

Thermodynamic and kinetic analysis of porphyrin binding to *Trichosanthes cucumerina* seed lectin

Roopa Kenoth, D. Raghunath Reddy, Bhaskar G. Maiya and Musti J. Swamy

School of Chemistry, University of Hyderabad, Hyderabad- 500 046, India

The interaction of several metallo-porphyrins with the galactose-specific lectin from *Trichosanthes cucumerina* (TCSL) has been investigated. Difference absorption spectroscopy revealed that significant changes occur in the Soret band region of the porphyrins upon binding to TCSL and these changes have been monitored to obtain association constants (K_a) and stoichiometry of binding (n). The dimeric lectin binds two porphyrin molecules and the presence of the specific saccharide lactose did not affect porphyrin binding significantly, indicating that the sugar and the porphyrin bind at different sites. The K_a values obtained for the binding of different porphyrins with TCSL at 25 °C were in the range of 2×10^3 – 5×10^5 M⁻¹. Association constants for *meso*-tetra(4-sulphonatophenyl)porphyrinato copper(II) (CuTPPS), a porphyrin bearing four negative charges and *meso*-tetra(4-methylpyridinium)porphyrinato copper(II) (CuTMPyP), a porphyrin with four positive

charges, were determined at several temperatures; from the temperature dependence of the association constants, the thermodynamic parameters change in enthalpy (ΔH°) and change in entropy (ΔS°) associated with the binding process were estimated. The thermodynamic data indicate that porphyrin binding to TCSL is driven largely by a favourable entropic contribution; the enthalpic contribution is very small, suggesting that the binding process is governed primarily by hydrophobic forces. Stopped-flow spectroscopic measurements show that binding of CuTMPyP to TCSL takes place by a single-step process and at 20 °C, the association and dissociation rate constants were 1.89×10^4 M⁻¹·s⁻¹ and 0.29 s⁻¹, respectively.

Keywords: haemagglutinin; photodynamic therapy; stopped-flow spectroscopy.

While carbohydrate-recognition by lectins is a functionally significant property, considerable evidence suggests that these proteins may exhibit other, physiologically relevant interactions. In particular, the fact that for most lectins endogenous carbohydrate ligands have not yet been found lends credence to the hypothesis that these proteins may have other, noncarbohydrate endogenous ligands that are physiologically relevant. This is generally true for most lectins of plant origin, for which endogenous carbohydrate ligands are yet to be identified.

Porphyrins are a group of biologically important molecules that have considerable hydrophobic character. In nature there are several examples where porphyrins are

bound to polypeptide chains, e.g. haemoglobin, myoglobin and cytochrome *c*. Binding of synthetic porphyrins to proteins such as human serum albumin, BSA and low-density lipoproteins has been investigated by several groups [1–3]. Recently porphyrins have been used as photosensitizers in photodynamic therapy (PDT), a new approach developed for the treatment of cancer [4,5]. In PDT, when excited by light of appropriate wavelength, the porphyrin photosensitizers interact with molecular oxygen and convert it to the triplet form, which then reacts with the surrounding tissue and leads to cell death. Porphyrins appear to be suitable for such an application, because in addition to being biocompatible, they have been found to exhibit a preferential localization in tumour tissue. However, in many cases the ratio of the photoactive drug in tumour tissues to that in the surrounding normal tissue is as low as 2 : 1 [5], thus making the use of porphyrins as such in tumour treatment less effective. Conjugating the porphyrin to another agent that can steer it towards the tumour is a possible approach to circumventing this limitation. In this regard it is of interest to investigate whether lectin-bound porphyrins can be used in PDT as several lectins have been shown to exhibit preferential binding to tumour cells. With the objective of exploring such a possibility, we have recently characterized the interaction of several free-base and metalloporphyrins with different plant lectins, viz., concanavalin A (Con A), pea lectin, Jacalin and the snake gourd (*Trichosanthes anguina*) seed lectin [6–8].

T. cucumerina seed lectin (TCSL) is a galactose-specific glycoprotein of $m \approx 62$ kDa that is made up of two nonidentical subunits of m 41 and 22 kDa. The two subunits of TCSL are linked by disulfide bridge(s) and chemical

Correspondence to M. J. Swamy, School of Chemistry, University of Hyderabad, Hyderabad 500 046, India. Fax: +91 40 301 2460/0145, Tel.: +91 40 301 0221, E-mail: mjssc@uohyd.ernet.in

Abbreviations: TCSL, *Trichosanthes cucumerina* seed lectin; SGSL, snake gourd (*Trichosanthes anguina*) seed lectin; PDT, photodynamic therapy; Jacalin, jack fruit (*Artocarpus integrifolia*) agglutinin; Con A, concanavalin A; ZnTPPS,

meso-tetra(4-sulphonatophenyl)porphyrinato zinc(II);

CuTPPS, *meso*-tetra(4-sulphonatophenyl)porphyrinato copper(II);

ZnTCPP, *meso*-tetra(4-carboxyphenyl)porphyrinato zinc(II);

CuTCPP, *meso*-tetra(4-carboxyphenyl)porphyrinato copper(II);

ZnTMPyP, *meso*-tetra(4-methylpyridinium)porphyrinato zinc(II);

CuTMPyP, *meso*-tetra(4-methylpyridinium)porphyrinato copper(II);

CTAB, cetyl trimethylammonium bromide.

Note: Author website can be found at <http://www.uohyd.ernet.in>

(Received 5 June 2001, revised 13 August 2001, accepted 29 August 2001)

modification studies indicate that the protein requires histidine residues for carbohydrate-binding and cell-agglutinating activities [9,10]. Agglutination-inhibition assays indicate that TCSL binds the β -anomer of galactose better than the α -anomer and *p*-nitrophenyl- β -D-galactopyranoside is the best saccharide inhibitor among a battery of sugars investigated [9]. The lectin exhibits immunological cross-reactivity with the snake gourd (*T. anguina*) seed lectin (SGSL), which is also a β -galactose-specific cucurbitaceae seed lectin [9,11]. This fact, together with the results of our previous study in which we observed that SGSL binds Cu- and Zn-porphyrins with considerable avidity [8], has prompted us to investigate the interaction of porphyrins with TCSL. In this manuscript, we report absorption spectroscopic studies on the interaction of different Cu- and Zn-porphyrins with TCSL. In order to delineate the forces that govern the interaction of porphyrins with this lectin, the binding experiments were performed at different temperatures with some of the porphyrins and the changes in enthalpy and entropy associated with the binding process were determined. Further, the kinetics of the interaction of one of the porphyrins was investigated by stopped-flow spectroscopy to elucidate the mechanism of binding of the porphyrin to the lectin. The results indicate that porphyrin binding to TCSL is governed primarily by hydrophobic forces. The binding kinetics indicate a simple, single-step association process.

