Pure & Appl. Chem., Vol. 70, No. 3, pp. 677–686, 1998.
Printed in Great Britain.

Elucidation of the combining site of *Coccinia indica agglutinin* (CIA) by thermodynamic analyses of its ligand binding

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Abstract Analyses of the ligand size dependence of the fluorescence spectra of CIA together with the thermodynamic parameters for the lectin reveal that its combining site spans the tetrasaccharide chitotetraose. Moreover the fourth sugar residue of chitoligosaccharide is proximal to a highly fluorescent tryptophan.

The highly specific binding of lectins to cell surface carbohydrate receptors is the event which leads to expression of their biological properties such as mitogenesis and mimicking of hormone action (1 & 2). In order to understand their structure-function relatioship and use them as cell surface probes, it is necessary to have a thorough understanding of the energetics of their interactions with carbohydrate ligands.

A large number of techniques can be used to study lectin-sugar interactions, of which fluorescence spectroscopy is the method of choice because of its sensitivity, ease in experimentation and the amount of information it yields about the association constants, the thermodynamics of binding and the number of binding sites on each protein molecule. This is possible because the fluorescence of a chromophore is more dependent on its environment than its absorption. In general the fluorescence intensity of a chromophore is altered upon sugar binding, and in cases where this does not occur, the fluorescence polarization (3 & 4), or anisotropy can be monitored instead.

There are two approaches in studying protein-sugar interactions using fluorescence spectroscopy. The first method involves the use of native, unlabeled sugars, and monitor the changes in intrinsic fluorescence of the protein i.e. the fluorescence of chromophores such as the tryptophan and tyrosine residues (5). On the other hand, a reporter group such as 4-methylumbelliferyl or dansyl can be coupled to the sugar, and the fluorescence of this label followed as the interaction between such sugars with protein take place (6 & 7).

In this paper, the thermodynamics of ligand binding as studied by following the intrinsic fluorescence of the protein are being reported.

MATERIALS AND METHODS

MATERIALS

Coccinia indica agglutinin (CIA) was purified from the fruits as described in (8). All the carbohydrates used were obtained from Seike-Gaku, Japan. All other chemicals used were of analytical grade, and obtained locally.

METHODS

Fluorescence Measurements

Fluorescence spectra were recorded on a Perkin-Elmer MPF 44A spectrofluorimeter. For the titrations, fluorescence measurements were taken using a Union Giken FS 501 fluorescence polarizer, equipped with photon counting multipliers. Samples in 1 x 1 x 4.5 cm quartz cuvettes were placed in a thermostatted copper holder, maintained at a constant temperature (±0.1°C) with a Lauda constant temperature bath. The samples were excited at 295 nm with a 7 nm slit width and emission monitored using a 320 nm cut off filter and a band pass (1/2 = 6.5 nm) centered at 335 nm. Titrations were performed under constant stirring, aliquots being added with micro-pipettes; measurements were taken 1 minute after addition. The fluorimeter is microprocessor controlled, and allows averaging of measurements. For every measurement the average of 10 readings was taken, the standard deviation being less than 0.5%. All solutions were filtered before use through 0.45 μm HAWP filters.

In the case of titraions with the sugars, protein concentrations were in the range of 1-4 μM, in PBS. The sugar solutions were also made in PBS. From the titration data, the association constants (K_a) were determined by the method of Chipman et al (5). The titrations were done at various temperatures (15°, 20°, 25°, 30° and 35°C), and the temperature dependence of the association constants was used to calculate the thermodynamic parameters of binding.

Concentration Determinations

Protein concentrations were determined by the method of Lowry et al (9) and all others by weight measurements.

RESULTS

FLUORESCENCE STUDIES

The fluorescence intensity of the protein increased on sugar binding. The percentage of enhancement depended on the ligand size. There was also a concommitant blue shift in λ_{max}, which was small in the case of binding of biose and triose (2 nm) and larger for all the other oligomers (5 nm) (Fig.1 and Table 1).

