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Thermodynamics of Monosaccharide and Disaccharide Binding to *Erythrina corallodendron* Lectin*

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Isothermal titration calorimetry measurements of the binding of 2′-fucosyllactose, lactose, *N*-acetyllactosamine, galactopyranose, 2-acetamido-2-deoxygalactopyranoside, methyl α-*N*-dansylgalactosaminide (Me-α-DNS-GalN), methyl α-D-galactopyranoside, methyl β-D-galactopyranoside, and fucose to *Erythrina corallodendron* lectin (ECorL), a dimer with one binding site per subunit, were performed at 283–286 and 297–299 K. The site binding enthalpies, ΔH_b, with the exception of Me-α-DNS-GalN, are the same at both temperatures and range from -47.1 ± 1.0 kJ mol^{-1} for *N*-acetyllactosamine to -4.4 ± 0.3 kJ mol^{-1} for fucose, and the site binding constants range from $3.82 \pm 0.9 \times 10^5$ M^{-1} for Me-α-DNS-GalN at 283.2 K to $0.46 \pm 0.05 \times 10^3$ M^{-1} for fucose at 297.2 K. The binding reactions are mainly enthalpically driven except for fucose and exhibit enthalpy-entropy compensation. The binding enthalpies of the disaccharides are about twice the binding enthalpies of the monosaccharides in contrast to concanavalin A where the binding enthalpies do not double for the disaccharides. Differential scanning calorimetry measurements show that denaturation of the ECorL dimer results in dissociation into its monomer subunits. The binding constants from the increase in denaturation temperature of ECorL in the presence of saccharides are in agreement with values from isothermal titration calorimetry results. The thermal denaturation of ECorL occurs around 333 K, well below the 344–360 K denaturation temperature of other legume lectins of similar size and tertiary structure, undoubtedly due to the difference in its quaternary structure relative to other legume lectins. This is also apparent from the independent unfolding of its two domains.

Lectins are proteins that bind carbohydrates with a high degree of specificity which makes them useful as probes for carbohydrate structure in various biological systems, such as cell membranes, and as model systems for elucidating protein-carbohydrate interactions (Goldstein and Hayes, 1978; Sharon and Lis, 1989). Previous investigations of the binding thermodynamics of the lectins with monosaccharides have shown that these reactions are enthalpically driven with little increase in the heat capacity change and that they exhibit enthalpy-entropy compensation (Schwarz *et al.*, 1991, 1993). These characteristics have also been observed for the binding of the trisaccharide methyl 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside to concanavalin A (Williams *et al.*, 1992) as well as for the binding of derivatives of galactose and lactose to the 14-kDa S-type lectin from sheep spleen (Ramkumar *et al.*, 1995).

Erythrina corallodendron lectin (ECorL)[1] consists of two 28,750-Da subunits, the tertiary structure of which is superimposable on that of other legume lectins (Shaanan *et al.*, 1991; Sharon, 1993). Like other legume lectins it contains one atom of Mn^{2+} and of Ca^{2+} in each subunit that are essential for its carbohydrate binding activity (Lis *et al.*, 1985). However, each subunit is glycosylated by two *N*-linked oligosaccharides (Young *et al.*, 1995) one of which is the heptasaccharide Manα6(Manα3)(Xyl1β2)Manβ4GlcNAcβ4(LFucα3)GlcNAcβ *N*-linked to Asn-17 at the monomer-monomer interface and which forces the ECorL dimer into a quaternary structure different from that of the other legume lectins (Shaanan *et al.*, 1991). The effect of this unusual structural feature of ECorL on its binding properties as well as its structural stability has not been determined.

In this investigation, isothermal titration calorimetry (ITC) was employed to determine the thermodynamics of the carbohydrate-ECorL binding reaction in terms of the site binding constant (K_b) and changes in the free energy (ΔG^0_b), the binding enthalpy (ΔH_b), and the binding entropy (ΔS_b).

In addition, the structural stability, particularly the effect of its unusual mode of dimerization as well as that of ligand binding on the thermal stability of ECorL, was investigated by differential scanning calorimetry (DSC). Previous DSC studies on the unfolding of concanavalin A, pea lectin, and lentil lectin (Schwarz *et al.*, 1993) have shown that the concanavalin A

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[1] The abbreviations used are: ECorL, *Erythrina corallodendron* lectin. All sugars have D configuration unless otherwise stated. Gal, galactose; GalNAc, *N*-acetylgalactosamine; Me-α-Gal, methyl α-galactoside; Me-β-Gal, methyl β-galactoside; Lac, lactose; LacNAc, *N*-acetyllactosamine; 2′-FL, 2′-fucosyllactose; Me-α-DNS-GalN, methyl α-*N*-dansylgalactosaminide; Man, mannose; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry.

tetramer dissociates into monomers while the pea and lentil lectin dimers dissociate into submonomer fragments. An earlier DSC study (Schwarz *et al.*, 1991) showed that, unlike the single transition exhibited by these lectins, denaturation of the basic lectin from winged bean consists of two transitions, with the higher temperature transition resulting in dissociation of the lectin dimer.

