Role of Water in the Specific Binding of Mannose and Mannooligosaccharides to Concanavalin A

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Received September 25, 1997

Abstract: We report here, the first solution state evidence for the role of water molecules in the specific interaction of carbohydrates with a legume lectin, concanavalin A. Concanavalin A from *Canavalia ensiformis* is a protein containing 237 amino acid residues with each monomer possessing one sugar binding site as well as sites for transition-metal ions, Mn^{2+} and Ca^{2+}. The lectin binds specifically to α-anomers of monosaccharides, D-glucopyranoside and D-mannopyranoside, and recognizes the trimannosidic core of *N*-linked glycoproteins, 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside with high specificity, which constitutes the minimum carbohydrate epitope that completely fills the sugar binding site. Sensitive isothermal titration microcalorimetry coupled with osmotic stress strategy on concanavalin A was used to dissect out the differential involvement of water molecules in the recognition of the branched trimannoside (3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside), the individual dimannosidic arms (3-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside and 6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside) as well as the monomer unit, D-mannopyranoside. The specific binding of concanavalin A to different sugars, is accompanied by differential uptake of water molecules during the binding process. These results not only complement the X-ray crystallographic studies of legume lectin−sugar complexes displaying structurally conserved water molecules mediating the specific ligation of the sugars with the corresponding sites in the binding pocket but also provide a rationale for the observed compensatory behavior of enthalpies with entropies in lectin−sugar interactions.

Introduction

The specificity and reversibility of carbohydrate recognition by lectins has made them useful as probes for the identification and characterization of carbohydrate structures during cell growth, differentiation, malignancies, and metastases.[1−3] Lectins also provide interesting paradigms for the understanding of protein−carbohydrate recognition. Despite a high degree of similarity in their recognition of monosaccharide ligands, plant lectins display exquisite specificities for binding to oligosaccharides. Concanavalin A (Con A), first isolated as bisphenoid crystals by Sumner,[4] has been one of the most extensively studied lectin. Pioneering studies of Goldstein and co-workers have shown that it binds to mono- and oligosaccharides with terminal non reducing α-D-mannopyranosyl (α-D-man) and α-D-glucopyranosyl (α-D-glc) residues, and to certain internal glucose or mannose residues, whereas other stereoisomers of these saccharides such as α-D-galactopyranosides (α-D-gal) do not inhibit its activities.[5] These studies led Goldstein to propose that the equatorial orientation of hydroxyl groups at C3 and C4 and the hydroxymethyl group at C5, as is the case with glucose and mannose, are indispensable for the binding of Con A with sugars and that these hydroxyl groups are involved in hydrogen-bonding interactions with the protein. These rules for Con A monosaccharide specificity though proposed in late 1960s were elucidated at atomic resolution only in 1989 when the crystal structure of Con A−methyl-α-D-mannopyranoside complex was determined.[6,7] These studies thus showed that C3, C4, and C6 hydroxyl groups of methyl-α-D-mannopyranoside bound in the C1 chair conformation are hydrogen bonded to the residues in the combining site of the protein while the hydroxyl group at C2 is linked through a water molecule to the adjacent subunit.

Elegant immunoprecipitation and NMR dispersion studies of Brewer and co-workers demonstrated that the most complementary ligand for Con A is methyl-3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside and postulated that the lectin has an extended binding site for the trisaccharide wherein certain groups from all of its constituent monosaccharide contribute to the interaction.[8] Indeed the recent crystallographic studies of Naismith and Field[9] and Loris et al.[10] have not only confirmed these postulates but have also provided a rationale for the high affinity interactions between Con A and the trimannoside.

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(1) Ben-Bassat, H.; Goldblum, N. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 1046−1049.

(2) Brown, J. C.; Hunt, R. C. *Int. Rev. Cytol.* **1978**, *52*, 277−349.

(3) Bittiger, H.; Schnebli, H. P. *Concanavalin A As A Tool*; John Wiley and Sons: New York, 1976.

(4) Sumner, J. B. *J. Biol. Chem.* **1919**, *37*, 137−142.

(5) Goldstein, I. J.; Poretz, R. D. *The Lectins: Properties, Functions and Applications in Biology and Medicine*; Liener, I. E., Sharon, N., Goldstein, I. J., Eds.; Academic Press Inc.: Orlando, FL, 1986; pp 33−247.

(6) Derewenda, Z.; Yariv, J.; Helliwell, J. R.; Kalb (Gilboa), A. J.; Dodson, E.; Paiz, M.; Wan, T.; Campbell, J. W. *EMBO J.* **1989**, *8*, 2189−2193.

(7) Naismith, J. H.; Emmerich, C.; Habash, J.; Harrop, S. J.; Helliwell, J. R.; Hunter, W. N.; Raferty, J.; Kalb (Gilboa), A. J.; Yariv, J. *Acta Crystallogr. Sect. D* **1994**, *50*, 847−858.

(8) Brewer, C. F.; Bhattacharyya, L.; Brown, R. D., III.; Koenig, S. H. *Biochem. Biophys. Res. Commun.* **1985**, *127*, 1066−1071.

(9) Naismith, J. H.; Field, R. A. *J. Biol. Chem.* **1996**, *271*, 972−976.

S0002-7863(97)03369-6 CCC: $15.00
Published on Web 05/13/1998

Lemieux and co-workers were the first to postulate the importance of water in mediating protein−sugar interactions.[11−14] Thermodynamic studies on Con A as well as other lectins have supported such a role of water in these reactions.[15−20]

During recent years the thermodynamics of carbohydrate interaction with Con A and other lectins has also been extensively characterized. A striking feature observed consistently in these studies is the compensatory changes in enthalpies and entropies. The enthlapy−entropy compensation has been suggested to originate primarily from the reorganization of the solvent water occurring during these reactions.[14] However, the precise nature of the reorganization of solvent which could involve either the uptake or the release of water molecules or the restructuring of the existing molecules has eluded an explanation. Studies reported here attempt to fill this lacuna in lectin−sugar interactions. For this purpose, we have employed the osmotic stress technique pioneered by Parsegian and co-workers[21] to control the water activity during the specific binding of mannose and manno-oligosaccharides to Con A.

The thermodynamics of the carbohydrate−Con A binding reaction were considered in terms of the site binding constant (K_b), and changes in the free energy ($\Delta G°_b$), the binding enthalpy ($\Delta H°_b$), and the binding entropy ($\Delta S°_b$). The binding reaction between the Con A binding site and the carbohydrate ligand (L) is

$$\text{Con A} + \text{L} \rightleftharpoons \text{Con A·L} \qquad (1)$$

in 0.05 M 3,3′-dimethylglutaric acid sodium hydroxide (DMG) buffer pH 5.2 ± 0.01 containing 250 mM sodium chloride, 1 mM manganous chloride, and 1 mM calcium chloride, and under various stressing conditions of neutral osmolytes glycerol and ethylene glycol constituting an "osmotic stress" effect.[21] This coupling of a sensitive isothermal titration microcalorimetric method[22] (ITC) with osmotic stress strategy is, to our knowledge, the first use of these two powerful techniques together, enabling us to dissect out the differential involvement of water molecules in the specific recognition of the branched trimannoside 3,6-di-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (mannotriose), the minimum carbohydrate epitope that completely fills the sugar binding site of Con A, the individual dimannosidic arms 3-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (manα1-3man) and 6-*O*-(α-D-mannopyranosyl)-α-D-mannopyranoside (manα1-6man), as well as the monomeric unit, D-mannopyranoside (α-D-man) in the solution state.

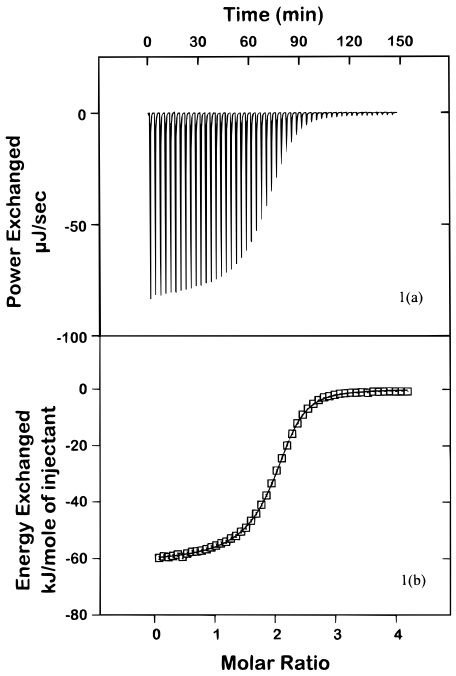

Figure 1. Calorimetric titration of mannotriose solution with Con A solution. (a) Raw data obtained from 49 automatic injections of 6.7 μL aliquots of 5.21 mM mannotriose solution into 0.34 mM Con A solution in 0.05 M DMG buffer, pH 5.2 containing 250 mM sodium chloride, 1 mM manganous chloride, and 1 mM calcium chloride at 283.2 K. (b) Non-linear least-squares fit (—) of the incremental heat per mole of added ligand (□) for the titration in (a) by injection number to eqs 3 and 4 in the text. The data points represent average values of four independent measurements. The standard deviations were well within the size of the data points.

Results

The results of a typical titration calorimetry measurement, which consisted of adding 6.7 μL aliquots of 5.21 mM mannotriose solution to 0.34 mM Con A solution in 0.05 M DMG buffer, pH 5.2 containing 250 mM sodium chloride, 1 mM manganous chloride, and 1 mM calcium chloride at 283.2 K is shown in Figure 1a. The results exhibit a monotonic decrease in the exothermic heat of binding with each successive injection until saturation is achieved. A non-linear least-squares fit of the ITC data to the identical site model described by eq 3, is also presented in Figure 1b. As shown by the solid curve in Figure 1b, the incremental heats per mole of added ligand follows closely the injection number. The results of a representative calorimetric titration of mannotriose solution into Con A solution in the presence of 1.66 Os (kg H_2O)$^{-1}$ ethylene glycol is shown in Figure 2. The close fit of the data to the identical site model shows that the ligand binds to each of the two binding sites of Con A independently, both in the absence as well as in the presence of osmolytes. The thermodynamic parameters $\Delta G°_b$, $\Delta H°_b$, and $\Delta S°_b$ at 283.2 K determined from the titration calorimetry measurements are presented in Table 1. The uncertainties in Table 1 are standard deviations of the mean values of K_b, $\Delta G°_b$, $\Delta H°_b$, and $\Delta S°_b$ determined from four independent measurements.