Table 1
Changes in the optical characteristcs of CIA on binding to chitoligosaccharides of different chain lengths

	Percentage enhancement in intrinsic fluorescence	Shift* in emission maximum (nm)
GlcNAc	--	--
Biose	23	2
Triose	25	2
Tetraose	30	5
Pentaose	30	5
Hexaose	31	5

* : all the shifts observed were blue shifts

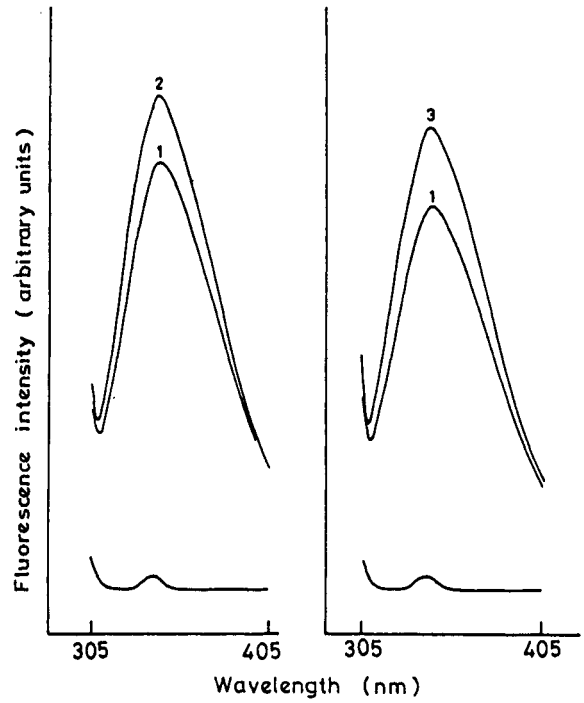

Fig. 1 Fluorescence emission spectra of CIA (3.0 μM) in the absence (1) and presence of (2) triose (215 μM), (3) tetraose (260 μM). The lower most spectra are of PBS alone, or with the corresponding sugars.

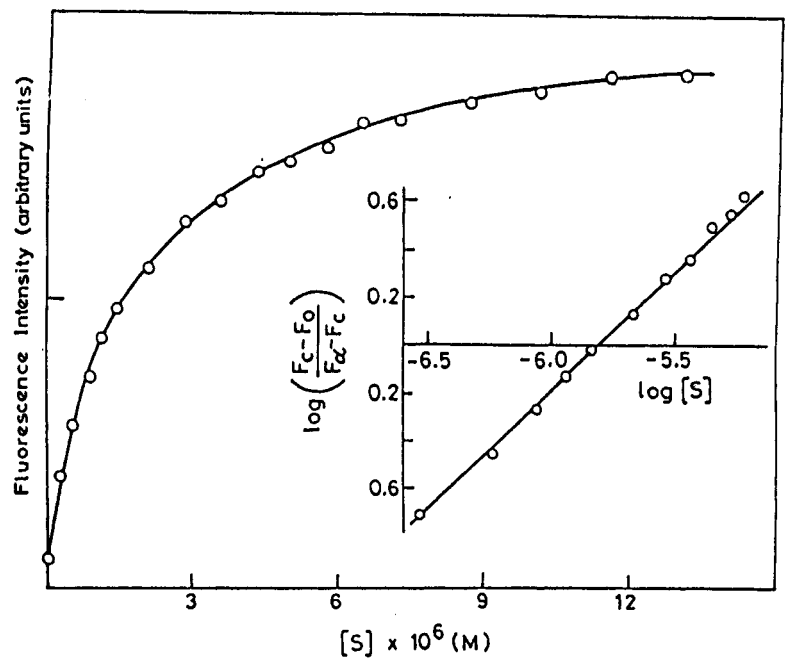

Fig. 2 Titration of CIA with tetraose at 25°C. A 1.5 μM solution of CIA was titrated with 0.2895 mM tetraose. Inset gives a graphical representation for the determination of association constant (K_a = 6.53 x 10^5 M^{-1})

The association constant (K_a) values obtained according to the method of Chipman et al (5) at various temperatures are given in Table 2. The change in the ratio of initial fluorescence F_o (in the absence of ligand) to the change in fluorescence ($\triangle F$), on addition of an aliquot of the ligand solution, was plotted against the inverse of the ligand concentration [S]. This plot, on extrapolation to the Y-axis, yielded the fluorescence at infinite ligand concentration, ($F\infty$) (when all the protein molecules are complexed with the ligand). This was used to plot $\log[(F_c-F_o)/F\infty-F_c)$ vs. log[S], where F_o, F_∞ and F_c are the fluorescence intensities at zero, infinite and at a particular concentration [S], of the ligand respectively. A representative plot of the binding of tetraose (at 25°C) by the lectin is shown in Fig. 2. The slope of this plot is unity in all cases, indicating the formation of a 1:1 complex of sugar and protein. Also, when $\log[(F_c-F_o)/(F_\infty-F_c)$ equals zero, pK_a for the complex equals log[S]. Thus, the X-intercept of this plot yields the K_a value at that temperature.