EXPERIMENTAL PROCEDURES

Materials—Gal, Me-β-Gal, Lac, LacNAc, GalNAc, and fucose were obtained from Sigma[2] and used without any further purification. The 2′-FL was a product of Dextra Laboratories, U. K., and Me-α-DNS-GalN was a gift of Dr. Willy Kinzy of Ciba-Geigy, Basel (Kinzy *et al.*, 1992). ECorL was prepared and purified to >98% purity as described (Lis *et al.*, 1985). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis performed according to Laemmli (1970) showed only one band with an M_r of 30,000. Deionized double-distilled water was used for all the solutions. All the other chemicals used were of the highest purity available.

Preparation and Analysis of Solutions—The ECorL solutions were prepared in the 0.02 M sodium phosphate and 0.15 M sodium chloride buffer at pH 7.4 (phosphate-buffered saline) by weight, dialyzed overnight against a large volume of the same buffer, and centrifuged to remove any insoluble material. The concentrations of the protein solutions were determined from extinction coefficient of ECorL *i.e.* $A^{1\%}_{280\ nm}$ = 15.3 (De Bock *et al.*, 1984). Solutions of the carbohydrates were prepared by weight in the dialysate to minimize differences between the protein buffer solution and ligand buffer solution in the ITC measurements. For DSC measurements, sugars (mg quantities) were added directly to 1.18 g of the lectin solution in the DSC cell (1.18 ml).

ITC Measurements and Analysis—The titration calorimetry measurements were performed with a Microcal Omega titration calorimeter as described previously by Wiseman *et al.* (1989) and Schwarz *et al.* (1991). Aliquots of the ligand solution at 10–20 × the site concentration were added via a 250-μl rotating stirrer-syringe to the solution cell containing 1.34 ml of the 0.4–0.9 mM protein solution. The heat of dilution was determined to be negligible in separate titrations of the ligand solution into just the buffer solution.

An identical site model, utilizing a site concentration = 2[ECorL], was the simplest binding model found to provide the best fit to the ITC data. A molecular mass of 57,500 Da was used for the ECorL dimer (Young *et al.*, 1995). The total heat content, Q_t, is related to the total ligand concentration, $[L]_t$, via the following equation (Wiseman *et al.*, 1989),

$$Q_t = 2n[\mathrm{ECorL}]_t\Delta H_b V\{1 + [L]_t/2n[\mathrm{ECorL}]_t + 1/2nK_b[\mathrm{ECorL}]_t - [(1 + [\mathrm{L}]_t2n[\mathrm{ECorL}]_tV + 1/2nK_b[\mathrm{ECorL}]_t)^2 - 4[\mathrm{L}]_t/2n[\mathrm{ECorL}]_t]^{1/2}\}/2 \quad \text{(Eq. 1)}$$

where n is the stoichiometry, and V is the cell volume. The expression for the heat released per the ith injection, $\Delta Q(i)$, is then (Yang, 1990)

$$\Delta Q(1) = \Delta Q(i) + dV_i/2V[Q(i) + Q(i-1)] - Q(i-1) \quad \text{(Eq. 2)}$$

where dV_i is the volume of titrant added to the solution.

The thermodynamic quantities, $\Delta G^0{}_b$ and ΔS_b, were obtained from the basic equation of thermodynamics,

$$\Delta G^0_b = \Delta H_b - T\Delta S_b \quad \text{(Eq. 3a)}$$

where

$$\Delta G^0_b = -nRT\ln\{K_b\} \quad \text{(Eq. 3b)}$$

and n = number of moles, T is the absolute temperature, and R = 8.3151 J mol^{-1} K^{-1}.

DSC Measurements and Analysis—DSC measurements were performed with a Microcal MC-2 DSC heat conduction scanning microcalorimeter which consists of two fixed 1.18-ml cells, a reference cell and a solution cell. The measurements were done usually at a scan rate of 20 Kh^{-1}. To determine any dependence of the parameters on scan rate, they were also performed at 45 and 90 Kh^{-1}. The best least squares fit of the two-state transition model, A ↔ nB, where A is the folded state and B is the unfolded state, to the data was obtained with n = 2 by the EXAM Program (Schwarz and Kirchhoff, 1988). This program utilizes a sigmoidal base line to yield a van't Hoff enthalpy (ΔH_v), a transition temperature (T_m, the temperature at half the peak area), and the transition peak area when divided by the number of moles of protein in the cell yields the calorimetric enthalpy (ΔH_c). The ratio of $\Delta H_c/\Delta H_v$ yields the cooperativity of the transition. T_p is the temperature where the transition peak is at maximum.

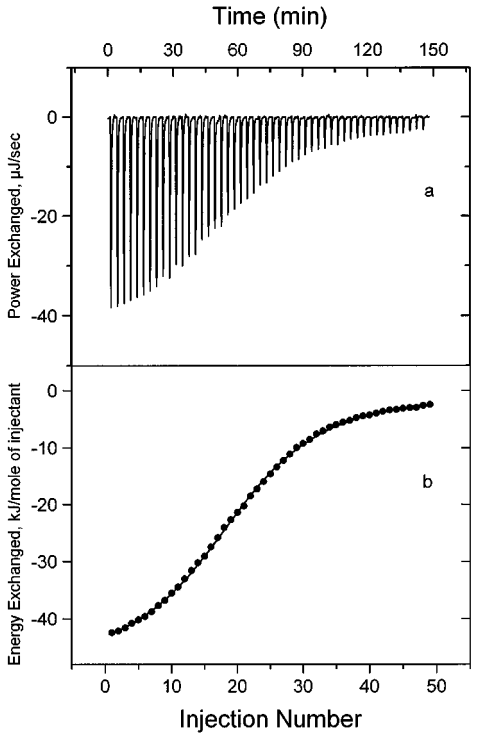

FIG. 1. *a*, titration calorimetry results from adding 6.0-μl aliquots of 4.0 mM *N*-acetyllactosamine to 0.2 mM ECorL in 0.02 M sodium phosphate buffer containing 0.15 M NaCl at pH 7.4 and 283.2 K. *b*, a plot of the energy released per mole of ligand as a function of injection number for the titration shown in *a*. The *solid line* is the result of the best least squares fit of the data to Equation 2.

RESULTS

The results of a typical titration calorimetry measurement for ECorL together with a least squares fit of the data to the identical site model described by Equation 2 is presented in Fig. 1. The close fit of the data to the identical site model shows that the ligand binds to each of the two sites of ECorL independently and with the same binding constant and enthalpy. The results of the carbohydrate-ECorL titrations at two different temperatures are presented in Table I. The binding reactions are mostly enthalpically driven with the exception of fucose which is mainly entropically driven. Values for ΔH_b range from −47.1 kJ mol^{-1} for *N*-acetyllactosamine to −4.4 kJ mol^{-1} for fucose and each ligand, with the exception of Me-α-DNS-GalN, is within the experimental error at both temperatures. Thus, there is very little dependence of the binding enthalpies on temperature over this temperature range. Values for K_b change by a factor of 500 between Me-α-DNS-GalN where $K_b = 3.81 \times 10^5$ M^{-1} and fucose where $K_b = 0.46 \times 10^3$ M^{-1}. Calculated values of the binding enthalpy from the van't Hoff equation,

$$\ln\{K_b(T)/K_b(T_0)\} = -\Delta H_{bv}[1/T_0 - 1/T]/R \quad \text{(Eq. 4)}$$

were in good agreement with the ITC binding enthalpies for

[2] Certain commercial equipment, instruments, and materials are identified in this paper in order to specify the experimental procedure as completely as possible. In no case does this identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the material, instrument, or equipment identified is necessarily the best available for the purpose.

TABLE I
Thermodynamic quantities of carbohydrate binding to ECorL in phosphate-buffered saline at pH = 7.4

Ligand	T [a]	K_b	$-\Delta G^0_b$	$-\Delta H^0_b$	$-\Delta S_b$
	K	$\times 10^3$ *liter mol*$^{-1}$	*kJ mol*$^{-1}$	*kJ mol*$^{-1}$	*J mol*$^{-1}$ *K*$^{-1}$
LacNac	283.2	17.5 ± 0.9	23.0 ± 0.1	46.1 ± 3.0	81.3 ± 9.5
	299.0	9.73 ± 0.6	22.7 ± 0.1	47.1 ± 2.6	83.2 ± 9.5
ΔH_{bv} [b] = −27.7 ± 2.0 kJ mol^{-1}		Average values:		46.6 ± 0.5	82.3 ± 1.0
Lac	285.7	4.05 ± 0.24	19.7 ± 0.1	43.4 ± 2.6	82.7 ± 9.1
	298.0	1.94 ± 0.12	18.8 ± 0.1	41.2 ± 2.5	75.4 ± 8.4
ΔH_{bv} = −42.5 ± 0.4 kJ mol^{-1}		Average values:		42.3 ± 1.1	79.0 ± 3.6
2′-FL	283.2	7.14 ± 0.28	20.9 ± 0.1	16.2 ± 0.8	−16.6 ± 2.0
	298.2	3.65 ± 0.23	20.3 ± 0.1	18.0 ± 0.9	−7.7 ± 1.2
ΔH_{bv} = −31.4 ± 2.5 kJ mol^{-1}		Average values:		17.1 ± 0.9	−12.2 ± 4.4
GalNAc	286.7	2.32 ± 0.19	18.5 ± 0.2	23.4 ± 1.6	17.2 ± 5.6
	298.2	1.34 ± 0.11	17.9 ± 0.2	23.0 ± 1.6	17.1 ± 5.3
ΔH_{bv} = −15.2 ± 1.7 kJ mol^{-1}		Average values:		23.2 ± 0.2	17.1 ± 0.1
Gal	286.0	2.41 ± 0.19	18.5 ± 0.2	14.9 ± 1.0	−12.5 ± 3.6
	298.2	1.57 ± 0.13	18.2 ± 0.2	13.7 ± 1.0	−15.3 ± 3.4
ΔH_{bv} = −24.7 ± 2.7 kJ mol^{-1}		Average values:		14.3 ± 0.6	−13.9 ± 1.4
Me-α-Gal	285.5	1.50 ± 0.12	17.4 ± 0.2	22.0 ± 1.5	16.2 ± 5.3
	297.6	1.40 ± 0.12	18.1 ± 0.2	21.6 ± 1.5	12.0 ± 5.0
ΔH_{bv} = −10.0 ± 1.1 kJ mol^{-1}		Average values:		21.8 ± 0.2	14.1 ± 2.1
Me-β-Gal	285.5	0.932 ± 0.075	16.2 ± 0.2	20.6 ± 1.4	15.2 ± 5.0
	297.2	0.720 ± 0.058	16.3 ± 0.2	18.2 ± 1.3	6.6 ± 4.4
ΔH_{bv} = −15.2 ± 1.7 kJ mol^{-1}		Average values:		19.4 ± 1.2	10.9 ± 3.3
Me-α-DNS-GalN	283.2	381.1 ± 10.0	30.5 ± 1.2	18.5 ± 0.6	−42.4 ± 0.7
	298.2	351.5 ± 12.0	31.7 ± 1.3	23.1 ± 0.7	−30.0 ± 0.8
D-Fucose	283.2	0.58 ± 0.06	15.0 ± 2.0	4.1 ± 0.4	−38.5 ± 5.0
	297.2	0.46 ± 0.05	15.2 ± 2.0	4.7 ± 0.4	−35.2 ± 4.0
ΔH_{bv} = −10.9 ± 1.1 kJ mol^{-1}		Average values:		4.4 ± 0.3	−36.9 ± 1.7

[a] T, error in the temperatures is +0.1.
[b] T, the ΔH_{bv} values were calculated from Equation 1 in the text.

Lac, GalNAc, and Me-β-Gal (Table I). The calculated van't Hoff enthalpies for 2′-fucosyllactose, *N*-acetyllactosamine, galactose, Me-α-Gal, and fucose differed substantially from the ITC binding enthalpies. Such differences between the ITC binding enthalpies and van't Hoff enthalpies are not uncommon and have been discussed in detail by Naghibi *et al.* (1995). Contributions of $T\Delta S_b$ to the binding reactions are negative with the exception of the binding of galactose, Me-α-DNS-GalN, and fucose. The binding reactions also exhibit enthalpy-entropy compensation (Fig. 2).

The ΔH_b for Me-α-DNS-GalN show a decrease with temperature from −18.5 ± 0.6 kJ mol^{-1} at 283.2 K to −23.1 ± 0.7 kJ mol^{-1} at 298.2 K. Assuming this increase to be linear with temperature, the heat capacity change ($d(\Delta H_b)/dT$) for this binding reaction is −0.31 ± 0.04 kJ mol^{-1} K^{-1}. Since the binding constants were determined only at two temperatures and an additional heat capacity term must be included in Equation 4, a van't Hoff binding enthalpy could not be calculated for Me-α-DNS-GalN with certainty.

A typical DSC scan of ECorL is shown in Fig. 3 along with the fit of the single transition peak data to the $A_2 \rightleftharpoons 2B$ two-state transition model. The peak did not reappear upon a re-scan of the sample indicating that the transition is irreversible. Results of the fit of the transition peak data to the two-state transition model are presented in Table II. Although the results at higher scan rates exhibit a slight transition dependence of T_m and T_p on the scan rate, the thermodynamic quantities, ΔH_v and ΔH_c, are independent of scan rate. Since the temperature increase is only about 2–3 K in increasing the scan rate by more than a factor of 4 and ΔH_v and ΔH_c for the

transitions are the same, the equilibrium two-state transition model was applied to these transitions instead of the irreversible model of Sanchez-Ruiz (1988). This analysis of the irreversible ECorL transitions in terms of a thermodynamic model is based on treating them as a sequence of two processes; the reversible unfolding of the protein described by the unfolding temperature and the calorimetric enthalpy followed by a slower irreversible process such as aggregation. This approach yields results for the overall process that are the same as for the reversible process (Manly *et al.*, 1985). In Table II, the ratio $\Delta H_c/\Delta H_v$ is close to 2 indicating that two domains are unfolding independently during denaturation. Since the shape of the transition peak indicates that the domains unfold at the same temperature, it is attractive to identify each domain with the lectin subunit and assume that as both subunits unfold they dissociate. Thus, in the unfolded conformation ECorL exists as two separate monomers whereas in the folded state it exists as a dimer. This is further substantiated by the increase of T_m and T_p with increase in the protein concentration. It has been shown that for the denaturation of an oligomer followed by dissociation into its monomers this dependence is given by the following equation from Fukada *et al.* (1983),

$$\ln[A_n] = -\Delta H_v(S)/\{RT_p(n-1)\} + \text{constant} \qquad \text{(Eq. 5)}$$

where n is the number of subunits in the oligomer, $[A_n]$ is the concentration of the protein oligomer in the native state, and $\Delta H_v(S)$ is a van't Hoff enthalpy obtained from the slope of $\ln[A_n]$ *versus* $1/T_p$. A plot of this dependence is shown in Fig. 4 and the slope of the plot for $n = 2$ yields $\Delta H_s(S) = 665 \pm 182$ kJ mol^{-1} which is the same as ΔH_v for the transition. It should be emphasized that T_p is not determined from fits of the two-state transition model to the data, and, thus, the agreement between ΔH_v and $\Delta H_v(S)$ is independent confirmation that denaturation of ECorL results in dissociation. DSC scans of ECorL in the presence of saturating amounts of carbohydrate ligands exhibit a single transition that is best fitted to a $A_2 \rightleftharpoons 2B$ two-state transition model as in the absence of the ligand. The results fitting the model to the DSC data for the unfolding of ECorL in the presence of *N*-acetyllactosamine, lactose and galactose, Me-α-Gal, and Me-β-Gal are presented in Table III. T_m and T_p increase with ligand concentration, whereas ΔH_v and ΔH_c remain the same. The increase in both T_m and T_p with ligand concentration arises from preferential binding of the ligand to the lectin in the folded form. The denaturation transition in the presence of bound ligand can be expressed as follows,

$$A_2L_m = 2B + \text{mL} \qquad \text{(Eq. 6)}$$

and at constant lectin concentration,

$$\ln[\text{L}] = \{-\Delta H_v(\text{L})/RT_pm\} + \text{constant.} \qquad \text{(Eq. 7)}$$

Plots of $\ln[\text{L}]$ *versus* $1/T_p$ are shown in Figs. 5 and 6, and the best fits of $\ln[\text{L}]$ to $1/T_p$ yield values with $m = 2$ for $\Delta H_v(\text{L})$ close to ΔH_v and ΔH_c as shown in Table III. Thus, one carbohydrate ligand binds to each of the two monomer units of ECorL. It has been shown (Schwarz, 1988) that the increase in the transition temperature with ligand concentration at [L]>>>[Protein] depends on K_b at the denaturation temperature and that $K_b(T_m)$

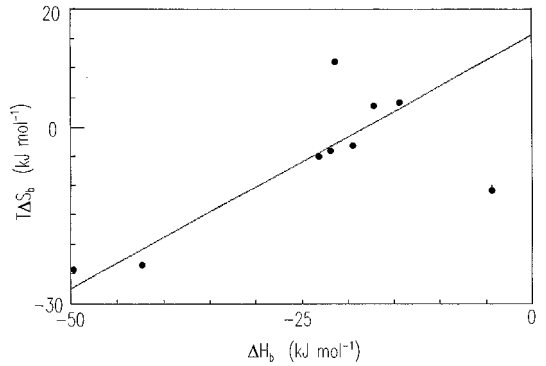

FIG. 2. **A plot of $T\Delta S_b$ *versus* ΔH_b for the binding of carbohydrates to ECorL at 298.2 K.** The *line* is the best least squares fit of the data to a straight line and is described by $T\Delta S_b$ (kJ mol^{-1})= 5.5 ± 2.1+ 0.86 ± 0.07ΔH_b (kJ mol^{-1}).

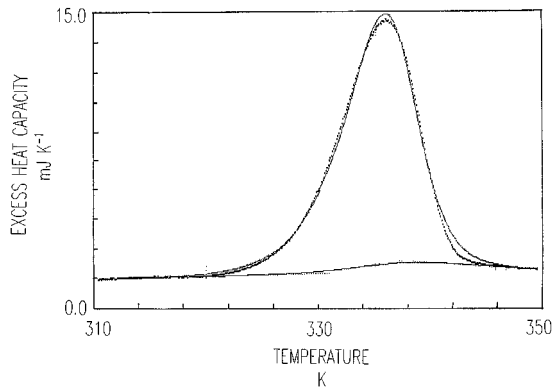

FIG. 3. **DSC scan of a 1.2-ml sample of 0.2 mM ECorL in 0.02 M sodium phosphate buffer containing 0.15 M NaCl at a scan rate of 20 K h^{-1}.** The *solid lines* are the best least squares fits of the DSC data to the A ↔ 2B two-state transition model. The extrapolated base line is also shown for the fit.

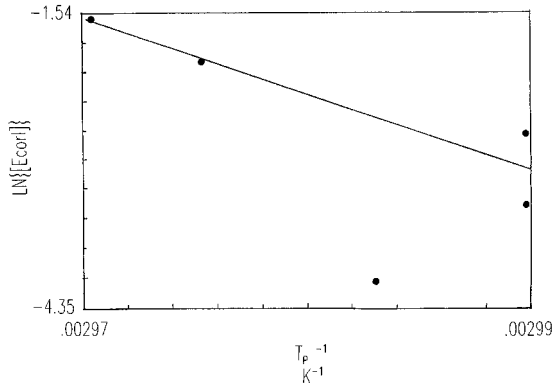

FIG. 4. **A plot of ln[ECorL] *versus* $1/T_p$.** The *line* is the best linear least squares fit of ln{[ECorL]} to $1/T_p$.