The dependence of the thermodynamic parameters for the binding of mannose, and the three manno-oligosaccharides, manα1-6man, manα1-3man, and mannotriose to Con A, on the osmolal concentration of the neutral solutes used, viz ethylene glycol are shown in Figure 3. For each solute, binding free energies show a linear dependence on solute osmolal concentration, consistent with an exclusion of these solutes from the water

(10) Loris, R.; Maes, D.; Poortmans, F.; Wyns, L.; Bouckaert, J. *J. Biol. Chem.* **1996**, *271*, 30614−30618.
(11) Lemieux, R. U.; Delbaere, L. T. J.; Beierbeck, H.; Spohr, U. *Ciba Found. Symp.* **1991**, *158*, 231−248.
(12) Spohr, U.; Paszkiewicz-Hnatiw, E.; Morishima, N.; Lemieux, R. U. *Can. J. Chem.* **1992**, *70*, 254−271.
(13) Hindsgaul, O.; Khare, D. P.; Bach, M.; Lemieux, R. U. *Can. J. Chem.* **1985**, *63*, 2653−2658.
(14) Lemieux, R. U. *Acc. Chem. Res.* **1996**, *29*, 373−380.
(15) Schwarz, F. P.; Puri, K. D.; Bhat, R. G.; Surolia, A. *J. Biol. Chem.* **1993**, *268*, 7668−7677.
(16) Puri, K. D.; Surolia, A. *Pure Appl. Chem.* **1994**, *66*, 497−502.
(17) Ramkumar, R.; Surolia, A.; Podder, S. K. *Biochem. J.* **1995**, *308*, 237−241.
(18) Surolia, A.; Sharon, N.; Schwarz, F. P. *J. Biol. Chem.* **1996**, *271*, 17697−17703.
(19) Toone, E. J. *Curr. Opin. Struct. Biol.* **1994**, *4*, 719−728.
(20) Chervenak, M. C.; Toone, E. J. *J. Am. Chem. Soc.* **1994**, *116*, 10533−10539.
(21) Parsegian, V. A.; Rand, R. P.; Rau, D. C. *Methods Enzymol.* **1995**, *259*, 43−94.
(22) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L.-N. *Anal. Biochem.* **1989**, *179*, 131−135.

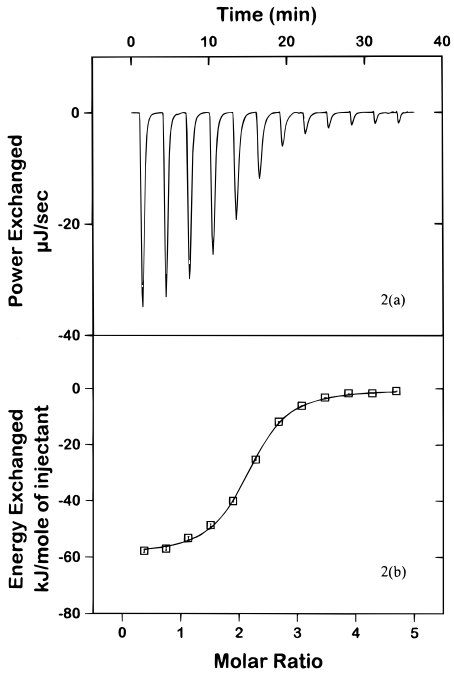

Figure 2. Calorimetric titration of mannotriose solution with Con A solution under osmotic stress. (a) Raw data obtained from 12 automatic injections of 12.0 μL aliquots of 1.50 mM mannotriose solution into 0.036 mM Con A solution in 0.05 M DMG buffer, pH 5.2 containing 250 mM sodium chloride, 1 mM manganous chloride, and 1 mM calcium chloride and 1.66 Os (kg H_2O)$^{-1}$ ethylene glycol at 283.2 K. (b) Non-linear least-squares fit (—) of the incremental heat per mole of added ligand (□) for the titration in (a) by injection number to eqs 3 and 4 in the text. The data points represent average values of four independent measurements. The standard deviations were well within the size of the data points.

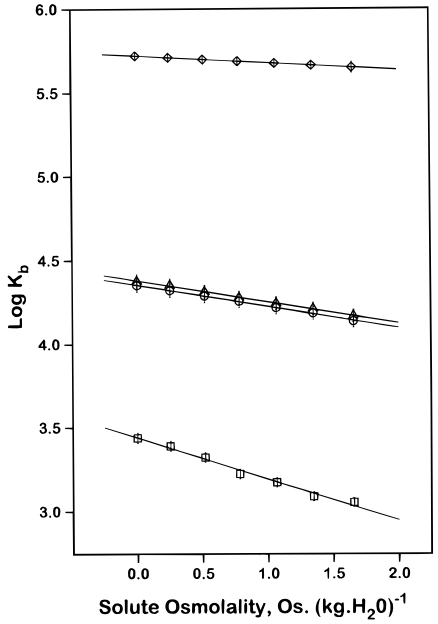

Figure 3. Osmotic sensitivity of logarithm of binding constant as a function of neutral solute osmolality for the binding of mannose (□), manα1-3man (△), manα1-6man (○), and mannotriose (◇) to Con A under ethylene glycol stress at 283.2 K. The straight lines were obtained by linear regression analysis of the data using Origin program and have slopes of −0.245 with a correlation coefficient to −0.993 for mannose; −0.127 with a correlation coefficient to −0.999 for manα1-3man; −0.127 with a correlation coefficient to −0.999 for manα1-6man; −0.043 with a correlation coefficient to −0.999 for mannotriose binding to Con A. The data points represent average values of four independent measurements. The standard deviations were well within the size of the data points.

surrounding the associating sugar and lectin surfaces. The negative sign of the slope defines a net uptake of water molecules by the Con A−sugar complex during the process of binding. The magnitude of the effect of osmotic stress depends both on the solute osmolal concentration, which is equivalent to the bulk water chemical potential, and on the difference in the number of solute-excluding water molecules associated with the Con A−sugar complex and the number associated with the free Con A and sugar molecules.

This osmotic effect can be quantitated analogous to the conventional analysis of the effect of salt activity on protein binding.[21] The slope of log K_a versus ln a_w, where a_w is the water activity, is $2.303\Delta n_w$. This value, Δn_w, gives the change in number of solute-excluding water molecules coupled to the binding process. Since ln $a_w = -[\text{solute}]_{\text{osmolal}}/55.56$, where $[\text{solute}]_{\text{osmolal}}$ is the solute osmolal concentration and 55.56 is the number of moles of water in 1 kg, the slope of the lines shown in Figure 3 is given by

$$d \log K_a / d\,[\text{solute}]_{\text{osmolal}} = -2.303\Delta n_w/55.56 \qquad (2)$$

The uptake of water molecules thus associated with the binding of man, manα1-3man, manα1-6man, and mannotriose to Con A as a function of the osmotic stress is shown in Table 2. The data show that a variable number of water molecules are taken up when different ligands interact with the lectin. Control experiments in which aliquots of glycerol or ethylene glycol were injected into Con A or sugar solutions did not result in any measurable heats of binding ruling out the possibility of specific interactions between the stressing solute and Con A or the sugars, affirming the neutrality of the chosen osmolytes.

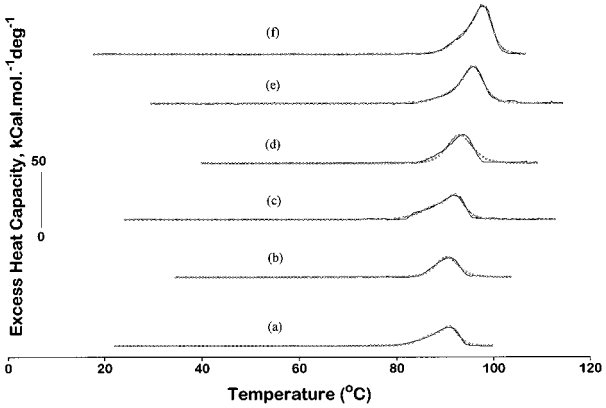

Figure 4. Differential scanning calorimetric scans at 20 K h^{-1} showing the apparent excess heat capacity for the thermal denaturation of Con A at a protein (dimer) concentration of 0.225 mM in 0.05 M DMG buffer, pH 5.2 containing 250 mM sodium chloride, 100 mM manganous chloride, and 100 mM calcium chloride (a); and increasing osmolal strengths of glycerol viz 6.25% (b); 12.5% (c); 25% (d); 37.5% (e); and 50% (f). The DSC data was best fitted to a single peak two-state transition (— ○ —), by the resolution of the progress baseline-subtracted and concentration-normalized DSC curve (—) for Con A using the Origin program.

A typical DSC scan of Con A in the presence and absence of osmolytes is shown in Figure 4 along with the fit of the single transition peak data to the $A_2 \leftrightarrow 2B$ two-state transition model. The transition peak with the osmolytes did not reappear upon a rescan of the sample, as is the case without them. Results of the fit of the transition peak data to the two-state transition model are presented in Table 3. Since the temperature increase is only about 2−3 K in increasing the scan rate by more than a factor

Table 1. Thermodynamic Quantities for Binding of Sugars to Con A at 283.2 K

sugar	osmolyte	osmolality (Os (kg-H_2O)$^{-1}$)	10^{-3} K_b (M^{-1})	$-\Delta G°_b$ (kJ mol^{-1})	$-\Delta H°_b$ (kJ mol^{-1})	$-\Delta S°$ (J mol^{-1} K^{-1})
man		0	2.75 (±0.08)[a]	18.67	21.04 (±0.19)	8
	glycerol	0.35	2.33 (±0.09)	18.25	20.49 (±0.18)	8
		0.73	1.94 (±0.04)	17.83	20.43 (±0.21)	9
		1.62	1.27 (±0.03)	16.82	19.95 (±0.25)	11
	ethylene glycol	0.25	2.46 (±0.11)	18.37	20.99 (±0.21)	9
		0.52	2.11 (±0.09)	18.04	20.90 (±0.20)	10
		0.78	1.68 (±0.07)	17.49	20.78 (±0.23)	12
		1.07	1.50 (±0.04)	17.20	20.29 (±0.14)	11
		1.35	1.24 (±0.03)	16.78	20.04 (±0.17)	12
		1.66	1.14 (±0.04)	16.57	19.85 (±0.12)	12
manα1-3man		0	22.38 (±0.26)	23.58	34.97 (±0.17)	40
	glycerol	0.35	20.32 (±0.15)	23.35	34.52 (±0.22)	39
		0.73	18.28 (±0.37)	23.10	33.51 (±0.14)	37
		1.62	13.89 (±0.09)	22.46	32.92 (±0.25)	37
	ethylene glycol	0.25	20.85 (±0.19)	23.41	34.43 (±0.12)	39
		0.52	19.32 (±0.13)	23.23	33.87 (±0.13)	38
		0.78	17.99 (±0.24)	23.06	33.14 (±0.21)	36
		1.07	16.45 (±0.38)	22.85	32.08 (±0.15)	33
		1.35	15.28 (±0.31)	22.68	31.42 (±0.19)	31
		1.66	13.71 (±0.16)	22.42	31.68 (±0.11)	33
manα1-6man		0	23.80 (±0.24)	23.72	30.22 (±0.17)	23
	glycerol	0.35	21.48 (±0.45)	23.48	29.54 (±0.39)	21
		0.73	19.34 (±0.58)	23.24	29.11 (±0.31)	21
		1.62	14.83 (±0.57)	22.61	28.76 (±0.28)	22
	ethylene glycol	0.25	22.44 (±0.38)	23.58	29.84 (±0.13)	22
		0.52	20.56 (±0.26)	23.38	29.57 (±0.37)	22
		0.78	18.96 (±0.29)	23.19	29.26 (±0.15)	21
		1.07	17.51 (±0.13)	23.00	29.82 (±0.26)	24
		1.35	16.25 (±0.14)	22.83	29.01 (±0.24)	22
		1.66	14.72 (±0.18)	22.59	28.84 (±0.14)	22
mannotriose		0	524.83 (±12.6)	31.01	59.49 (±0.37)	101
	glycerol	0.35	507.11 (±11.2)	30.93	59.37 (±0.43)	100
		0.73	488.47 (±11.7)	30.84	59.31 (±0.42)	101
		1.62	447.25 (±11.1)	30.63	59.24 (±0.46)	101
	ethylene glycol	0.25	512.17 (±10.2)	30.97	59.45 (±0.34)	101
		0.52	498.69 (±10.1)	30.89	59.38 (±0.31)	101
		0.78	485.93 (±10.4)	30.85	59.31 (±0.33)	101
		1.07	472.34 (±10.2)	30.75	59.32 (±0.32)	101
		1.35	459.46 (±11.3)	30.72	59.29 (±0.34)	101
		1.66	445.67 (±11.6)	30.63	59.19 (±0.34)	101

[a] The uncertainties in parantheses represent standard deviations of the mean values determined from four independent measurements.

Table 2. Changes in the Number of Solute-Excluding Water Molecules (Δn_w) Coupled to the Binding of Sugars to Con A as a Function of Osmotic Stress

sugar	glycerol	ethylene glycol
man	4.99 (±0.01)[a]	5.90 (±0.29)
manα1-3man	3.09 (±0.06)	3.06 (±0.04)
manα1-6man	3.06 (±0.03)	3.06 (±0.05)
mannotriose	1.03 (±0.01)	1.04 (±0.01)

[a] The uncertainties in parantheses represent standard deviations of the mean values determined from four independent measurements.

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 as has been done earlier in the absence of osmolytes,[15] instead of the irreversible model of Sanchez-Ruiz.[23] As evident from Figure 4, an increase in osmolality of the solution results in the increase of both T_m and ΔH values. The thermodynamic data thus obtained both with and without the osmolytes are shown in Table 3. The values of the van't Hoff enthalpies obtained from a plot of ln[P] vs $1/T_p$, where [P] is the lectin concentration and T_p is the temperature of the peak maximum, both of which are independent of the model used to fit the transition data are also tabulated. It may be noted that though

Table 3. Effect of Glycerol on the Thermodynamic Quantities from DSC Measurements on the Thermal Transition of Con A at a Scan Rate of 20 K h^{-1}

glycerol (%)	T_p (K)	T_m (K)	ΔH_c (kJ mol^{-1})	ΔH_v (kJ mol^{-1})	$\Delta H_c/\Delta H_v$
	363.8	363.0 (±0.03)[a]	1193 (±30)	799 (±26)	1.49
6.25	366.4	366.1 (±0.04)	1239 (±18)	824 (±42)	1.50
12.5	367.5	366.7 (±0.06)	1264 (±68)	854 (±31)	1.48
25	369.1	368.5 (±0.03)	1302 (±37)	875 (±38)	1.49
37.5	371.2	370.7 (±0.02)	1335 (±23)	887 (±29)	1.51
50	372.8	372.4 (±0.03)	1364 (±39)	900 (±22)	1.52

[a] The uncertainties in parantheses represent standard deviations of the mean values determined from four independent measurements.

T_m, T_p, ΔH_c, and ΔH_v increase with osmolytes, the cooperativity of the unfolding transition viz $\Delta H_c/\Delta H_v \approx 1$, remains unaltered.

Discussion

Intense attempts are being made to determine the exact number of functionally critical "structural" water molecules in proteins through X-ray crystallography. Loris et al.[24] have found that despite protein hydration being mainly dependent on the detailed local surface characteristics of the protein, significant portion of the water positions appear to be conserved in all

(23) Sanchez-Ruiz, J. M.; Lopez-Lacomba, J. L.; Cortijo, M.; Mateo, P. L. *Biochemistry* **1988**, *27*, 1648−1652.

(24) Loris, R.; Stas, P. P. G.; Wyns, L. *J. Biol. Chem.* **1994**, *269*, 26722−26733.

independent crystal structures of proteins of a given family. They found seven water sites completely conserved in all legume lectin crystal structures including Con A, independent of their degree of sequence homology or carbohydrate specificity.

It is noteworthy that although the overall conformational similarity between D-mannopyranoside and D-glucopyranoside and the configuration of hydroxyl groups at their C3, C4, and C6 positions are identical, the origin of specific discrimination among these saccharides by Con A is not readily evident, notwithstanding the availability of X-ray crystallographic structures of Con A complexed with methyl-α-mannopyranoside as well as those complexed with derivatives of D-glucopyranoside.[6,7,25−27] More so as the hydroxyl group at C2 of D-glucopyranoside is not known to interact with Con A; whereas, the axially oriented hydroxyl group at the corresponding position in D-mannopyranoside interacts with residues on the adjacent subunits in the crystals, such an interaction is unlikely to occur in solution. The positions of water molecules assigned in crystal structures refined to a high resolution either using X-ray diffraction, which reveals the water oxygen positions, or neutron diffraction, which unveils water hydrogen positions are still the best views of structural water molecules in proteins. NMR, on the other hand, provides information about the positions of the most slowly exchanging water molecules in the solution state.[28] However the observation of water-mediated hydrogen-bonding interactions between the conserved binding site amino acid residues and the corresponding hydroxyl group in the bound monosaccharide molecule in high-resolution crystal structures underscore the importance of water in determining the binding specificity of these reactions. Since neither Con A nor its sugar ligands undergo any appreciable alterations during their interactions,[9] we have chosen to utilize osmotic stress to evaluate the uptake or loss of waters involved in these reactions.

Glycerol and ethylene glycol are solutes of different chemical nature, yet their effect on Con A−saccharide interactions reported here is the same. Though the dielectric constant of the solution decreases slightly with polyols such as glycerol and decrease significantly with oligoethylene glycols, a common osmotic mechanism of solute action emerges, clearly distinguishing it from an electrostatic one. Linear plots of logarithm of binding constants versus neutral solute's osmolality for the binding of mannose, manα1-3man, manα1-6man, and mannotriose to Con A clearly indicate a linkage to a well defined and constant difference in the number of water molecules involved in ligand binding (Figure 3). Since the slopes are nearly the same for two chemically different classes of neutral solutes, the observed changes can indeed be rationalized in terms of a true osmotic stress effect. It is also known that, ethylene glycol at a concentration of 20% (v/v) appears to have little effect on protein conformation and does not specifically interact with proteins.[29] It is well-known that ethylene glycol and glycerol are preferentially excluded from the tiny spaces and from the envelope around the macromolecular surface and do not interact with the proteins or alter their conformation.[30−33] The free energy for the interaction of two hydrated species, the lectin Con A and the corresponding sugar molecule coming into contact, is the difference between the complex of Con A and the sugar and that between Con A and water as well as between the sugar and water. The measurement of these changes in numbers of associated water molecules for binding of mannose and mannooligosaccharides to Con A is directly connected to the energetics of these hydration/dehydration reactions. The interaction of the tested sugars to Con A is enthalpically driven and accompanied by water uptake (Table 2). The osmotic release of water molecules from the binding site causes a concomitant decrease in the free energy of binding (Figure 3), suggesting that the water molecules are assisting substantially the binding of sugars with the lectin.

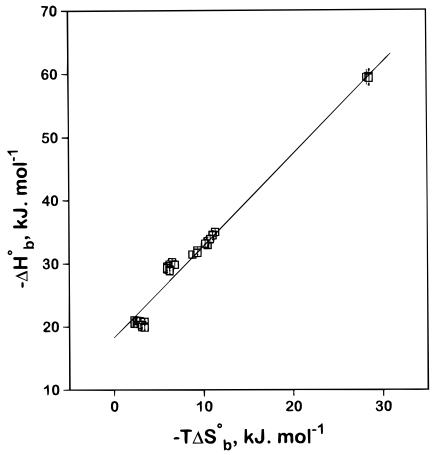

Figure 5. Enthalpy−entropy compensation plot of $-\Delta H^\circ_b$ as a function of $-T\Delta S^\circ_b$ for the binding of mannose and mannooligosaccharides to Con A in the absence and presence of osmolytes. The plot was generated using Origin program and shows a linear relationship with a slope of 1.45 with a correlation coefficient to 0.994. The data points represent average values of four independent measurements. The standard deviations were well within the size of the data points.

The thermodynamics of binding of mannose and mannooligosaccharides to Con A shows compensatory changes in ΔH°_b and $T\Delta S^\circ_b$ as shown in Figure 5. Enthalpy−entropy compensation has been associated with solvent reorganization accompanying protein−ligand interactions[11−20,34,35] and also appears to be a general feature of weak intermolecular reactions.[36] A reduction in soft vibrational modes leading to the restriction of water molecules in the interface between the interacting lectin and sugar molecules could account for these observations. An efficient displacement of water molecules from the interacting surfaces of the lectin binding site as well as the sugar is the source of the favorable entropic contribution. This gain in entropy from the removal of previously imposed motional restrictions gets compensated by a loss of a certain amount of enthalpic interactions such as water-mediated hydrogen bonds and van der Waals interactions in the lectin binding site. If a group of analogous species interact by the same mechanism, then a linear relationship between enthalpy and entropy can be expected, with the slope exactly equal to unity being a result of complete compensation. Our results with and without osmolytes also show a linear relationship between ΔH°_b and $T\Delta S^\circ_b$ with a slope of 1.45 (correlation coefficient = 0.99),

(25) Harrop, S. J.; Helliwell, J. R.; Wan, T. C. M.; Kalb (Gilboa), A. J.; Tong, L.; Yariv, J. *Acta Crystallogr., Sect. D* **1996**, *52*, 143−155.
(26) Kanellopoulos, P. N.; Pavlou, K.; Perrakis, A.; Agianian, B.; Vorgias, C. E.; Mavrommatis, C.; Soufi, M.; Tucker, P. A.; Hamodrakas, S. J. *J. Struct. Biol.* **1996**, *116*, 345−355.
(27) Hamodrakas, S. J.; Kanellopoulos, P. N.; Pavlou, K.; Tucker, P. A. *J. Struct. Biol.* **1997**, *118*, 23−30.
(28) Levitt, M.; Park, B. H. *Structure* **1993**, *1*, 223−226.
(29) Donovan, J. W. *J. Biol. Chem* **1969**, *244*, 1961−1967.
(30) Gekko, K.; Timasheff, S. N. *Biochemistry* **1981**, *20*, 4667−4676.
(31) Lee, L. C.; Gekko, K.; Timasheff, S. N. *Methods Enzymol.* **1979**, *61*, 26−49.
(32) Timasheff, S. N. *Biochemistry* **1992**, *31*, 9857−9864.
(33) Timasheff, S. N. *Annu. Rev. Biophys. Biomol. Struct.* **1993**, *22*, 67−97.
(34) Lumry, R.; Rajender, S. *Biopolymers* **1970**, *9*, 1125−1227.
(35) Fukada, H.; Sturtevant, J. M.; Quiocho, F. A. *J. Biol. Chem.* **1983**, *258*, 13193−13198.
(36) Dunitz, J. D. *Chem. Biol.* **1995**, *2*, 709−712.

indicative of an underlying common mechanism in the mode of specific recognition of the sugars by Con A, namely solvent reorganization (Figure 5). This is substantiated by the evidence that the slope of the enthalpy−entropy compensation plot is greater than unity, suggesting that the free energy of binding is more sensitive to changes in enthalpy both in the presence as well as in the absence of osmolytes. We propose that this strong isoequilibrium relationship of enthalpy with entropy during the recognition of saccharides by Con A studied under osmotic stress be considered as diagnostic of a true osmotic effect.

These results suggest that the disruption of specific interactions by water molecules at the lectin−sugar interface may contribute to the decreased binding affinity upon osmotic stress. To rule out the possibility of a decrease in the stability of the protein by the osmolytes, we have determined the thermal stability of the lectin using differential scanning calorimetry, in which the thermal unfolding of Con A was monitored in the absence and presence of glycerol. That the decrease in binding free energies upon osmotic stress is not due to destabilizing effect on Con A is succinctly highlighted by the DSC thermograms of Con A in the absence and in the presence of increasing osmolal concentrations of osmolytes (Figure 4), which show increase in the peak transition temperature T_p. From the observation that the thermodynamic transition parameters T_p, T_m, ΔH_c, and ΔH_v are increasing functions of concentrations of glycerol (Table 3), it can be inferred that the stability of Con A is increased by the osmolytes. Hence, the decrease in the affinity of the lectin for its ligand in the presence of osmolytes is not related to the decreased stability of the protein. The stabilizing effect results from an additional enthalpy of interaction in the presence of glycerol. These osmolytes apparently stabilize Con A by preferential hydration effects[30−33] and also by reducing the volume of the voids at the protein core.[37−39]

Our results are consistent with the recently obtained crystal structure of mannotriose bound to tetrameric Con A[9,10] in which interactions of the reducing mannose with the binding site includes a strong hydrogen bond of 2.6 Å between O2 and an invariant bridging water molecule bound by protein residues Asn14, Asp16, and Arg228 which is also present in both native Con A and the Con A−methyl-α-D-mannopyranoside complex. It appears from our solution state experiments that the net uptake of a solitary water molecule is involved in the process of binding of mannotriose to Con A (Table 2). It is tempting to speculate that this water molecule, at least in part, determines the specific interactions of Con A with the monosaccharide mannose, the two disaccharides manα1-3man and manα1-6man that constitute the two arms of mannotriose, and mannotriose. In mannotriose, sugar hydroxyls of α1−3-linked mannose and the reducing end mannose substitute the water molecules necessary for the binding of the monosaccharide and mannobioses. This interpretation is also supported by crystallographic studies on Con A where binding of mannotriose displaces several water molecules from the extended binding site (Figure 6). The C3, C4, and C6 hydroxyls of the α1−3-linked arm and the C2 and C4 hydroxyls of the reducing end mannose are the most likely ones replacing these solvent molecules implicated in the binding of mannose and mannobioses. The differential uptake of water molecules associated with the binding of saccharides to Con A could account, in part, for the non-linear thermodynamic effects[40] observed in these reactions.

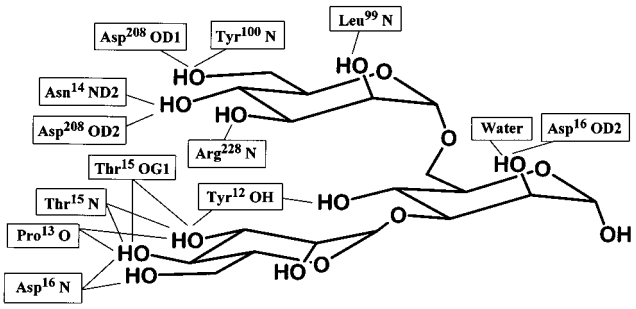

Figure 6. A schematic representation of the extended combining site of Con A complexed with mannotriose. The amino acid residues of Con A participating in sugar binding[10] are enclosed in rectangular boxes and are denoted by three-letter abbreviation; superscripts refer to their positions in the sequence of the protein. The thin lines represent the hydrogen bonds between the sugar and the protein. The stucturally conserved bridging water molecule is also shown. The figure was generated using ISIS/Draw version 1.2.

Conclusion

In conclusion, this paper provides the first solution state evidence for the role of water molecules in the specific recognition of mannose and mannooligosaccharides by Con A and demonstrates the feasibility of coupling the osmotic stress strategy with a powerful and senstive microcalorimetric technique, thus enabling the measurement of the overall number of water molecules involved in the specific binding process, the relevance of which in the structure−function analytical context need not be overstated.

Experimental Section

Materials. All reagents were of analytical or ultrapure grade. α-D-man was purchased from Sigma Chemical Co., while manα1-3man, manα1-6man, and mannotriose were obtained from Dextra Laboratories. Dimethylglutaric acid, glycerol, and ethylene glycol were of ultrapure grade from Sigma. All other reagents were recrystallized or repurified before use. Deionized Milli-Q water was used for all the studies. Con A was prepared from jack bean seeds according to previously described methods.[41]

Preparation and Analysis of Solutions. Lyophilized powder of Con A was dissolved in 0.05 M DMG buffer at pH 5.2, containing 250 mM sodium chloride and 1 mM each of $MnCl_2$ and $CaCl_2$ by weight, dialyzed overnight in a large volume of the same buffer, and centrifuged to remove any insoluble lectin. 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. The concentration of Con A was determined spectrophotometrically at 280 nm using $A^{1\%,1cm} = 12.4$ at pH 5.2 and expressed in terms of dimer ($M_r = 53\,000$).[5,42] For osmotic stress studies, the lectin solution was dialyzed extensively against glycerol and ethylene glycol solutions in the above buffer. Sugar solutions were then prepared with the dialysate. Care was taken that the neutral solute osmolalities used were not significantly different from their ordinary molal concentrations.[43] The solution osmolalities were measured on a Herman Roebling automatic Type II micro-osmometer.

ITC Measurements. The titration calorimetric measurements were performed with a Microcal Omega titration calorimeter as described

(37) Goldanskii, V. I.; Krupyanskii, Y. F. *Protein−Solvent Interactions*; Gregory, R. B., Ed.: Marcel Dekker: New York, 1995; pp 289−326.
(38) Oliveira, A. C.; Gaspar, L. P.; Da Poian, A. T.; Silva, J. L. *J. Mol. Biol* **1994**, *240*, 184−187.
(39) Priev, A.; Alamgor, A.; Yedgar, S.; Gavish, B. *Biochemistry* **1996**, *35*, 2061−2066.
(40) Gupta, D.; Dam, T. K.; Oscarson, S.; Brewer, C. F. *J. Biol. Chem* **1997**, *272*, 6388−6392.
(41) Agrawal, B. B. L.; Goldstein, I. J. *Biochim. Biophys. Acta* **1967**, *147*, 262−271.
(42) Yariv, J.; Kalb, A. J.; Levitzki, A. *Biochim. Biophys. Acta* **1968**, *167*, 303−305.
(43) Weast, R. C. *CRC Handbook of Chemistry and Physics*, 61st ed.; CRC Press Inc.: Boca Raton, FL, 1980; pp D229−D276.

previously.[22] Briefly, the titration calorimeter consists of 1.347 mL cell containing the protein solution and a matched reference cell in an adiabatic enclosure. Aliquots of ligand solution at 15−40 times the Con A concentration are added via a 250 μL rotating stirrer microsyringe operated with a plunger driven by a stepping motor under computer control. The heat absorbed or released accompanying the addition of aliquots of the ligand solution to the protein solution is measured by a thermocouple sensor between the cells. Samples were carefully scrutinized for precipitate after the titration. No precipitate was observed after the titration either in the presence or in the absence of the osmolytes. The quantity $c = K_b[\text{Con A}]_0$, where $[\text{Con A}]_0$ is the initial macromolecule, Con A, concentration and K_b is the association constant was in the range of $2 < c < 200$ as required for isothermal titration microcalorimetry studies.[22] The total concentration of the lectin, $[\text{Con A}]_t$, was from 0.036 to 2 mM, whereas the total concentration of ligand, $[\text{L}]_t$, was from 1.5 to 80 mM. The titration of ligand solution in this concentration range with the buffer solution alone gave negligible values for the heat of dilution both in the presence as well as absence of osmolytes. Nonetheless, for every experiment the heat of dilution of the ligand were measured and subtracted from the runs conducted with the lectin. The time duration between the injections was at least 3 min to allow the peak to return to baseline, and the number of additions of the sugar titrant were fixed such that the area below the peak is reduced by at least an order of magnitude or until protein binding sites are saturated ≫85%. All measurements were made at 283.2 K, and the stirrer speed was kept constant at 395 rpm.

ITC Analyses. The titration data were analyzed using a single-site fitting model. The identical site model was fitted to the data utilizing a dimeric Con A concentration. The heat content of a solution Q_t, has been shown to be related to the total concentrations of the protein and ligand through the following equation[22]

$$Q_t = n[\text{Con A}]_t\Delta H^\circ_b V\{1 + [\text{L}]_t/n[\text{Con A}]_t + 1/nK_b[\text{Con A}]_t - [(1 + [\text{L}]_t/n[\text{Con A}]_t + 1/nK_b[\text{Con A}]_t)^2 - 4[\text{L}]_t/n[\text{Con A}]_t]^{1/2}\}/2 \quad (3)$$

where n is the stoichiometry, K_b is an intrinsic binding constant, ΔH°_b is an intrinsic heat of binding, $[\text{Con A}]_t$ is the total Con A concentration, and V is the cell volume. The expression for the heat released per ith injection, ΔQ_i, is then[44]

$$\Delta Q_i = Q_i + dV_i/2V[Q_i + Q_{i-1}] - Q_{i-1} \quad (4)$$

where dV_i is the volume of titrant added to the solution. A least-squares fit of ΔQ_i to eq 4 obtained by each run of titration calorimetry gives values for stoichiometry (n), ΔH°_b, and K_b. Since Con A at pH 5.2 exists as a homodimeric protein with one binding site per subunit, an identical site model utilizing a concentration of the lectin dimer was the simplest binding model found to provide the best fit to the ITC data. Values for ΔS°_b were obtained from the basic equation of thermodynamics

$$\Delta G^\circ_b = \Delta H^\circ_b - T\Delta S^\circ_b \quad (5)$$

where

$$\Delta G^\circ_b = -RT \ln K_b \quad (6)$$

and n = number of moles, T is the absolute temperature, and R = 8.315 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 1.187 mL cells, a reference cell, and a solution cell. The measurements were usually made at a scan rate of 20 K h^{-1}. To determine any dependence of the parameters on scan rate, scans were also performed at 10 K h^{-1}. The best least-squares fit of the two-state transition model, $A_n \leftrightarrow nB$ where A is the folded state and B is the unfolded state, to the data was obtained with $n = 2$ by the Origin program. Analyses of the normalized data utilizing a progress baseline connection of the pre- and post-transition baselines of the DSC thermogram yields van't Hoff enthalpy (ΔH_v), transition temperature (T_m, the temperature at half the peak area), and the transition peak area which, 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.

Acknowledgment. This work has been supported by a grant from the Department of Science and Technology, Government of India to A.S. The authors thank Dr. M. Vijayan and Dr. R. Varadarajan for discussions and a critical reading of the manuscript.

JA9733696

(44) Yang, C. P. *Omega Data in Origin*; Microcal Inc.: Northampton, MA, 1990; p 66.