Table 2
Association constants for the binding of chitooligosaccharides by CIA (in M^{-1})

	15°C	20°C	25°C	30°C	35°C
Biose ($K_a \times 10^{-3}$)	10.715	7.41	5.25	3.67	2.48
Triose ($K_a \times 10^{-5}$)	10.35	6.31	4.03	2.69	1.799
Tetraose ($K_a \times 10^{-5}$)	14.9	9.33	6.53	3.43	1.8
Pentaose ($K_a \times 10^{-5}$)	22.1	14.8	6.84	6.607	3.55
Hexaose ($K_a \times 10^{-5}$)	32.7	17.78	12.74	9.016	6.025

The $\triangle H$ values for the binding of different sugars were determined from Van't Hoff plots, where log K_a is plotted vs. the inverse of absolute temperature. The changes in free energy $\triangle G$, at 15°C, were calculated from the equation

$$\triangle G = -RT \ln K_a$$

where R is the gas constant, T the absolute temperature and K_a the association constant at that temperature.

Entropy changes ($\triangle S$), were calculated from the equation

$$\triangle S = (\triangle H - \triangle G)/T$$

The values of $\triangle H$, $\triangle G$ and $\triangle S$ as obtained above, are given in Table 3. Van't Hoff plots for the binding of chitooligosaccharides by the lectin are given in Fig. 3.

Table 3

Thermodynamic parameters for the binding of chitooligosaccharides to CIA

	$-\Delta H$ (kJ mol^{-1})	$-\Delta G$ (15°C) (kJ mol^{-1})	$-\Delta S$ (15°C) ($Jmol^{-1}$ K^{-1})
Biose	51.06	22.22	100.14
Triose	64.62	33.17	109.2
Tetraose	73.64	34.05	137.46
Pentaose	60.92	34.99	90.03
Hexaose	59.83	35.93	82.99

DISCUSSION

The absence of a peak at 315 nm and the λ_{max} at 335 nm in the emission spectrum of the protein implies that the fluorescence of the protein is largely due to the presence of tryptophan residues (Table 3, Fig. 3). The extent of the shifts in the emission maxima are ligand size dependent, and this has also been reported in the case of other chitooligosaccharide binding proteins, notably WGA (10) and *Luffa* lectin (11).

The enhancement of fluorescence and the concommitant blue shift that occur upon ligand binding are strongly indicative of the involvement of tryptophan residues in binding activity and are presumed to occur as a result of its perturbation. Since the sugars themselves are not intrinsically hydrophobic, this increased hydrophobicity as sensed by the tryptophans could arise out of one or a combination of the following factors. Firstly, there could be a conformational change on binding - subtle or otherwise, resulting in a more hydrophobic environment around the tryptophanyl residue(s). It has been established in the case of lysozyme by x-ray techniques (12), that the position of the tryptophan changes by 0.75 Å on ligand binding. Secondly, ligand binding could result in an exclusion of water molecules from around the tryptophan(s), leading to increased hydrophobicity. Lastly, the orientation of the sugar ring(s) on binding, could result in the clustering of the CH_2 group and / or protons at certain topological distributions in the sugar binding site close to the tryptophan(s), with the same effect on the tryptophan side chain(s).

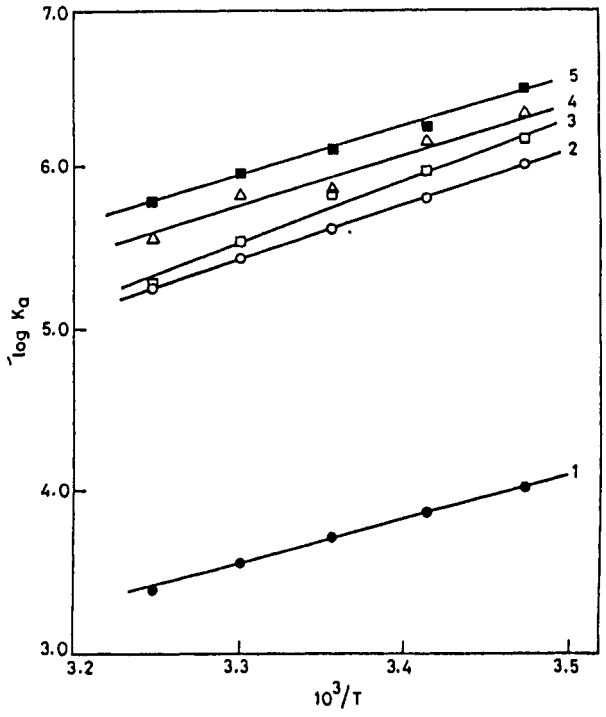

Fig. 3 Van't Hoff plots for the association of chitooligosaccharides with CIA. The symbols used are (●) Biose ; (o) Triose; ( ) Tetraose ; ( ) Pentaose and ( ) Hexaose.

Ligand size dependent enhancement has also been observed in other cases - WGA (10), rice germ lectin (13) and *Luffa* lecin (11). This implies a unique orientation of the tryptophan(s) with respect to the differnt saccharide units of the ligand, which in turn suggests that the tryptophan(s) involved in the binding process is(are) placed to one side of the centre of the binding site.

The K_a values increase with ligand size, the minimum unit required for binding being a disaccharide (GlcNAc does not bind). This increase in the association constants with increase in ligand size is not related to a statistical increase of binding probability of the combining site accomodating a single sugar residue, since the magnitude of affinities is much higher than can be ascribed to statistical effects (14). Hence, the increase in affinities with increase in ligand size can be explained by the fact that the site is an extended one and consists of subsites each of which accomodates a single sugar residue. Moreover, the association of a given subsite with a sugar residue is independent of other sugar residues of the ligand.

The association constants for all the ligands decreased markedly with increase in temperature (Table 2). For triose, K_a decreased by a factor of 5.75 on raising the temperature from 15° to 1.799 x 10^5 M^{-1} at 35°C). CIA is thus very different from WGA, where the association constant does not change much on increasing the temperature from 6 to 50°C (15), and rice germ lectin, where it decreases to abouthalf, from 4 to 37°C (13). It is, on the other hand, similar to *Luffa* lectin (11), where it decreases by about 4 times, on raising the temperature from 15° to 35°C.

At this point, it is interesting to note that though closely related, *Luffa* lectin and CIA differ markedly. While *Luffa* lectin binds to both biose and triose with association constants of the same order of magnitude (11), CIA binds triose 75 times better than biose. CIA differs from WGA too in this sense, where triose and higher oligosaccharides are not significantly better inhibitors than biose (10) and rice germ lectin, which binds triose only 5 times better than biose (13). Thus, comparing the binding of triose by *Luffa* lectin and CIA, CIA binds much better than *Luffa* lectin, probably because of a better complementarity of the third sugar ring in the binding site.

On going to tetraose from triose, the association constant increases by only about 45% in the case of CIA. It is thus very different from *Luffa* lectin, where it increases by a factor of 9 (11). It is on the other hand, similar to potato lectin, where tetraose is bound only 1.3 times better than triose (16).

Since the association constants increase by only about 45% on going from triose to the higher oligosaccharides in succession, the increase in ΔG is not marked. This is in contrast to lysozyme where values of ΔG are 9.3,, 20.7 and 30.3 kJ mol^{-1} for the binding of GlcNAc, biose and triose respectively (17), while in the case of *Luffa* lectin (11) the value of $-\Delta G$ increases till the pentasaccharide, and remains the same on further increase in ligand size.

The values of $-\Delta H$ are fairly high as compared to the reported values of lysozyme (17), rice germ lectin (13) and *Luffa* lectin (11). The numbers in the top row of the following table (Table 4) refer to the chain length of the ligand; $-\Delta H$ values are in kJ mol^{-1}.