TABLE II

Thermodynamic quantities from DSC measurements on the thermal transition of ECorL in PBS at pH 7.4

Concentration (mM)	T_m (K)	ΔH_v	ΔH_c	$\Delta H_c/\Delta H_v$	T_p	$\Delta H(S)$
		kJ mol^{-1}			*K*	*kJ mol^{-1}*
At scan rate = 20 K h^{-1}						
0.017–0.200	333.9–335.5	633 ± 19	1338 ± 152	2.12 ± 0.28	334.4–336.4	665 ± 182
At scan rate = 45 K h^{-1}						
0.033	335.7	655 ± 20	1465 ± 161	2.23 ± 0.25	336.0	
At scan rate = 90 K h^{-1}						
0.033	336.8	616 ± 11	1507 ± 70	2.45 ± 0.05	338.2	

TABLE III

Thermodynamic quantities from DSC measurements on the thermal transition of ECorL in the presence of ligands in PBS

K_b at T_m of 333.9 was determined from Equation 5 in text for LacNAc, Lac and Me-β-Gal and for Me-α-Gal by using ΔH_b for ΔH_{bv} in the equation. K_b (DSC) for LacNAc was calculated for ΔH_c = 1348 kJ/mol, the lower limit of ΔH_c.

Ligand	Ligand concentration	T_m	ΔH_v	ΔH_c	$\Delta H_c/\Delta H_v$	T_p	$\Delta H(L)$	K_b (DSC)/K_b
	mM	*K*	*kJ mol*$^{-1}$			*K*	*kJ mol*$^{-1}$	
LacNAc	1–15	334.9–340.1	713 ± 14	1555 ± 207	2.18 ± 0.30	335.9–341.8	506 ± 66	1.24 ± 0.80
Lac	3–24	334.0–338.2	689 ± 17	1475 ± 60	2.14 ± 0.12	334.1–339.2	538 ± 76	1.80 ± 0.38
Me-α-Gal	4–24	335.6–338.8	723 ± 40	1048 ± 178	1.46 ± 0.31	336.3–339.7	419 ± 36	1.55 ± 0.31
Me-β-Gal	4–24	335.4–337.9	697 ± 11	1123 ± 36	1.60 ± 0.04	335.9–338.7	545 ± 69	1.09 ± 0.35

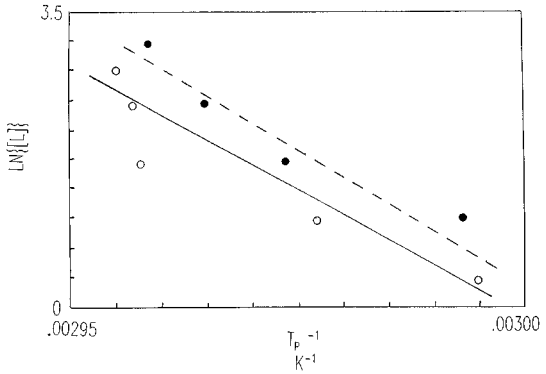

FIG. 5. **Plots of ln[ligand] *versus* $1/T_p$ for lactose (○) and for LacNAc (●).** The *lines* are the best linear least squares fits of ln{[L]} *versus* $1/T_p$ for Lac (−) and for LacNAc (− −).

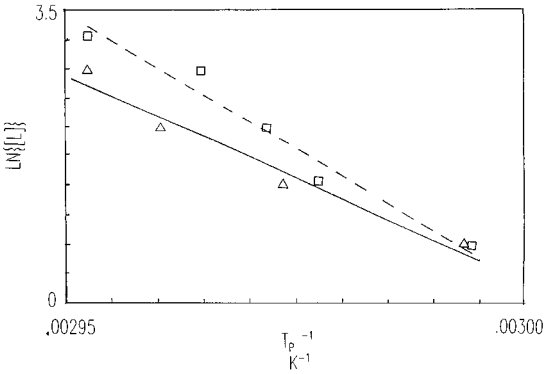

FIG. 6. **Plots of ln[ligand concentration] *versus* $1/T_p$ for Me-α-Gal (△) and for Me-β-Gal (□).** The *lines* are the best linear least squares fits of ln{[L]} *versus* $1/T_p$ for Me-α-Gal (−) and for Me-β-Gal (− −).

can be determined from this increase. For ligand dissociation from the two independent, identical sites of a protein upon denaturation, Schellman (1975) showed that the free energy of binding at the T_m of a ligated protein, T_l, is

$$\Delta G(T_1)/RT_1 = -\ln\{1 + K_b[L]\}^2 = \{(T_1 - T_m)\Delta H_c(T_c)/RT_1T_m\} \quad \text{(Eq. 8)}$$

so that

$$K_b(T_1) = [\exp\{(T_1 - T_m)\Delta H_c(T_c)/2.0\ RT_1T_m\} - 1]/[\mathrm{L}], \quad \text{(Eq. 9)}$$

where [L], the free ligand concentration, is approximated as the difference between the total ligand concentration and 2 × the protein concentration. In Table III, values for K_b(l) are compared with values for K_b determined from ITC, $K_b(T_l)$, using Equation 5. The comparison in terms of the ratio K_b(DSC)/K_b(ITC) in Table III shows that the ITC results are in reasonable agreement with the DSC results for the determination of K_b.