MATERIALS AND METHODS

Materials

Seeds of *T. cucumerina* were obtained from United Chemicals and Allied Products (Kolkata, India). Guar gum, SDS, BSA, lactose and the reagents for PAGE were from Sigma. Epichlorohydrin was from SD's Chemicals (Mumbai, India). *Meso*-tetra(4-sulphonatophenyl) porphyrinato zinc(II) (ZnTPPS), *meso*-tetra(4-sulphonatophenyl)porphyrinato copper(II) (CuTPPS), *meso*-tetra(4-carboxyphenyl) porphyrinato zinc(II) (ZnTCPP), *meso*-tetra(4-carboxyphenyl)porphyrinato copper(II) (CuTCPP), *meso*-tetra(4-methylpyridinium) porphyrinato zinc(II) (ZnTMPyP) and *meso*-tetra(4-methylpyridinium)porphyrinato copper(II) (CuTMPyP) were prepared and characterized as described previously [12–16].

Purification and characterization of TCSL

TCSL was purified by affinity chromatography on cross-linked guar gum [17] as described previously [9]. The purity of the protein was assessed by PAGE in the absence and in the presence of SDS [18]. The activity of the protein was checked by haemagglutination and haemagglutination-inhibition assays as described previously [9].

Absorption spectroscopy

Absorption measurements were made on a Shimadzu model UV-3101PC UV-Vis-NIR double-beam spectrophotometer using 1.0-cm path-length cells. Temperature was maintained constant (± 0.5 °C) by means of a Peltier device supplied by the manufacturer.

Concentration determination

Concentration of TCSL was determined by the method of Lowry *et al.* [19], using BSA as the standard. Concentrations of the porphyrins were determined spectrophotometrically using their molar absorption at the λ_{\max} of the Soret band, as described by Komath *et al.* [8].

Binding of porphyrins to TCSL

Porphyrin binding to TCSL was investigated by absorption titration essentially as described earlier for studies with SGSL [8]. All binding experiments were performed in 20 mM phosphate buffer, containing 0.15 M NaCl pH 7.4 (NaCl/P_i). Porphyrin samples (2 mL) at 2.0–4.0 μ M were titrated by adding small aliquots of the lectin from a concentrated stock solution. The UV-visible spectra were recorded after an equilibration period of 2 min following each addition. All titrations were repeated at least three times and the mean values of K_a and number of binding sites per lectin molecule (n) were calculated.

Stopped-flow spectroscopy

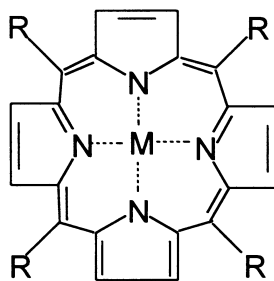
Kinetics of the interaction of CuTMPyP with TCSL were investigated by the stopped-flow method. Experiments were performed on an Applied Photophysics SX-18 MV stopped-flow apparatus (Leatherhead, UK). The dead time of the instrument was estimated by the method described by Hiromi and coworkers [20] and found to be 1.2 ms [21]. Concentration of CuTMPyP was fixed at 2.55 μ M and the protein concentration was varied from 50 μ M to 175 μ M (both concentrations after mixing) and the change in absorbance at 424 nm was monitored. The sample syringes and the cell were maintained at 20 °C by circulating water through jackets surrounding the cell and the syringes, by means of a constant-temperature bath. Measurements were performed in NaCl/P_i. All traces are mean values calculated from 5–10 independent kinetic profiles. The stopped-flow traces were analysed by curve fitting using the Marquardt algorithm based on the routine curves. The curve-fitting and further analyses were carried out on an ARCON 5000, RISC workstation.

RESULTS

Difference absorption spectra and micro-environment of porphyrin-binding site on TCSL

A schematic diagram depicting the structures of various porphyrins used in this study is shown in Fig. 1. It was observed that all of the porphyrins used in this study obeyed Beer's law in the concentration range 0–5 μ M, indicating that under the experimental conditions used, the porphyrins were not aggregated (see, for example [8]).

Absorption spectra in the Soret band region for CuTMPyP, ZnTPPS and ZnTCPP in the absence as well as in the presence of different concentrations of TCSL are given in Fig. 2A, B and C, respectively. In these spectra, the λ_{\max} of the Soret band is at 424.9, 423.3 and 422.5 nm for CuTMPyP, ZnTPPS and ZnTCPP, respectively. Addition of TCSL results in a red shift of the λ_{\max} by $\approx 1 \pm 0.2$ nm in



Porphyrin	R	M
CuTPPS	PhSO ₃ ⁻²	Cu ⁺²
ZnTPPS	PhSO ₃ ⁻²	Zn ⁺²
CuTCPP	PhCOO ⁻	Cu ⁺²
ZnTCPP	PhCOO ⁻	Zn ⁺²
CuTMPyP	Methyl-pyridinium	Cu ⁺²
ZnTMPyP	Methyl-pyridinium	Zn ⁺²

Fig. 1. Structures of the porphyrins used in this study.

the spectra of CuTMPyP, ZnTPPS and ZnTCPP at the highest concentration of TCSL added in the titration. Similar shifts were also observed in the Soret band region of the absorption spectra of CuTPPS, CuTCPP and ZnTMPyP in the presence of TCSL (spectra not shown).

To characterize further the nature of the interaction of these porphyrins with TCSL, difference spectra were obtained by subtracting the spectrum of the porphyrin alone from those of the porphyrin–lectin mixtures. Difference absorption

spectra thus obtained for CuTMPyP, ZnTPPS and ZnTCPP are shown in Fig. 3A, B and C, respectively. The difference spectra obtained for the titration of the zinc(II) porphyrins, namely ZnTPPS and ZnTCPP with TCSL were found to be qualitatively similar to those obtained for the titration of the corresponding copper(II) analogues (spectra not shown). Similarly, the difference spectra obtained with CuTMPyP and ZnTMPyP were found to be qualitatively very similar.

Kadish and coworkers [13,22] have characterized the interaction of different porphyrins used in this study with neutral, cationic and anionic micelles, namely Triton X-100, cetyltrimethylammonium bromide (CTAB) and SDS and concluded that the tetra-anionic porphyrins such as H₂TPPS and its metal derivatives interact with CTAB and Triton X-100 via Coulombic as well as hydrophobic interactions, whereas the interaction of tetra-cationic porphyrins such as H₂TMPyP and its metal derivatives interact with SDS via Coulombic interactions only and that hydrophobic interactions do not play a significant role in the TMPyP–micelle interaction. A significant shift in the Soret band region was interpreted as indicative of an interaction between the porphyrin and the micelles. To compare our results on the interaction of porphyrins with TCSL with the results obtained on the interaction of porphyrins with micelles, we recorded difference spectra with ZnTPPS and ZnTMPyP in the presence of SDS, Triton X-100 and CTAB, at concentrations well above their critical micellar concentrations. Difference spectra for ZnTPPS obtained in the presence of SDS, Triton X-100 and CTAB are shown in Fig. 4. A comparison clearly shows that the difference spectra of ZnTPPS and ZnTCPP in the presence of the lectin are very similar to the difference spectra of ZnTPPS obtained in CTAB and Triton X-100 (Figs 3 and 4). On the other hand, only minor changes were noticed in the difference spectra of ZnTMPyP in the presence of CTAB and Triton X-100,

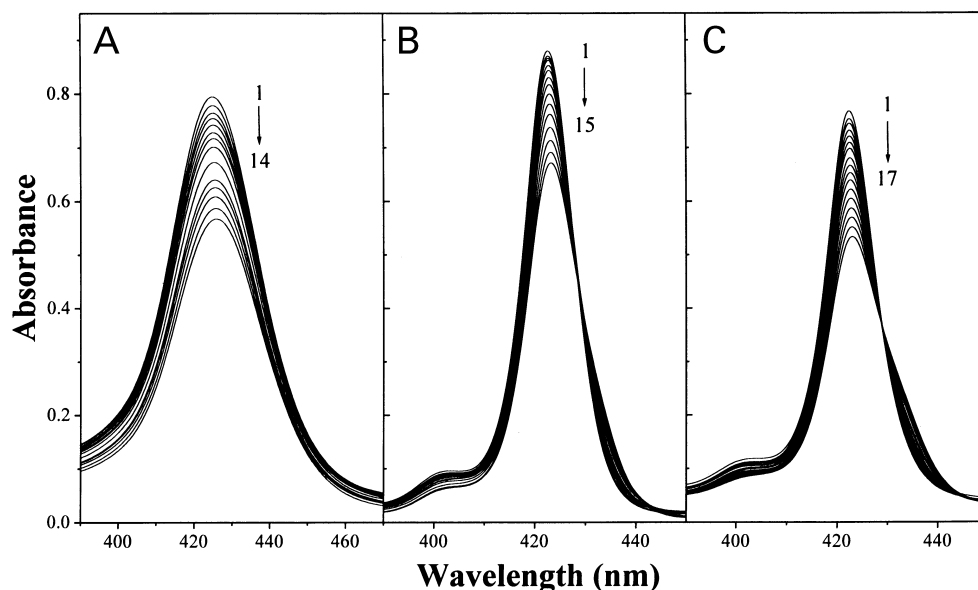


Fig. 2. Absorption spectral titration of porphyrin binding to TCSL. (A) CuTMPyP (B) ZnTPPS (C) ZnTCPP. The porphyrin absorption spectra were recorded in the absence and presence of increasing concentrations of TCSL. Spectrum 1 in each panel corresponds to the porphyrin alone and the remaining spectra of decreasing intensity at the Soret band maximum were obtained in the presence of increasing concentrations of TCSL. See text for details.

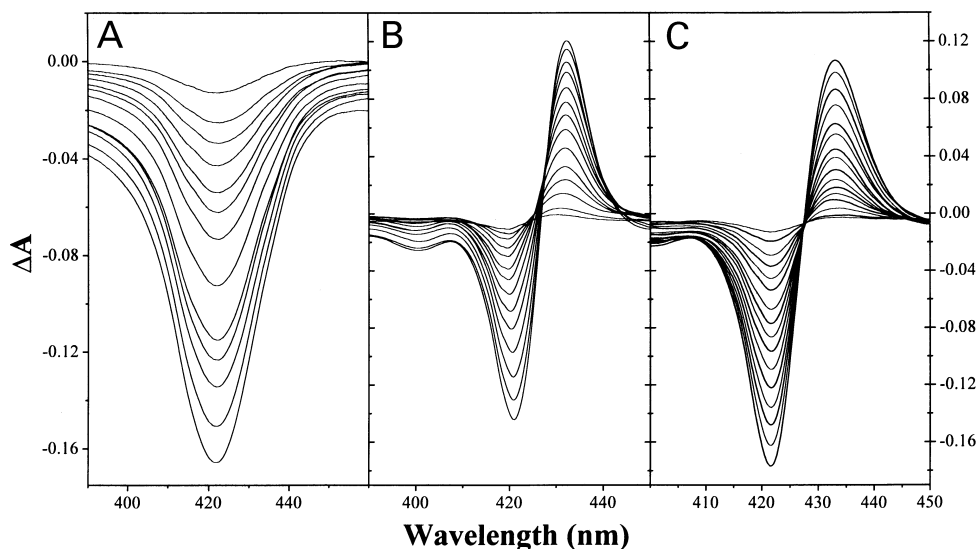


Fig. 3. Difference absorption spectra for porphyrin binding to TCSL. (A) CuTMPyP (B) ZnTPPS (C) ZnTCPP. The difference spectra shown in panels A, B, and C were obtained by subtracting the spectrum of the porphyrin alone from the spectra of lectin–porphyrin mixtures, shown in the corresponding panel of Fig. 2. The Y-scale on the left relates to panel A and that on the right relates to panels B and C.

whereas considerably larger changes were noticed in the difference spectra obtained in the presence of SDS (not shown). These observations are consistent with the results of Kadish and coworkers [13,22] described above.

Association constants and thermodynamics of porphyrin binding to TCSL

The data obtained from the absorption titrations was analysed in the following manner to obtain the association constants (K_a) for TCSL–porphyrin interactions. A plot of $(A_0/\Delta A)$ vs. $[P]_t^{-1}$, where ΔA refers to the change in absorbance of the porphyrin at a given TCSL concentration during the titration and A_0 corresponds to the absorbance of

the sample in the absence of protein, yielded a straight line (data not shown). From the ordinate of the plot, A_∞ , the absorbance of the sample at infinite protein concentration was calculated. From the titration data the K_a values were obtained according to the method of Chipman *et al.* [23] as described earlier for the binding of porphyrins to Con A and pea lectin [6]. Briefly, a plot of $\log [P]_f$ against $\log[(\Delta A)/(A_c - A_\infty)]$ where $[P]_f$ is the free protein concentration, was obtained. A representative plot for the interaction of CuTPPS with TCSL is given in Fig. 5. The abscissa intercept of this plot yielded the pK_a value of the TCSL–CuTPPS interaction according to the relationship [23]:

$$\log[(\Delta A)/(A_c - A_\infty)] = \log K_a [[P]_t - [L]_t (\Delta A / \Delta A_\infty)] \quad (1)$$

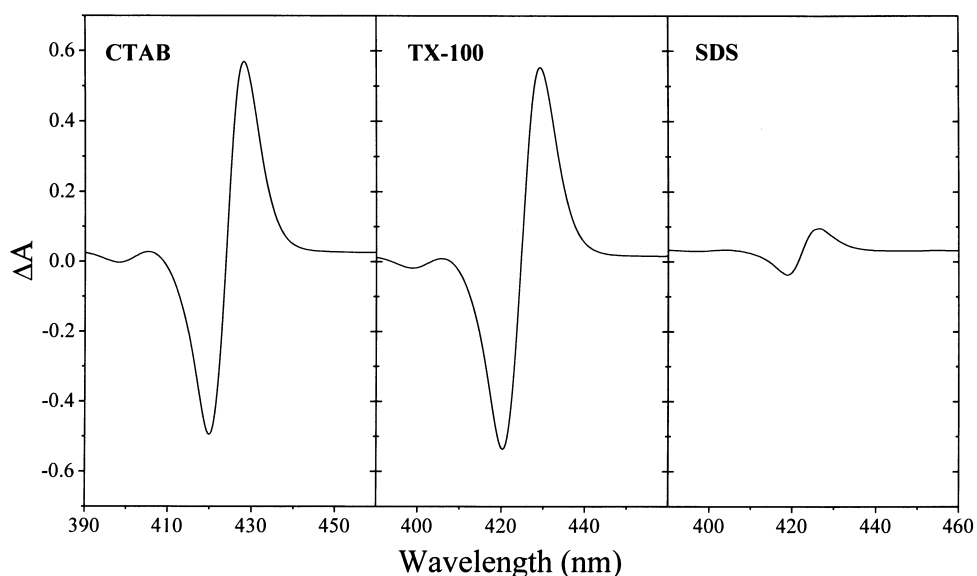


Fig. 4. Difference absorption spectra of ZnTPPS in the presence of different surfactants. The surfactants used are indicated in the figure.

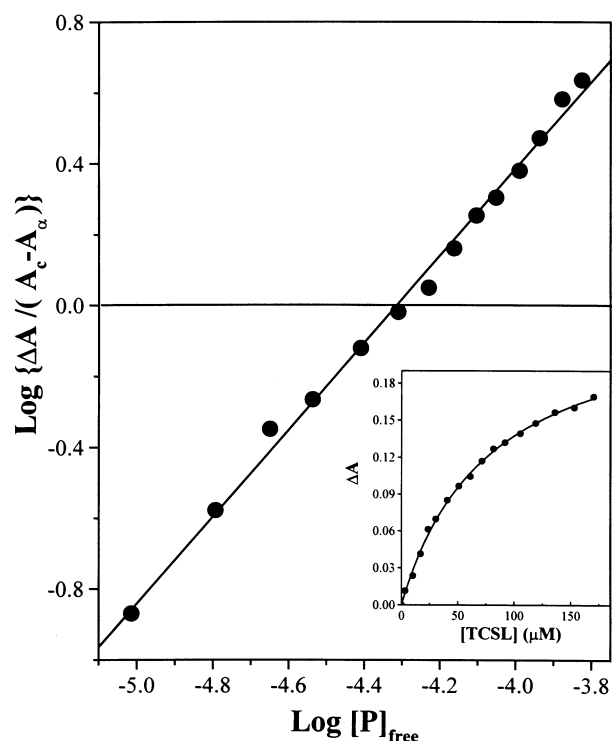


Fig. 5. Chipman plot for CuTPPS binding to TCSL. The antilog of the intercept on the abscissa gives the association constant (K_a) for the interaction. The inset gives a binding curve for the interaction, obtained by plotting the increase in absorption intensity at 411 nm as a function of the lectin concentration.

where A_c is the absorption intensity of the sample at any point during the titration, $[L]_t$ is the total concentration of porphyrin, ΔA_∞ is the change in absorption intensity at saturation binding, and $[P]_f$ is given by:

$$[P]_f = [[P]_t - [L]_t(\Delta A / \Delta A_\infty)] \quad (2)$$

The slopes of such plots, obtained for the binding of different porphyrins to TCSL, were generally found to be in the range 0.8–1.2, indicating that each lectin subunit has one porphyrin-binding site. Furthermore, the linearity of the plots indicates that the two binding sites present on the dimeric lectin interact with the porphyrin with comparable binding strength and that binding at one site does not affect

binding at the other. The slopes, maximal change in the absorption intensity at infinite protein concentration and the corresponding association constants for porphyrin binding to TCSL, are listed in Table 1. The data obtained with different porphyrins indicates that TCSL–porphyrin interaction is characterized by association constants in the range of 2×10^3 – $5 \times 10^5 \text{ M}^{-1}$ and binding stoichiometry of $\approx 1 : 1$ per subunit, assuming an average M_r of 30 000 per subunit (Table 1).

Association constants for representative examples of an anionic porphyrin (CuTPPS) and a cationic porphyrin (CuTMPyP), were also obtained in the presence of 0.1 M lactose, in order to assess the effect of the specific sugar on the porphyrin binding characteristics of TCSL (see Table 1). The data obtained clearly show that while considerable differences could be seen in the K_a values obtained in the absence and in the presence of the lectin, the stoichiometry of binding (as obtained from the slopes of the double-logarithmic plots) remains unchanged, indicating that sugar binding does not inhibit porphyrin binding. In other words, the sugar and porphyrin bind to the lectin at distinctly different sites under the experimental conditions used here.

Binding of CuTPPS and CuTMPyP was investigated at different temperatures and the K_a values obtained are listed in Table 2. At each temperature, at least three independent titrations were performed for each lectin–porphyrin system and the mean values are given. From the temperature-dependent K_a values for CuTPPS and CuTMPyP, the Gibbs' free energies (ΔG°), the enthalpy of binding (ΔH°) and entropy of binding (ΔS°) have been obtained by means of van't Hoff plots using the expressions:

$$\Delta G^\circ = -RT \ln K_a \quad (3)$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (4)$$

For CuTPPS binding to TCSL, the values obtained at 25 °C are: $\Delta G^\circ = -29.0 \text{ kJ}\cdot\text{mol}^{-1}$, $\Delta H^\circ = -15.06 (\pm 18.41) \text{ kJ}\cdot\text{mol}^{-1}$, $\Delta S^\circ = 43.93 (\pm 62.34) \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$, whereas the corresponding values for CuTMPyP binding to TCSL are, $-27.32 \text{ kJ}\cdot\text{mol}^{-1}$, $-7.53 (\pm 11.72) \text{ kJ}\cdot\text{mol}^{-1}$ and $67.78 (\pm 39.33) \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$, respectively.

Stopped-flow kinetics

As the absorption intensity of CuTMPyP in the Soret band region decreases upon binding to TCSL (see Fig. 2A), the

Table 1. The maximal change in porphyrin absorbance (ΔA_∞) at infinite lectin concentration, the average slopes from double logarithmic plots and the association constants, K_a , for TCSL–porphyrin complexes at 25 °C. Experiments were performed in the absence and in the presence of 0.1 M lactose.

Porphyrin	Without sugar			With 0.1 M lactose		
	ΔA_∞ (%)	Slope	$K_a (\times 10^{-4} \text{ M}^{-1})$	ΔA_∞ (%)	Slope	$K_a (\times 10^{-4} \text{ M}^{-1})$
CuTPPS	25.8 ± 2.4	1.05 ± 0.02	12.11 ± 6.23	26.9 ± 3.5	1.16 ± 0.23	1.39 ± 0.15
ZnTPPS	32.2 ± 9.7	0.99 ± 0.10	1.9 ± 1.086	–	–	–
CuTCPP	16.9 ± 2.9	1.03 ± 0.22	0.22 ± 1.5	–	–	–
ZnTCPP	40.0 ± 4.3	1.06 ± 0.23	0.58 ± 0.3	–	–	–
CuTMPyP	30.7 ± 7.4	0.86 ± 0.06	6.11 ± 0.5	28.5 ± 5.0	0.92 ± 0.12	17.9 ± 0.58
ZnTMPyP ^a	22.0	0.7	46.8	–	–	–

^a Data correspond to a single titration.

Table 2. Association constants, K_a , obtained at different temperatures for the interaction of CuTPPS and CuTMPyP with TCSL. ND, not determined.

T (°C)	K_a ($\times 10^{-4} \text{ M}^{-1}$)	
	CuTPPS	CuTMPyP
20	7.85 ± 2.70	10.48 ± 0.35
25	12.11 ± 6.23	6.11 ± 0.50
27.5	13.61 ± 4.96	ND
30	4.85 ± 0.25	6.18 ± 0.49
32.5	7.38 ± 2.10	ND
35	5.1 ± 1.12	8.4 ± 3.25
40	8.4 ± 4.26	7.0 ± 2.12

kinetics of the corresponding interaction were monitored at 424 nm by the stopped-flow method. The apparent rate constant obtained from the absorption change of the porphyrin, k_{app} , is then related to the elementary steps involved in the binding process in a concentration-dependent manner. Several possible mechanisms have been suggested for the protein–ligand interactions that do not involve covalent transformations on the protein (P) or the ligand (L) (cf [24]). Here we consider the most commonly invoked model which involves the rapid formation of an intermediate, PL_i , which isomerizes to give the final complex, PL^* (Eqn 5). This model has been found to explain satisfactorily the stopped-flow kinetic data obtained by us.



In this case, k_{app} is related to the different rate constants and $[P]_o$ by

$$k_{app} = k_{-2} + k_2[P]_o / (K_{-1} + [P]_o) \quad (6)$$

where $K_{-1} = k_{-1}/k_1$ and $K_{-2} = k_{-2}/k_2$. Eqn (6) predicts

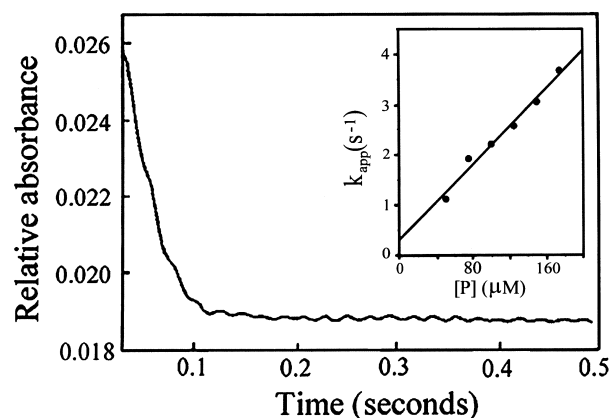


Fig. 6. Stopped-flow trace for the binding of CuTMPyP to TCSL. The change in absorption intensity at 424 nm was monitored as a function of time. Temperature = 20 °C. The inset gives the dependence of the apparent rate constant, k_{app} , on the lectin concentration. The slope and the intercept on the ordinate gave the k_1 and k_{-1} values as $1.89 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and 0.29 s^{-1} , respectively.

that k_{app} would increase linearly with $[P]_o$ but tends to saturate as $[P]_o$ increases from a value much lower than $1/K_1$ to $P \gg 1/K_1$.

Stopped-flow experiments were performed under pseudo-first order conditions by keeping the porphyrin concentration fixed at 2.55 μM and varying the TCSL concentration in the range 50–175 μM (subunit concentration). A representative stopped-flow trace recorded at 20 °C is given in Fig. 6. The k_{app} values obtained were found to increase linearly with increasing protein concentration. A plot of the dependence of k_{app} on the protein concentration yielded a straight line (Fig. 6 inset). The values of k_{+1} and k_{-1} for the CuTMPyP–TCSL interaction at 20 °C were obtained as $1.89 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and 0.29 s^{-1} , from the slope and intercept, respectively, of this plot.

DISCUSSION

In view of the preferential agglutination of tumour cells and the hydrophobic ligand binding exhibited by the plant lectins, and also the low tumour tissue localization exhibited by porphyrins [5], we envisaged that lectins may potentially be useful in the targeting of porphyrins to tumour tissues in PDT. In light of this, we have previously investigated the interaction of several lectins such as Con A, pea lectin, jacalin and SGSL with different porphyrins [6–8]. In those explorative studies, the association constants for the lectin–porphyrin interaction were determined at room temperature only; no information was sought on the forces that stabilize the lectin–porphyrin interaction and the kinetics of the association were not investigated either. In the present study, the interaction of different porphyrins with TCSL was investigated by performing the binding experiments at different temperatures to seek information on the thermodynamic forces that govern binding. In addition, the kinetics of binding of a porphyrin were investigated by the stopped-flow technique in order to unravel the elementary steps involved in the binding process.

Our previous studies on the interaction of porphyrins with SGSL and Jacalin indicated that positively and negatively charged porphyrins bind to these lectins with comparable affinities, suggesting that the charge on the porphyrin does not play any significant role in the interaction [7,8]. Therefore, it appears that hydrophobic interactions largely mediate the binding interaction between these two lectins and the porphyrins. As the tetra-anionic porphyrin, CuTPPS and the tetra-cationic porphyrin, CuTMPyP bind to TCSL with comparable affinities at different temperatures (see Table 2), it may be argued that porphyrin binding to TCSL also occurs via hydrophobic interactions. However, the possibility of polar interactions such as hydrogen bonding between the substituents on the porphyrin with certain functional groups on the protein cannot be ruled out in the absence of specific structural data on the porphyrin–lectin complex.

Similarity in the difference spectra obtained when ZnTPPS and ZnTCPP were titrated with TCSL (Fig. 3) with those obtained for ZnTPPS in the presence of cationic and neutral surfactants, CTAB and Triton X-100 (Fig. 4), suggests that the forces involved in the interactions of these porphyrins with micelles and the protein are likely to be very similar. As the difference spectra obtained for CuTPPS and CuTCPP in the presence of TCSL are qualitatively rather

similar to the corresponding spectra of their Zn-analogues, it is likely that the interaction of the former two porphyrins with TCSL is also mediated by both hydrophobic and electrostatic interactions. Similarly, the difference spectra obtained when CuTMPyP is titrated with TCSL resembles the difference spectrum obtained with the anionic surfactant, SDS. This result suggests that forces governing the binding of CuTMPyP to TCSL are rather similar to those that come into play when this tetra-cationic porphyrin interacts with SDS.

The data presented in Table 1 clearly show that slopes of the double-logarithmic plots were close to unity within the limits of experimental error for all of the TCSL–porphyrin combinations investigated, suggesting that each lectin subunit has one binding site for the porphyrin. This is also true for experiments carried out in the presence of 0.1 M lactose, indicating that sugar binding does not interfere with porphyrin binding. Thus, it is most likely that porphyrin binding to TCSL takes place at a site that is different from the saccharide-binding site. The K_a values obtained in the presence of lactose, however, differ somewhat from those obtained in its absence (Table 1). A possible reason for this is that ligand binding leads to certain changes in the conformation of the protein, resulting in an alteration in the affinity of the protein towards the porphyrins.

The association constants obtained here for the TCSL–porphyrin complexes are in the range of 2×10^3 to $2 \times 10^5 \text{ M}^{-1}$ (Table 1) and are comparable to those observed generally for lectin–monosaccharide complexes [25] as well as those obtained for porphyrin–serum protein interactions [1]. Other hydrophobic ligands and plant growth hormones, such as 2,6-toluidinylnaphthalene-sulphonic acid, adenine, auxins and cytokinins also bind to lectins with comparable affinity ($K_a \approx 1.0 \times 10^3$ to $1.0 \times 10^6 \text{ M}^{-1}$), suggesting that some of them can also be considered as potential endogenous ligands for the lectins *in vivo* [26–30]. This postulate is especially attractive in view of the high concentrations of plant lectins in growing tissues, where plant growth hormones too exert their action most. Pertinently, it has been postulated that Con A could be involved in the regulation of plant cell division or germination via binding to nonpolar growth factors [31]. Similarly association of adenine or other hydrophobic ligands with the *Dolichos biflorus* lectin has been suggested to be biologically relevant [29]. In the light of these observations and the considerably strong interaction between TCSL and the porphyrins, it is possible that there may be some endogenous hydrophobic ligands for this lectin also.

The thermodynamic parameters, ΔH° and ΔS° associated with the binding of CuTPPS and CuTMPyP, which are obtained from the temperature dependence of the association constants, indicate that the interaction of both these porphyrins with TCSL is governed primarily by entropic forces, though there is some enthalpic contribution to the binding process. It is thus interesting to note that though the K_a values for the binding of different porphyrins to TCSL are in the same range as those obtained for the interaction of mono- and disaccharides with different lectins, the corresponding enthalpy and entropy changes are considerably different.

Due to their preferential agglutination of tumour cells, lectins have been suggested as carriers for the targeted

delivery of drugs and pharmaceuticals to tumour tissues. A number of lectin–drug conjugates have also been prepared and some were successful when tested on cultured cells and animal models [32–34]. In view of the considerable affinity of TCSL for Cu(II)- and Zn(II)-porphyrins investigated in this study, it is possible to use the lectin as a vehicle for targeting porphyrin-based drugs to tumour tissues in PDT. Studies using cultured cells/animal models are required to investigate this possibility further.

Kinetic studies

The stopped-flow studies on the binding of CuTMPyP to TCSL indicate that this binding reaction is a relatively slow process. The k_{+1} value of $1.89 \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$ is approximately four orders of magnitude slower than diffusion-controlled processes, the k_{+1} values for which are in the order of 10^8 – $10^9 \text{ M}^{-1}\cdot\text{s}^{-1}$ [35]. However, the association rate constant obtained here for the CuTMPyP–TCSL interaction is in the same range as that observed for several lectin–saccharide systems (5×10^3 to $5 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$ [24,36–42]). Such slow second-order rate constants are usually explained by invoking the formation of an intermediate $[\text{PL}_i]$, see Eqn (5) that isomerizes to form the final complex (PL^*). Our failure to observe PL_i could be attributed to the low K_1 value, so that significant quantities of it do not accumulate during the course of the reaction. Because the k_{app} vs. $[\text{P}]_0$ plot is linear up to 175 μM of the latter, K_1 has to be $< 5700 \text{ M}^{-1}$. As the K_a value obtained from the kinetic data is in reasonable agreement with the value obtained from the equilibrium titrations, it is very unlikely that there are additional steps in the binding process that contribute significantly to the binding enthalpy.

The dissociation rate constant of 0.29 s^{-1} indicates that the dissociation reaction is a rather slow process and is comparable to the dissociation rate constants reported for the interaction of fluorescently labelled saccharides to different lectins, viz., *N*-dansylgalactosamine binding to soybean agglutinin, 4-methylumbelliferyl-*N*-acetyl- α -galactosaminide binding to WBA-I and the interaction of 4-methylumbelliferyl β -D-galactopyranosyl (1 \rightarrow 3)-*N*-acetyl β -D-galactosaminide with peanut agglutinin [39,40,42].

CONCLUSIONS

The thermodynamic and kinetic parameters that characterize lectin–porphyrin interaction have been elucidated for the first time. The association constants obtained for the TCSL–porphyrin interaction are comparable to those observed in general for mono- and disaccharide binding to lectins and the binding appears to be favoured predominantly by entropic factors. The association and dissociation rate constants for TCSL–porphyrin interaction are in the same range as that observed for lectin–saccharide interaction. Further studies are required to determine if these observations could be applicable to other lectin–porphyrin systems also.

ACKNOWLEDGEMENTS

This work was supported by research grants from the Department of Science and Technology, Government of India, to M. J. S. and to

B. G. M. The authors are grateful to A. Surolia for the use of stopped-flow spectrophotometer and to C. J. Thomas for help in carrying out the kinetic experiments.

REFERENCES

- Davila, J. & Harriman, A. (1990) Photochemical and radiolytic oxidation of a zinc porphyrin bound to human serum albumin. *J. Am. Chem. Soc.* **112**, 2686–2690.
- Beaven, G.H., Chen, S.-H., D'Albis, A. & Gratzer, W.B. (1974) A spectroscopic study of the haemin-human-serum-albumin system. *Eur. J. Biochem.* **41**, 539–546.
- Reyftmann, J.P., Morliere, P., Goldstein, S., Santus, R., Dubertret, L. & Lagrange, D. (1984) Interaction of human serum low density lipoproteins with porphyrins: a spectroscopic and photochemical study. *Photochem. Photobiol.* **40**, 721–729.
- Levy, G.J. (1995) Photodynamic therapy. *Trends Biotechnol.* **13**, 14–18.
- Klyashchitsky, B.A., Nechaeva, I.S. & Ponomaryov, G.V. (1994) Approaches to targeted photodynamic tumor therapy. *J. Controlled Release* **29**, 1–16.
- Bhanu, K., Komath, S.S., Maiya, B.G. & Swamy, M.J. (1997) Interaction of porphyrins with concanavalin A and pea lectin. *Current Sci.* **73**, 598–602.
- Komath, S.S., Bhanu, K., Maiya, B.G. & Swamy, M.J. (2000) Binding of porphyrins by the tumor-specific lectin, jacalin [jack fruit (*Artocarpus integrifolia*) agglutinin]. *Biosci. Reports* **20**, 265–276.
- Komath, S.S., Kenoth, R., Giribabu, L., Maiya, B.G. & Swamy, M.J. (2000) Fluorescence and absorption spectroscopic studies on the interaction of porphyrins with snake gourd (*Trichosanthes anguina*) seed lectin. *J. Photochem. Photobiol. B: Biol.* **55**, 49–55.
- Padma, P., Komath, S.S., Nadimpalli, S.K. & Swamy, M.J. (1999) Purification in high yield and characterisation of a new galactose-specific lectin from the seeds of *Trichosanthes cucumerina*. *Phytochemistry* **50**, 363–371.
- Kenoth, R., Padma, P., Sarada, S. & Swamy, M.J. (2000) Role of histidine residues in the sugar-binding activity of *Trichosanthes cucumerina* seed lectin. *J. Biochem. Mol. Biol. Biophys.* **4**, 423–431.
- Komath, S.S., Kenoth, R. & Swamy, M.J. (2001) Thermodynamic analysis of saccharide binding to snake gourd (*Trichosanthes anguina*) seed lectin. Fluorescence and absorption spectroscopic studies. *Eur. J. Biochem.* **268**, 111–119.
- Fleishcher, E.B., Palmer, J.M., Srivastava, T.S. & Chatterjee, A. (1971) Thermodynamic and kinetic properties of an iron-porphyrin system. *J. Am. Chem. Soc.* **93**, 3162–3167.
- Kadish, K.M., Maiya, G.B., Araullo, C. & Guillard, R. (1989) Micellar effects on the aggregation of tetraanionic porphyrins. Spectroscopic characterization of free-base meso-tetrakis (4-sulfonatophenyl) porphyrin, (TPPS) H₂ and (TPPS) M (M = Zn (II), Cu (II), VO²⁺) in aqueous micellar media. *Inorg. Chem.* **28**, 2725–2731.
- Longo, F.R., Finarelli, M.G. & Kim, J.B. (1969) The synthesis and some physical properties of *ms*-tetra(pentafluorophenyl)-porphyrin and *ms*-tetra(pentachlorophenyl)-porphyrin(1). *J. Heterocycl. Chem.* **6**, 927–931.
- Pasternack, R.F., Huber, P.R., Boyd, P., Engasser, G., Francesconi, L., Gibbs, E., Fasella, P., Ventura, G.C. & Hinds, L.D. (1972) On the aggregation of meso-substituted water-soluble porphyrins. *J. Am. Chem. Soc.* **94**, 4511–4517.
- Pasternack, R.F., Francesconi, L., Ratt, D. & Spiro, E. (1973) Aggregation of nickel (II), copper (II), and zinc (II) derivatives of water soluble porphyrins. *Inorg. Chem.* **12**, 2606–2611.
- Appukkuttan, P.S., Surolia, A. & Bachhawat, B.K. (1977) Isolation of two galactose-binding proteins from *Ricinus communis* by affinity chromatography. *Ind. J. Biochem. Biophys.* **14**, 382–384.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* **227**, 680–685.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–273.
- Tonomura, B., Nakatani, H., Ohnishi, M., Yamaguchi-Ito, J. & Hiromi, K. (1978) Test reactions for a stopped-flow apparatus. Reduction of 2,6-dichlorophenolindophenol and potassium ferricyanide by L-ascorbic acid. *Anal. Biochem.* **84**, 370–383.
- Thomas, C.J., Gangadhar, B.P., Surolia, N. & Surolia, A. (1998) Kinetics and mechanism of the recognition of endotoxin by polymyxin B. *J. Am. Chem. Soc.* **120**, 12428–12434.
- Kadish, K.M., Maiya, B.G. & Araullo-McAdams, C. (1991) Spectroscopic characterization of meso-tetrakis (1-methylpyridinium-4-yl) porphyrins, [(TmPyP) H₂]⁴⁺ and [(TmPyP) M]⁴⁺, in aqueous micellar media, where M = VO²⁺, Cu (II), and Zn (II). *J. Phys. Chem.* **95**, 427–431.
- Chipman, D.M., Grisaro, V. & Sharon, N. (1967) The binding of oligosaccharides containing N-acetylglucosamine and N-acetylmuramic acid to lysozyme. *J. Biol. Chem.* **242**, 4388–4394.
- Gupta, D., Rao, N.V.S.A.V.P., Puri, K.D., Matta, K.L. & Surolia, A. (1992) Thermodynamic and kinetic studies on the mechanism of binding of methylumbelliferyl glycosides to jacalin. *J. Biol. Chem.* **267**, 8909–8918.
- Goldstein, I.J. & Poretz, R.D. (1986) Isolation, physicochemical characterization and carbohydrate-binding specificity of lectins. In *The Lectins. Properties, Functions and Applications in Biology and Medicine* (Liener, I.E., Sharon, N. & Goldstein, I.J., eds), pp. 33–247. Academic Press, New York, USA.
- Roberts, D.D. & Goldstein, I.J. (1982) Hydrophobic binding properties of the lectin from lima beans (*Phaseolus lunatus*). *J. Biol. Chem.* **257**, 11274–11277.
- Roberts, D.D. & Goldstein, I.J. (1983) Binding of hydrophobic ligands to plant lectins: titration with arylaminonaphthalenesulfonates. *Arch. Biochem. Biophys.* **224**, 479–484.
- Maliarik, M.J. & Goldstein, I.J. (1988) Photoaffinity labelling of the adenine binding sites of the lectins from lima bean, *Phaseolus lunatus* and the kidney bean, *Phaseolus vulgaris*. *J. Biol. Chem.* **263**, 11274–11279.
- Gegg, C.V., Roberts, D.D., Segel, I.H. & Etzler, M.E. (1992) Characterization of the adenine binding sites of two *Dolichos biflorus* lectins. *Biochemistry* **31**, 6938–6942.
- Puri, K.D. & Surolia, A. (1994) Amino acid sequence of the winged bean (*Psophocarpus tetragonolobus*) basic lectin. *J. Biol. Chem.* **269**, 30917–30926.
- Hardman, K.D. & Ainsworth, C.F. (1973) Binding of nonpolar molecules by crystalline concanavalin A. *Biochemistry* **12**, 4442–4448.
- Kitao, T. & Hattori, K. (1977) Concanavalin A as a carrier of daunomycin. *Nature* **265**, 81–82.
- Gilliland, D.G., Collier, R.J., Moehring, J.M. & Moehring, T.J. (1978) Chimeric toxins: Toxic, disulfide-linked conjugate of concanavalin A with fragment A from diphtheria toxin. *Proc. Natl Acad. Sci. USA* **75**, 5319–5323.
- Yamaguchi, T., Kato, R., Beppu, M., Terao, T., Inoue, Y., Ikawa, Y. & Osawa, T. (1979) Preparation of concanavalin A-ricin A-chain conjugate and its biologic activity against various cultured cells. *J. Natl Cancer Inst.* **62**, 1387–1395.
- Voet, D. & Voet, J.G. (1995) *Biochemistry*, 2nd edn. Wiley, New York
- Gray, R.D. & Glew, R.H. (1973) The kinetics of carbohydrate binding to concanavalin A. *J. Biol. Chem.* **248**, 7547–7551.
- Brewer, C.F., Sternlicht, H., Marcus, D.M. & Grollman, A.P. (1973) Interaction of saccharides with concanavalin A. Mechanism of binding of α - and β -methyl D-glucopyranoside to concanavalin A

- as determined by ^{13}C nuclear magnetic resonance. *Biochemistry* **12**, 4448–4457.
38. Podder, S.K., Surolia, A. & Bachhawat, B.K. (1978) Dynamics of carbohydrate–lectin interaction: The interaction of *p*-nitrophenyl- β -D-galactose with a lectin from *Ricinus communis*. *FEBS Lett.* **85**, 313–316.
39. Loontjens, F.G. (1983) Slow dissociation of 4-methylumbelliferyl β -D-galactosyl-(1 \rightarrow 3)-*N*-acetyl β -D-galactosaminide from its complex with peanut agglutinin. *FEBS Lett.* **162**, 193–196.
40. Swamy, M.J., Sastry, M.V.K., