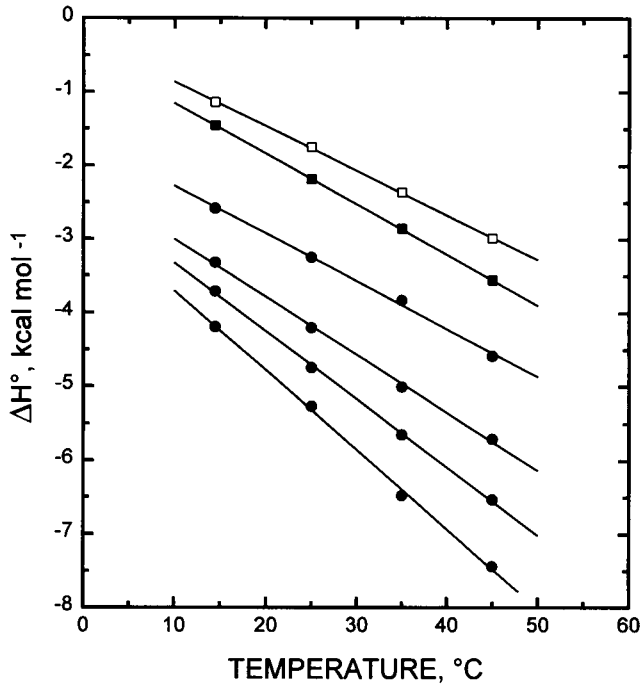

FIGURE 3 $\Delta H°$ versus T behavior of ligand CH_3- and -CH_2- group interactions with cyclodextrins. Reactions of 3-phenylpropionate (□) and of 3-phenylbutyrate (■) with β-cyclodextrin. ●, Reactions of normal amines with α-cyclodextrin. Top to bottom: *n*-pentylamine, *n*-hexylamine, *n*-heptylamine, and *n*-octylamine. Best-fit regression lines have an enhanced negative slope for the added CH_3- or -CH_2- interaction, and within each chemically similar group display a tendency toward convergence of $\Delta H°$ values at low temperature.

data taken over the limited temperature range of 15°C to 45°C. Thus

$$\Delta H°(T) = \Delta H°(\theta) + \Delta C°_p(T - \theta). \quad (4)$$

For the best representation of our calorimetric results consistent with the 1:1 binding model, we have performed a multidimensional least-squares fit of Eqs. 3 and 4 to the experimental data in Tables 1 to 3. The data were weighted according to the inverse of their variances deduced from the reported uncertainties. For these data, the assignment of equal or unequal weights does not appreciably alter the outcome. The global values of the thermodynamic quantities at 25°C are presented in Table 4 for each cyclodextrin complex as the top line entry for each reaction and are denoted "cal." These best-fit values are close to the individual experimental results reported in Tables 1 to 3.

For calculating thermodynamic quantities at other temperatures, we have applied the integrated van't Hoff equation (Eq. 3) to our K versus T data or fitted the expression for $\Delta G°(T)$, obtained upon multiplication of Eq. 3 by $-T$. These van't Hoff quantities at 25°C are presented in Table 4 in the second line for each reaction, labeled "vH." The van't Hoff values, $\Delta H°_{vH}$ and $\Delta C°_{p\ vH}$, are often very different from the experimentally determined calorimetric quantities, $\Delta H°_{cal}$ and $\Delta C°_{p\ cal}$; however, when introduced into Eq. 3, they best represent our experimental results of K and $\Delta G°$. Examination of the variance-covariance matrix of the least-squares fits shows that extremely large uncertainties are associated with the van't Hoff quantities, $\Delta H°_{vH}$ and $\Delta C°_{p\ vH}$. It is shown in Table 4 that these vH uncertainties may be as large as ± 1 kcal mol^{-1} for $\Delta H°_{vH}$, and in about one-half of the examples, the uncertainty in $\Delta C°_{p\ vH}$ is greater than the value of $\Delta C°_{p\ vH}$ itself, i.e., the value of $\Delta C°_{p\ vH}$ is not significantly different from zero. The large statistical uncertainties associated with the van't Hoff parameters, $\Delta H°_{vH}$ and $\Delta C°_{p\ vH}$, are well known (King, 1965) and arise from the propagation of error. The magnitude of these uncertainities depends upon the precision, temperature range, and sampling interval of the equilibrium constant data (King, 1965). These uncertainties may reconcile many cases in which the van't Hoff and calorimetric quantities are only moderately disparate.

Insertion of the best-fit calorimetric ("cal") values of Table 4 into Eq. 3 generates a predicted line that in all cases falls within two standard deviations of the estimate of the fit of the van't Hoff equation used alone. The largest discrepancies between $\Delta G°$ values predicted by these two methods are always less than 100 cal mol^{-1} and occur at the extremities of the temperature range of 15°C to 45°C. In a few cases, the lines predicted from the calorimetric results appear to exhibit systematic deviations from the experimental data. The van't Hoff parameters, despite their very large uncertainities, give a closer representation of the experimental K data between 15°C and 45°C. Extrapolation beyond these limits using either set of values is not advisable. Because data were not obtained over a sufficiently wide temperature range to establish the temperature dependence of $\Delta C°_p$, extrapolated predictions could be in further error from this source.

The calorimetric and van't Hoff quantities may be compared by their ratio. The last column of Table 4 shows that $\Delta H°_{cal}/\Delta H°_{vH}$ varies from 0.5 for cyclohexanol to 3 for 3-phenylbutyrate in their respective complexes with β-cyclodextrin. In two cases, the $\Delta H°$ are of opposite sign. The ratio, $\Delta C°_{p\ cal}/\Delta C°_{p\ vH}$, varies between 0.4 to 3 for the reactions in Table 4. These departures from unity are beyond combined statistical error and reflect inherent properties of the systems studied. It is not likely that these discrepancies arise from difficulties in the evaluation of binding constants by titration calorimetry, because agreement in values of K for cyclodextrins have been found by calorimetric, spectrophotometric, kinetic, and potentiometric techniques (Szejtli, 1988). Recently, Naghibi et al. (1995) called attention to similar discrepancies between calorimetric and van't Hoff quantities in about 10 systems that included binding processes of proteins and nucleic acids. The range of K values for which this widespread disagreement exists extends from $K = 10^1$ for the cyclodextrins to $K = 10^7$ for the macromolecules; thus this discord is not a property associated with the smaller K of the cyclodextrin complexes. Therefore, for the approximately two dozen systems examined, close agreement between calorimetric and van't Hoff quantities for binding equilibria in aqueous solutions appears to be the exception rather than the rule. Naghibi et al. (1995) pointed out that calorimetry measures all of the thermally detectable

TABLE 4 Best-fit calorimetric and van't Hoff thermodynamic quantities at 25°C

Reaction	Fit*	ΔG° (kcal mol^{-1})	ΔH° (kcal mol^{-1})	ΔC_p° (cal mol^{-1} K^{-1})	$\Delta H^\circ_{cal}/\Delta H^\circ_{vH}$
n-Pentylamine$^+$ + α-CD	cal	−2.69 ± 0.02	−3.25 ± 0.05	−64 ± 6	1.07
	vH	−2.70 ± 0.04	−3.03 ± 0.94	−113 ± 152	
n-Hexylamine$^+$ + α-CD	cal	−3.53 ± 0.01	−4.18 ± 0.02	−81 ± 2	1.00
	vH	−3.53 ± 0.01	−4.17 ± 0.11	−75 ± 20	
n-Heptylamine$^+$ + α-CD	cal	−4.13 ± 0.01	−4.71 ± 0.03	−94 ± 2	1.00
	vH	−4.13 ± 0.02	−4.68 ± 0.24	−83 ± 54	
n-Octylamine$^+$ + α-CD	cal	−4.58 ± 0.02	−5.32 ± 0.04	−107 ± 4	0.95
	vH	−4.57 ± 0.05	−5.56 ± 0.86	−59 ± 148	
n-Hexanoate$^-$ + α-CD	cal	−3.40 ± 0.02	−3.38 ± 0.06	−73 ± 6	1.04
	vH	−3.37 ± 0.02	−3.24 ± 0.29	−25 ± 56	
n-Heptanoate$^-$ + α-CD	cal	−3.98 ± 0.01	−4.21 ± 0.03	−83 ± 4	1.04
	vH	−3.98 ± 0.01	−4.06 ± 0.19	−60 ± 48	
Phenethylamine$^+$ + β-CD	cal	−2.02 ± 0.07	−1.53 ± 0.29	−59 ± 20	−1.40
	vH	−1.89 ± 0.02	1.09 ± 0.41	−59 ± 20#	
Tyramine$^+$ + β-CD	cal	−2.52 ± 0.02	−3.32 ± 0.04	−37 ± 2	1.16
	vH	−2.52 ± 0.01	−2.87 ± 0.21	−48 ± 37	
Hydrocinnamate$^-$ + β-CD	cal	−2.99 ± 0.06	−1.76 ± 0.10	−60 ± 10	−2.35
	vH	−2.96 ± 0.02	0.75 ± 0.75	−149 ± 106	
3-(4-Hydroxyphenyl)propionate$^-$ + β-CD	cal	−3.37 ± 0.01	−3.45 ± 0.04	−44 ± 4	1.07
	vH	−3.37 ± 0.01	−3.21 ± 0.13	−39 ± 28	
3-(2-Hydroxyphenyl)propionate$^-$ + β-CD	cal	−2.61 ± 0.03	−3.65 ± 0.11	−43 ± 12	1.38
	vH	−2.60 ± 0.01	−2.65 ± 0.50	−49 ± 68	
3-Phenylbutyrate$^-$ + β-CD	cal	−3.56 ± 0.03	−2.18 ± 0.04	−69 ± 4	2.95
	vH	−3.53 ± 0.01	−0.74 ± 0.21	−98 ± 32	
Cyclohexanol + β-CD	cal	−3.86 ± 0.03	−1.54 ± 0.02	−80 ± 2	0.51
	vH	−3.87 ± 0.01	−2.99 ± 0.28	25 ± 34	
4-Methylcyclohexanol + β-CD (*cis*)	cal	−4.34 ± 0.02	−2.27 ± 0.02	−87 ± 2	0.97
	vH	−4.33 ± 0.03	−2.33 ± 0.90	−71 ± 128	

*cal, Simultaneous fit of Eqs. 3 and 4 to experimental K and ΔH° as a function of T. vH, Fit of Eq. 3 to K and T.
Two standard deviations of the uncertainty of each parameter are reported.
#Assigned value.

processes taking place in solution and that there may be more processes occurring than described by the simple single equilibrium constant of the van't Hoff equation, which assumes the reaction to be exactly the same at each temperature. It is of interest that the normal hexyl (and heptyl) amines and acids studied here exhibit nearly perfect agreement between the van't Hoff model and the calorimetric results. The hydrocarbon chains of these molecules should just about completely occupy the approximately 175 Å^3 α-cyclodextrin cavity and exclude the estimated six water molecules from the interior of the complex.

The x-ray crystal structures show two hydrogen-bonded water molecules in the α-cyclodextrin cavity (Manor and Saenger, 1974) and 6.5 water molecules distributed over eight sites in the β-cyclodextrin cavity (Lindner and Saenger, 1978), which has room for 11 water molecules. We rewrite Eq. 1, where n represents these tightly bound hydration waters, m the water molecules interacting with the ligand, and x the net displacement of water in the complexation reaction

$$CD \cdot n(H_2O) + L \cdot m(H_2O) = CD \cdot L(m + n - x)(H_2O) + x\,H_2O. \quad (5)$$

We have no knowledge of the values of n, m, or x in solution. We see in Table 4 that all of the aliphatic ligands reacting with α-cyclodextrin and those ligands with substituent groups capable of entering the β-cyclodextrin cavity have a $\Delta H^\circ_{cal}/\Delta H^\circ_{vH}$ not differing greatly from unity. We offer the speculation that displacement of or severe interference with the hydrated water molecules in the interior of the cyclodextrin-ligand complex may be the feature required to obtain agreement between the calorimetric results and the derived van't Hoff thermodynamic quantities.

In this investigation we have sought to examine the effect of defined structural perturbations upon thermodynamic quantities by comparison of the results of calorimetric measurements of carefully selected ligands binding to cyclodextrins. This approach is based upon the assumption that other effects common to the similar molecules being compared will largely cancel. Therefore, we report the directly measured difference thermodynamic quantities in Tables 6 and 7 and illustrate some of these in Fig. 4. Because these difference thermodynamic quantities are quite small, the reader is again cautioned regarding further application of thermodynamic formulae to these results.

DISCUSSION

Basis of changes in thermodynamic parameters upon binding

The small molecule ligands used in this study form soluble 1:1 complexes with cyclodextrin in which we visualize the nonpolar portion of the ligand to be inserted via the wide end, so as to make maximum contact with the cyclodextrin cavity while the charged polar residue remains in the bulk solution. This picture emerges from NMR solution spectra of these complexes (Bergeron, 1984; Szetjtli, 1988), which are consonant with the mode of binding determined by x-ray crystallography (e.g., Wood et al., 1977). In crystal structures, the guest molecule is often found to be disordered (Hamilton et al., 1976), indicating that it is not rigidly bound. Appreciable and different molecular motion of cyclodextrin and ligand in complexes in solution has been demonstrated by NMR relaxation studies (Behr and Lehn, 1976). These results imply that weakly directed forces are involved in complex formation. In α-cyclodextrin complexes, when hexyl or longer aliphatic chains are introduced, it is likely that all of the water molecules are displaced from the cavity. Some water molecules most likely remain present in the β-cyclodextrin complexes. Dehydration of ligand and cyclodextrin, hydrogen-bond and van der Waals interactions between them, and conformational changes have been cited as processes possibly contributing to the changes in the thermodynamic quantities upon binding (Hallén et al., 1992).

The initial state of the ligand in these associations is determined by binary solute-solvent interactions, which are reflected in the infinite dilution values of the partial molar volumes, V_2° (Høiland, 1986a), the partial molar compressibilities, K_2° (Høiland, 1986b), and the partial molar heat capacity, $C_{p,2}^\circ$ (Nichols et al., 1976). These quantities primarily scale with molecular size and hence with the number of heavy atoms for the monoamines discussed below. Alkyl chain branching up to iso-propyl or *N*-methyl substitution results in an insignificant (<2%) increase in V_2° (Høiland, 1986a) and $C_{p,2}^\circ$ (Nichols et al., 1976).

The effect upon the binding thermodynamics of the positioning of an additional CH_3 group (boldface) such that it will reside inside or outside the cyclodextrin cavity is shown in Table 5. The stippled area above the heading "Ligand" in Table 5 denotes the approximate position of the top of the cyclodextrin cavity. Irrespective of the number of carbon atoms in the molecule, when the additional (boldfaced) CH_3 group is introduced on the left-hand side, where it can enter the cyclodextrin cavity, there is about a 2.6-fold increase in the binding constant K, and ΔH° is $\sim$0.5 kcal mol^{-1} more negative. When the additional (boldfaced) CH_3 group is introduced on the right-hand side, in proximity to the charged amino group, which is thought to remain in the bulk solution before and after association (Bergeron, 1984; Szetjtli, 1988), the values of ΔG°, ΔH°, and ΔS° are the same irrespective of the number of carbon atoms in the molecule. These molecules, hexylamine, *N*-methylhexylamine, and 2-aminoheptane, also have identical ΔH° at other temperatures (Table 5); thus their ΔC_p° is the same.

In summary, the thermodynamic values in Table 5 show a correlation with the extent of penetration of the ligand into the cyclodextrin cavity and a lack of correlation with the

TABLE 5 IN versus OUT: effect of position of CH_3 group added to ligand on binding thermodynamics

α-Cyclodextrin ▒* bulk solution Ligand	$K^\#$	ΔG° (kcal mol^{-1})$^\#$	ΔH° (kcal mol^{-1})$^\#$	ΔS° (cal mol^{-1} K^{-1})$^\#$
T = 25°C				
C-C-C-C-C-C-NH_3^+	383 ± 5	−3.52 ± 0.01	−4.20 ± 0.02	−2.3 ± 0.1
C-C-C-C-C-C-NH_2^+-**CH_3**	378 ± 4	−3.52 ± 0.01	−4.20 ± 0.01	−2.3 ± 0.1
C-C-C-C-C-**CH_3** 'NH_3^+	439 ± 6	−3.61 ± 0.01	−4.27 ± 0.02	−2.2 ± 0.1
CH_3-C-C-C-C-C-C-NH_3^+	1070 ± 38	−4.13 ± 0.02	−4.75 ± 0.05	−2.1 ± 0.2
CH_3-C-C-C-C-C-C 'NH_3^+	1130 ± 17	−4.17 ± 0.01	−4.74 ± 0.02	−1.9 ± 0.1
CH_3-CH_2-C-C-C-C-C-C-NH_3^+	2330 ± 240	−4.59 ± 0.07	−5.27 ± 0.13	−2.3 ± 0.5
T = 14.5°C				
C-C-C-C-C-C-NH_3^+	484 ± 7	−3.53 ± 0.01	−3.32 ± 0.02	0.7 ± 0.1
C-C-C-C-C-C-NH_2^+-**CH_3**	466 ± 11	−3.51 ± 0.01	−3.34 ± 0.03	0.6 ± 0.1
C-C-C-C-C-**CH_3** 'NH_3^+	543 ± 7	−3.60 ± 0.01	−3.29 ± 0.01	1.1 ± 0.1
T = 45°C				
C-C-C-C-C-C-NH_3^+	227 ± 7	−3.43 ± 0.02	−5.71 ± 0.08	−7.2 ± 0.3
C-C-C-C-C-C-NH_2^+-**CH_3**	236 ± 10	−3.45 ± 0.03	−5.56 ± 0.11	−6.6 ± 0.4
C-C-C-C-C-**CH_3** 'NH_3^+	300 ± 16	−3.61 ± 0.03	−5.64 ± 0.12	−6.4 ± 0.4

*Stippled region, ▒, denotes approximate top of cyclodextrin cavity. Added group indicated in boldface.

$^\#$There are no significant differences in the thermodynamic quantities for each member of a comparison group, with the exception of K and ΔG° for 2-aminoheptane, which forms a marginally more stable complex. In the hexylamine group, at each temperature examined, the values of ΔH° are the same, so ΔC_p° is the same for each group member.

number of carbon atoms in the ligand (size), the principal determinant of solute-solvent interaction. These results lead to the important conclusion that 1) any differences in solute-solvent interactions of the isolated ligands before reaction do not contribute measurably to the thermodynamics of binding in these reactions, and 2) only those parts of a molecule that undergo a change in their environment upon association contribute to the thermodynamic parameters of binding.

Finally, we note that for phenethylamine and hydrocinnamate binding to β-cyclodextrin, the values of ΔC_p° are nearly identical, despite widely different extents of electrostriction about the COO^- and NH_3^+ groups, as estimated by values of 14.5 versus 4.5 cm^3mol^{-1}, respectively (Shahidi, 1980). This result is consistent with the NH_3^+ and COO^- being outside the binding site. According to the conclusion of the previous paragraph, if these obviously different electrostrictive solute-solvent interactions are remote from the binding site, they would not measurably affect the thermodynamics. Thus the ΔC_p° of ~ -60 cal $K^{-1}mol^{-1}$ would primarily reflect the interaction of the identical aromatic portion of these two ligands with β-cyclodextrin. It is also possible, but less likely, that these interactions make different but compensating contributions so that ΔC_p° remains the same.

Thermodynamics of hydrogen bond interaction

There are only a limited number of studies of the thermodynamics of hydrogen bond formation in aqueous solution by simple model compounds. Particular attention has been directed toward the amide hydrogen bond because of its occurrence in proteins. Schellman (1955) analyzed the nonideality of aqueous urea solutions in terms of an indefinite association model, obtaining a value of $K = 0.041\ M^{-1}$ at 25°C. For the dimerization of urea, he estimated $\Delta H^\circ = -1.5$ kcal mol^{-1}, which was a compromise value based upon the possibility of forming both linear and cyclic dimers. Using additional thermochemical data, Kresheck and Scheraga (1965) extended this analysis to examine the temperature dependence of K and ΔH° from 0 to 40°C. Making assumptions about ΔS°, they found a small positive ΔC_p° for hydrogen bond formation, which they regarded as barely significant. Other thermodynamic studies of model hydrogen bonding associations include the dimerizations of *N*-methylacetamide (Klotz and Franzen, 1962), of lactams (Susi et al., 1964; Susi and Ard, 1966), and of carboxylic acids (Schrier et al., 1964), and the dissolution of diketopiperazine (Gill and Noll, 1972) and other cyclic dipeptides containing amino acid side chains (Murphy and Gill, 1989). Although these investigations have been informative, the above systems usually have additional complicating features contributing to the thermodynamics, such as hydrophobic effects arising from the organic side chains and, in the solid-state studies, the involvement of crystal forces.

In this study of hydrogen bonding to β-cyclodextrin, the ligand substituted with a phenolic -OH group enabling hydrogen bonding differs by only a single oxygen atom from its parent compound, which is incapable of forming a hydrogen bond at that site. Comparison of the binding thermodynamics of two such similar ligands should give a good measure of the thermodynamic properties associated with the formation of an O-H-O hydrogen bond.

The thermodynamic characteristics accompanying hydrogen bond formation are given by the difference quantities reported in Table 6. These quantities are the net contribution of the differences of ligand-cyclodextrin and ligand-water hydrogen bond interactions, solvation changes about the oxygen atom, and van der Waals interactions. The hydrogen bond makes a positive contribution of $\Delta\Delta C_p^\circ \approx 18$ cal $K^{-1}mol^{-1}$ (Table 6) to the overall negative heat capacity change of association (Fig. 2). It is seen that, as the temperature increases, the hydrogen-bonded complex decreases in stability ($\Delta\Delta G^\circ$ becomes more positive; Table 6) while the stability of the non-hydrogen-bonded parent compound increases (Table 1). These results show, not surprisingly, that hydrogen bonding is a temperature-dependent equilibrium or, colloquially, that hydrogen bonds "melt." Negative $\Delta\Delta H^\circ$ values that range from $\Delta\Delta H^\circ \approx -2$ kcal mol^{-1} at 15°C to $\Delta\Delta H^\circ \approx -1.4$ kcal mol^{-1} at 45°C are characteristic values accompanying the formation of these spectroscopically demonstrated hydrogen bonds (Table 6).

For the three examples of hydrogen bond formation studied, the average value of $\Delta\Delta C_p^\circ = 18$ cal $K^{-1}mol^{-1}$ is

TABLE 6 Thermodynamic contribution from a hydrogen bond interaction in β-cyclodextrin

T (°C)	$\Delta\Delta G^\circ$ (cal mol^{-1})			$\Delta\Delta H^\circ$ (cal mol^{-1})			$\Delta\Delta S^\circ$ (cal mol^{-1} K^{-1})			$\Delta\Delta C_p^\circ$ (cal mol^{-1} K^{-1})		
pair* =	A	B	C	A	B	C	A	B	C	A	B	C
14.5		−570	300		−1860	−2120		−4.5	−8.0			
25.0	−630	−400	370	−1770	−1650	−1870	−3.9	−4.2	−7.5			
										22	16	17
35.0	−520	−320	460	−1530	−1570	−1840	−3.3	−4.0	−7.5			
45.0	−400	−250	540	−1340	−1350	−1550	−2.9	−3.5	−6.6			

*The thermodynamic characteristics of the hydrogen bond between ligand and β-cyclodextrin are taken from the difference in the corresponding thermodynamic quantity for the hydrogen-bond-forming ligand and the reference ligand lacking this additional hydrogen-bond-forming capability. Ligand pairs: A = {tyramine–phenethylamine}; B = {[3-(4-hydroxyphenyl)propionate–3-phenylpropionate]}; C = {[3-(2-hydroxyphenyl)propionate–3-phenylpropionate]} complexes with β-cyclodextrin.

TABLE 7 Thermodynamic contribution from CH_3- and -CH_2- group interactions in α- and β-cyclodextrin

T (°C)	$\Delta\Delta G°$ (cal mol^{-1})			$\Delta\Delta H°$ (cal mol^{-1})			$\Delta\Delta S°$ (cal mol^{-1} K^{-1})			$\Delta\Delta C_p^\circ$ (cal mol^{-1} K^{-1})		
pair* =	A	B	C	A	B	C	A	B	C	A	B	C
14.5	−630	−410	−600	−320	−660	−390	1.1	−0.9	0.8			
25.0	−560	−450	−610	−440	−750	−550	0.4	−1.0	0.2			
										−8	−8	−14
35.0	−570	−500	−590	−500	−780	−650	0.2	−0.9	−0.2			
45.0	−540	−470	−610	−570	−910	−820	−0.1	−1.4	−0.6			

*The thermodynamic characteristics of the CH_3- and -CH_2- group interactions between ligand and cyclodextrin are taken from the difference in the corresponding thermodynamic quantity for the ligand with the additional CH_3- or -CH_2- group and the reference ligand lacking those groups. Pairs: A = {3-phenylbutyrate–3-phenylpropionate} and B = {*cis*-4-methylcyclohexanol–cyclohexanol} complexes with β-cyclodextrin; C = {*n*-heptylamine–*n*-hexylamine} complexes with α-cyclodextrin.

greater than the value of 14 cal $K^{-1}mol^{-1}$ used by Murphy and Gill (1991) in their analysis of protein denaturation. This difference may arise from the different environments of the hydrogen bond in the interior of the organic crystal and in the hydrated β-cyclodextrin cavity and/or differences in the proximity of neighboring heavy atoms in the soluble complex and in the crystal. Another possible source of the difference is that we have examined an O-H-O hydrogen bond that is usually shorter and presumably stronger than the N-H-O hydrogen bond (Pimentel and McClellan, 1960).

An additional hydrogen bond does not necessarily lead to enhanced stability of the complex, as shown by the interesting case of 3-(2-hydroxyphenyl)propionate. Here the spectroscopically demonstrated hydrogen bond displays $\Delta H°$ values typical of a hydrogen bond (Case C, Table 6), yet a weaker ($\Delta\Delta G°$ is positive) complex with β-cyclodextrin is formed than by the parent compound, hydrocinnamate (Table 1). This destabilization arises from an unfavorable entropy change associated with a substitution at the 2 position of the aromatic ring. This destabilization effect depends solely on position and not on the chemical nature of the substituent. Substitution of a methoxy group at the 2 position halves the binding constant to β-cyclodextrin at 25°C from $K = 76$ for 3- methoxyphenethylamine to $K = 39$ for 2–5-dimethoxyphenethylamine (Rekharsky et al., 1995). Unsubstituted phenethylamine and phenylacetate form complexes with β-cyclodextrin at pH 6.9; 2-methoxyphenethylamine and 2- methylphenylacetate do not (Rekharsky et al., 1995, and manuscript in preparation). Thus 2-OH, 2-OCH_3, and 2-CH_3 substitutions all destabilize the complex, indicating that this is probably a steric effect.

Thermodynamics of a hydrophobic interaction

The thermodynamic characteristics of a hydrophobic interaction in which an additional CH_3- or -CH_2- group is introduced into the interior of the cyclodextrin cavity are reported in Table 7 as difference values. These quantities represent the net effect of solvation changes (hydrophobic hydration) and van der Waals interactions of the CH_3- or -CH_2- group interaction. The quantity $\Delta\Delta C_p^\circ$ is negative, the hallmark effect of the transfer of a hydrophobic group from an aqueous to a nonpolar environment. The values of $\Delta\Delta C_p^\circ$ are such that the stability enhancement of $\Delta\Delta G° \approx -600$ cal mol^{-1} per added CH_2 group in these cyclodextrin complexes is essentially independent of temperature. This result would be consistent with the predominance of dispersion interactions.

The values for all of the thermodynamic parameters in Table 7 agree, within combined experimental error, with the -CH_2- group effect found in a potentiometric and calorimetric study of butyl- and pentyl-substituted polyacids, which undergo a cooperative hydrophobic collapse upon neutralization (Martin et al., 1980; Martin and Strauss, 1980).

The value of $\Delta\Delta C_p^\circ = -14$ cal $K^{-1}mol^{-1}$ (Table 7) for the -CH_2- effect in α-cyclodextrin, upon reversing the sign, agrees with $\Delta C_{p\,soln}^\circ$ values for the transfer of the methylene group of amines, amides, acids (Konicek and Wadsö, 1971), and alcohols (Hallén et al., 1986) to water. The hydrophobic -CH_2- effect in α-cyclodextrin, for the four examples reported in Table 3, has an average value of $\Delta\Delta C_p^\circ = -13.3$ cal $K^{-1}mol^{-1}$, which upon dividing by 2 for the methylene hydrogens and reversing the sign, is in exact agreement with the "apolar hydrogen" value of 6.7 cal $K^{-1}mol^{-1}$ used by Murphy and Gill (1991) in their analysis of protein unfolding.

For the CH_3 substitution in β-cyclodextrin (in which two "apolar hydrogen" atoms are also involved) the heat capacity change, $\Delta\Delta C_p^\circ = -8$ cal $K^{-1}mol^{-1}$ (Table 7), is smaller, possibly because the CH_3 group is considerably more exposed to solvent in the larger β-cyclodextrin cavity and may be less efficiently buried in a nonpolar environment than in the α-cyclodextrin complex.

The values of $\Delta H°$ converge toward a common value at low temperatures, as shown for the amines and hydrocinnamates in Fig. 3. Similar behavior is evident in data for *n*-alcohol–α-cyclodextrin complexes (Hallén et al., 1992). Convergence toward a common value of $\Delta H°$ at low temperatures was also found in the associations of Cro protein with closely related DNAs (Takeda et al., 1992). We suggest that this low-temperature enthalpy convergence in a family of compounds may be a phenomenon of some generality.

SUMMARY AND IMPLICATIONS FOR BIOPOLYMERS

The temperature dependence of the contributions of the additional hydrogen bond and hydrophobic interaction from

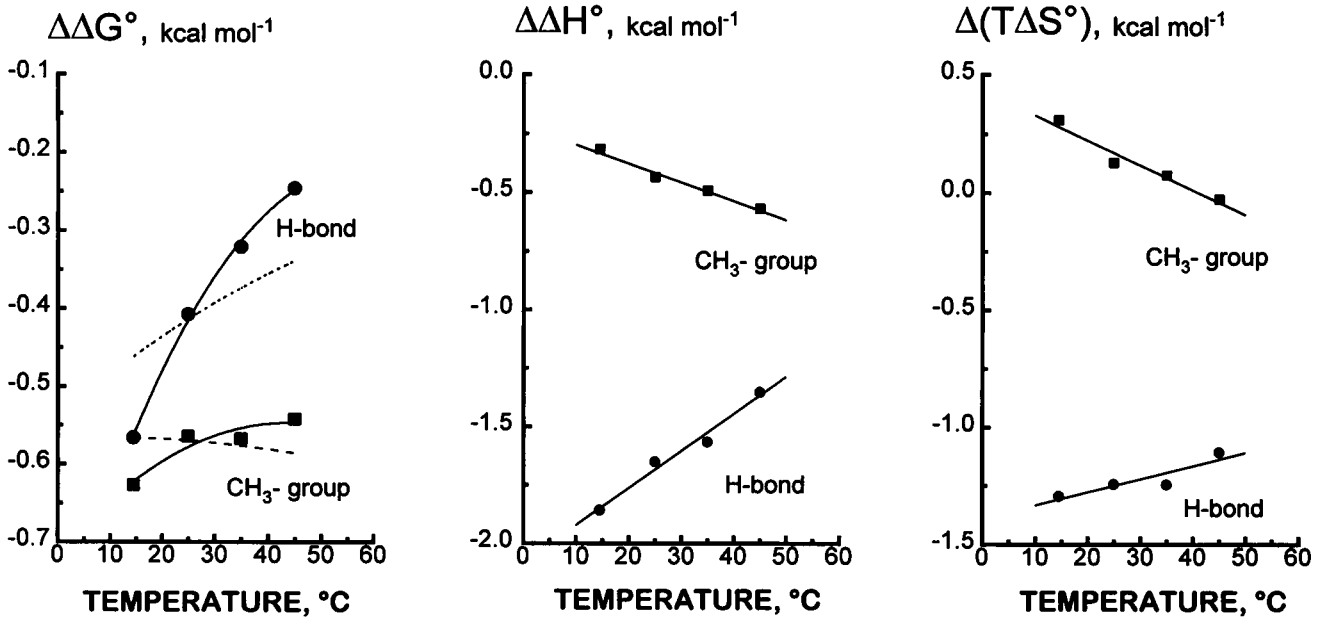

FIGURE 4 Temperature dependence of the thermodynamics of hydrogen bond and hydrophobic interactions from the binding of three hydrocinnamates to β-cyclodextrin. ●, H-bond [(3(4-hydroxyphenyl)propionate-3-phenylpropionate)] (Case B, Table 6). ■, CH_3- group [(3-phenylbutyrate-3-phenylpropionate)] (Case A, Table 7). (*A*) Stability of H-bond diminishes with temperature, CH_3- effect remains nearly constant. The van't Hoff equation (Eq. 3) gives an excellent fit to the $\Delta\Delta G°$ data (solid lines), but the derived van't Hoff parameters are associated with large uncertainties (Table 4). The plot of $\Delta\Delta G°$ generated from the value of $\Delta\Delta G°$ at 25°C and the calorimetrically determined $\Delta H°$ and ΔC_p° (dashed and dotted lines) do not represent the data as well; however, the measured points are all within ± 2 SD of these curves. (*B* and *C*) The lines indicate the trends in the $\Delta\Delta H°$ and $\Delta(T\Delta S°)$ data.

a −OH and a CH_3 group substitution to hydrocinnamate, respectively, is shown in Fig. 4. This picture is representative of the difference thermodynamic values reported in Table 6 and Table 7. The principal result is that the stability of the hydrogen bond decreases as the temperature is raised, while the contribution of the CH_3- group hydrophobic interaction remains essentially constant ($\Delta\Delta G°$ in Fig. 4). This behavior arises from the opposite sign of the contributions to $\Delta\Delta C_p^\circ$ (slopes of $\Delta\Delta H°$ versus T; Fig. 4) and the interplay of compensating $\Delta\Delta H°$ and $\Delta(T\Delta S°)$ contributions in these two kinds of interactions. The destabilization of the hydrogen bond with increasing temperature arises from a more positive $\Delta H°$. For the hydrophobic CH_3- and -CH_2- interaction, the enthalpic and entropic contributions balance, resulting in little change in free energy with temperature.

Let us examine the implications of this model compound study for macromolecules, such as proteins and nucleic acids, which for the sake of discussion we assume to be solely stabilized by a mixture of these hydrogen bond and hydrophobic interactions. Depending upon the details of composition and structure, the stability of such a molecule would be a weighted average of these hydrogen bond and hydrophobic contributions. The stability as a function of temperature would follow a course between the hydrogen bond and CH_3- group curves shown in the $\Delta\Delta G°$ panel of Fig. 4. At room temperature, both hydrogen bond and hydrophobic interactions stabilize this macromolecule. As the temperature is raised, the stabilizing forces are reduced primarily because of the breaking of hydrogen bonds. At some elevated temperature, where $\Delta\Delta G° = 0$, the disruptive effects of conformational entropy and hydration interactions that increase with temperature have overcome the residual stability of the hydrophobic interactions and the hypothetical macromolecule denatures. This approach, including only the contributions of the hydrogen bond and hydrophobic interactions, obviously neglects the portions of any real macromolecule not directly involved in these specific interactions that could make significant solvation or other contributions to the overall thermodynamics. Nevertheless, the view presented above is consonant with the conclusion of Shirley et al. (1992) from mutational studies that hydrophobic effects and hydrogen bonding make comparable contributions to the stability of the protein ribonuclease T1 at room temperature.

The phenolic hydrogen bond we have studied bears obvious similarity to tyrosyl hydrogen bonds that play an important role in proteins, for example, the breaking of two tyrosine-to-valine oxygen hydrogen bonds in the conformational change accompanying the oxygenation of hemoglobin (Dickerson and Geis, 1983). The smaller ΔC_p° values obtained for the hydrophobic effect in β-cyclodextrin (compared to α-cyclodextrin) may be a fair approximation of the interactions taking place at the more solvent-exposed polar interfaces in protein-DNA and in protein-protein associations and in some ligand-macromolecule binding reactions. The hydrophobic effect observed with α-cyclodextrin approximates that used to model the dense organic interior of a globular protein from which water is excluded. The values

we have obtained for the ΔC_p° of hydrophobic and hydrogen bond interactions agree reasonably well with the values of these key experimental parameters used by Murphy and Gill (1991) and subsequently by Murphy and Freire (1992) in their analysis of protein stability. The opposite signs of the heat capacity effects of the hydrophobic and hydrogen bond interactions found in this model system study are also consonant with other current treatments of the problem of protein unfolding (Makhatadze and Privalov, 1993; Privalov and Makhatadze, 1993). The results described in this paper point to the important role of the melting of hydrogen bonds in determining the stability of biopolymers.

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