DISCUSSION

The binding site observed in x-ray crystallography studies of ECorL (Fig. 7) complexed with lactose is very similar to that of concanavalin A and pea lectin. It consists of a shallow depression on the surface of the protein that shares residues with a calcium binding site (Shaanan *et al.*, 1991). However, concanavalin A and pea lectin are specific for manno- and glucopyranoside derivatives where C4-OH is equatorial whereas ECorL is specific for galactopyranoside derivatives where the C4-OH is axial. In addition to the predominance of the usual hydrogen bonds between a carbohydrate and the lectin, hydrophobic interactions have also been identified for galactose binding to ECorL and involve Ala-88, Tyr-106, Phe-131, and Ala-218 at the binding site (Shaanan *et al.*, 1991). Galactose in ECorL specifically stacks on Phe-131 just as mannose stacks on a tyrosine in concanavalin A. Hydrophobic interactions with concanavalin A and pea lectin have also been identified for the phenyl group of phenyl α-glucopyranoside and the methyl group of 3-*O*-methyl glucose (Schwarz *et al.*, 1996). Differences in the specificity of ECorL arise from rotation of galactose so that its C3-OH and C4-OH groups hydrogen bond to Asn-133 and Asp-89, whereas the equivalent residues in concanavalin A and pea lectin hydrogen bond to the C4-OH and C6-OH groups of the pyranose ring (Shaanan *et al.*, 1991; Adar and Sharon, 1996).

Observation of enthalpy-entropy compensation is not surprising as thermodynamic studies on several lectins strongly emphasize the importance of water as a mediator in protein-carbohydrate recognition (Lemieux *et al.*, 1994; Puri and Surolia, 1994; Toone, 1994; Ramkumar *et al.*, 1995). This fact is also succinctly brought out by the high resolution structural analysis of *Lathyrus ochrus* lectin I complexed with its complementary oligosaccharide ligand (Bourne *et al.*, 1992). Other factors, although less likely, such as subtle shifts in protein conformational states cannot be ignored as a source of the observed enthalpy-entropy compensation (Eftink *et al.*, 1983).

The binding reactions are predominantly enthalpically driven, and entropy-enthalpy compensation is observed. Therefore, differences in the binding affinities can be discussed in terms of differences in the binding enthalpies if it is assumed that the initial interaction of the isolated saccharide ligands with surrounding water molecules is the same for all the ligands. The binding enthalpies of *N*-acetyllactosamine, Me-α-Gal, and Me-β-Gal are all close to −20 kJ mol^{-1} whereas that of galactose is −14 kJ mol^{-1}. A similar stronger interaction between concanavalin A and manno- and glucopyranose is observed upon converting the OH of C1 to OCH_3 (Schwarz *et al.*, 1993). Possible hydrophobic interactions between the C1 methoxy groups and the neighboring aromatic ring of Tyr-12 in concanavalin A and Phe-123 in pea lectin could account for a stronger interaction with these derivatives. Despite the proximity of the Phe-131 aromatic ring and Ala-218 in ECorL to C1 of galactose in the binding site, such hydrophobic interactions with this lectin appear less tenacious as compared with concanavalin A.

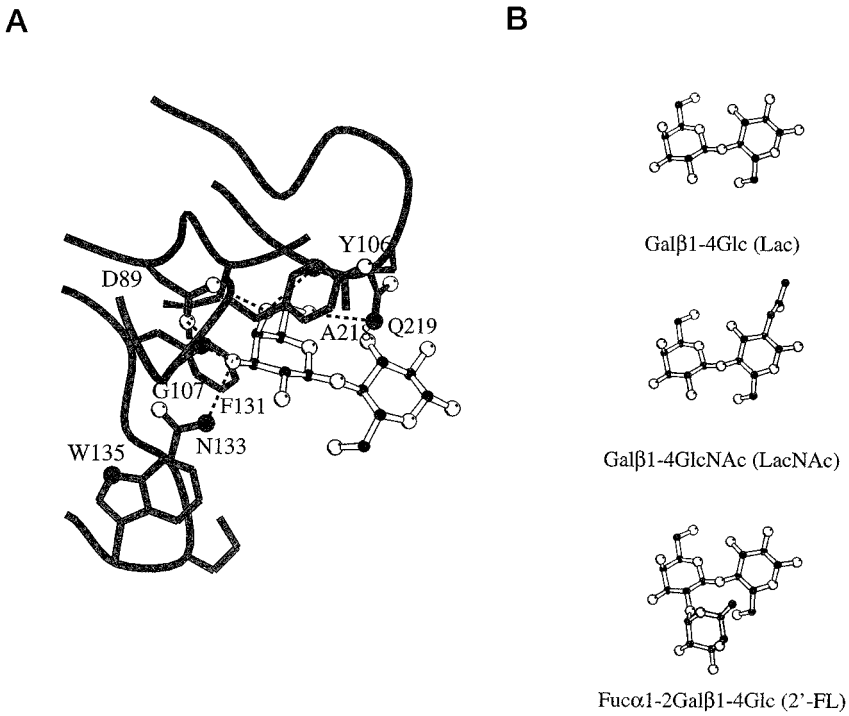

FIG. 7. **MOLSCRIPT (Kraulis, 1991) representation of the combining site of ECorL-lactose complex (*A*), lactose, *N*-acetyllactosamine, and 2′-fucosyllactose (*B*) ECorL.** *A*, the hydrogen bonds between the galactose and the side chains of D89, N133, and Q219 and of the main chain amides of G107 and A218 displayed on the basis of structure by Shaanan *et al.* (1991). *B*, oligosaccharide structures are adapted from Acharya *et al.* (1990).

Binding of fucose, which has a methyl group at its C5 position instead of a hydroxymethyl group at the equivalent carbon of the galactose, suggests that C5 substituent of galactose is primarily involved in van der Waals interaction with the lectin. The fact that *N*-acetylgalactosamine is as potent a ligand as galactose indicates that an acetamido group at C2-OH of the latter does not interfere with the interaction with the ECorL (Kinzy *et al.*, 1992). Addition of L-fucose to lactose as in 2′-fucosyllactose enhances the binding affinity due to a favorable entropic contribution. In 2′-fucosyllactose, perhaps the L-fucose residue at the C2 of galactose encounters a hydrophobic locus in the binding site of ECorL. This is not surprising as L-fucose differs from other pyranoses in having a $1C_4$ configuration, and the surface presented by its C4 and C5 region of the molecule is distinctly nonpolar and might abut to within 3.5 Å of Trp-135, Tyr-106, and Tyr-108 side chains in the binding site. Likewise, the strong and entropically driven nature of the binding of Me-α-DNS-GalN to EcorL also appears to be due to enhanced nonpolar contacts (<3 Å) between the aromatic dansyl moiety at C2 of the ligand and Trp-135 in the binding site of ECorL. The contribution of nonpolar contacts in the interaction between Me-α-DNS-GalN and ECorL is further evident in the negative heat capacity change observed for this interaction.

For the disaccharides, the binding enthalpy to ECorL (Table I) increases by a factor of 2 upon the addition of glucopyranoside to the galactopyranose to form lactose, in contrast to the binding enthalpies of the dimannopyranosides to concanavalin A which are the same as for mannopyranose (Van Landschoot *et al.*, 1980; Williams *et al.*, 1992). Since crystal structures of both lectins show that the second pyranoside ring projects out of the pocket into the solvent for ECorL (Shaanan *et al.*, 1991) and presumably also for concanavalin A, the binding interaction should be the same for both the mono- and disaccharide as is observed for the concanavalin A complex. Closer inspection of the structure of the ECorL complex indicates an additional possible hydrogen bond formation between the glucopyranoside ring and Gln-219 (Adar and Sharon, 1996) which would account for a small negative increase in ΔH_b but not a doubling of the binding enthalpy. Since, in the crystal structure of the Lac-ECorL complex, there are three localized water molecules trapped between the glucopyranoside ring and the surface surrounding the binding cavity, it is possible that a network of hydrogen bonds involving these water molecules between the glucopyranoside moiety of lactose and the surface of ECorL could account for this enhanced binding interaction. Such networks of water-mediated hydrogen bonds seem to account for the large binding enthalpy (-90 kJ mol^{-1}) between lysozyme antigen and the D1.3 IgG antibody binding site (Bhat *et al.*, 1994) relative to the enthalpy change of -39.4 kJ mol^{-1} for lysozyme binding to the D44.1 IgG which has only a few less water molecules in the binding interface (Braden *et al.*, 1994). There is also a marked enhancement in the binding interaction with the acetamido derivative of lactose, namely *N*-acetyllactosamine, which would further promote water-mediated hydrogen bonding at this interface in addition to the enhanced interactions between its acetamido group and Gln-219 residue in the combining site of ECorL.

The importance of water molecules in the binding interaction between ECorL and the disaccharides is also evident by the fact that there is a net uptake of 2–3 additional water molecules upon binding of lactose and *N*-acetyllactosamine as compared with that observed for galactose or Me-β-Gal.[3]

Thermal denaturation results in dissociation of the ECorL dimer into its two monomer subunits. Dissociation into monomeric units is also observed for the concanavalin A tetramer (Schwarz *et al.*, 1993). The ratio of $\Delta H_c/\Delta H_v$ of 2 indicates that two domains of the protein unfold independently with the subsequent dissociation of the protein. This is similar to the basic lectin from winged bean (Schwarz *et al.*, 1991) where two

[3] C. P. Swaminathan and A. Surolia, unpublished observations.

domains unfold independently, but at different temperatures, and dissociate into two subunits. Although the monomeric units of concanavalin A have a similar molecular mass and tertiary structure as ECorL (Shaanan *et al*., 1991), the thermal denaturation of ECorL occurs at 333 K whereas that of concanavalin A occurs at 363 K (Schwarz *et al*., 1993) reflecting the much greater stability of the latter. The structures of the pea and lentil lectins also exhibit greater stability than that of ECorL. The greater stability of concanavalin A is also reflected in the $\Delta H_c/\Delta H_v$ ratio of 1 indicating that concanavalin A tetramer unfolds as a single entity because of strong interactions between the subunits. On the other hand, ECorL where the contact region at monomer-monomer interface (700 Å) is smaller than in concanavalin A (1000 A) (Shaanan *et al*., 1991) exhibits independent unfolding of two identical domains which may be identified as its monomer subunits. This difference in the stability thus results from the difference in the quaternary structure of ECorL induced by the bulky carbohydrate protruding from Asn-17 (Shaanan *et al*., 1991). Therefore, the mode of dimerization in legume lectins seems to have a profound consequence on their thermal stability as well as the mode of their thermal unfolding.

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Thermodynamics of Monosaccharide and Disaccharide Binding to *Erythrina corallodendron* Lectin

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