Looking at the increments in ΔH with increase in ligand size, the maximum change is observed on going from biose to triose, -13.56 kJ mol^{-1}. Thus, subsite C contributes maximally to binding. The value is not as large when comparing triose and tetraose, -9.02 kJ mol^{-1}; further increase in ligand size results in a decrease in $-\Delta H$. All these increments are much higher than in the case of rice germ lectin (13) where the maximum increase in ΔH is 5.5 kJ mol^{-1}, and *Luffa* lectin (11) where the maximum change is from biose to the ligand being bound by CIA, the maximum change is from biose to triose, thus confirming that subsite C is the strongest binding subsite.

Table 4

Dependence of changes in enthalpies for the binding of proteins on the chain length of chitooligosaccharides

		Chito-oligosaccharide length					
		1	2	3	4	5	6
Lysozyme	ΔH (kJ mol^{-1})	26	47.7	59.9	0	0	0
Rice germ lectin	ΔH (kJ mol^{-1})	12.1	17.6	20.6	0	0	0
Luffa lectin	ΔH (kJ mol^{-1})	0	41	47.9	55.9	56	50
CIA	ΔH (kJ mol^{-1})	0	51.06	64.62	73.64	60.92	59.83

There is a continuous increase in $-\Delta S$ from biose to tetraose, and a decrease on further increase in ligand size. This is similar to *Luffa* lectin where $-\Delta S$ increases till the tetrasaccharide and falls thereafter (11), but the increments observed for *Luffa* are much less than in the case of CIA. The values of $-\Delta S$ are much larger than those observed in lysozyme -56.1, 90.5 and 99.7 J mol^{-1} K^{-1} for GlcNAc, biose and triose respectively (17) and rice germ lectin, where the corresponding values are 19.4, 31.4 and 34.9 J mol^{-1} K^{-1} respectively (13).

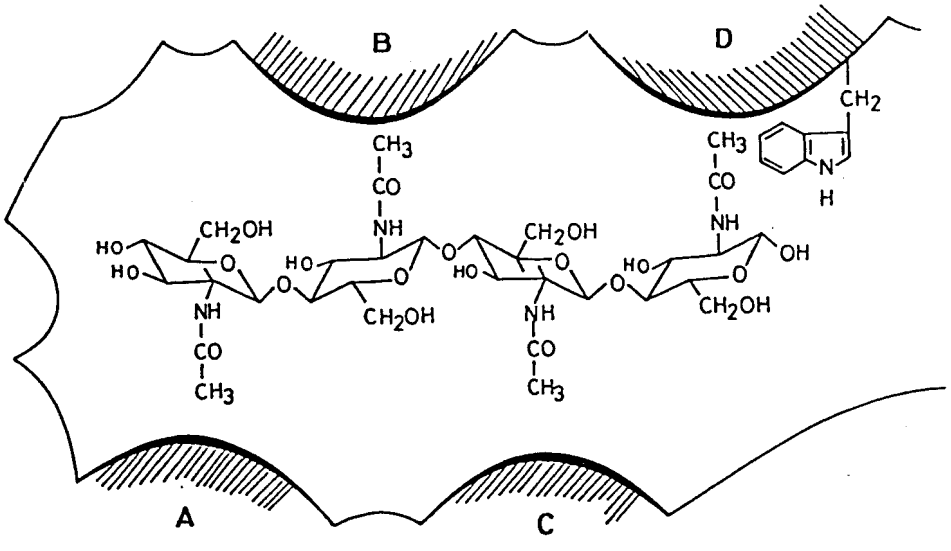

Fig. 4 Model of the binding site of CIA with bound chitotetraose. A, B, C and D refer to the different subsites

In conclusion, it is possible to schematize the binding of chitooligosaccharides to CIA. Since the maximum perturbation of the emission maximum occurs only on binding to the tetrasaccharide and higher oligomers, the position of the tryptophan in the binding site can be ascribed to the fourth subsite, or in close proximity of it. The modes of binding of the different oligosaccharides are shown below, and a model of the binding site of CIA complexed to chitotetraose, its most complementary ligand is given in Fig. 4.

	SUBSITES			
	A	B	C	D
Biose	GlcNac	GlcNac		
Triose	GlcNAc	GlcNAc	GlcNAc	
Tetraose	GlcNAc	GlcNAc	GlcNAc	GlcNAc

where A, B, C and D refer to the subsites.

Acknowledgements

This work was supported by a grant from the Department of Science and Technology, Government of India to AS. ARS and VA were research associates in the above grant.

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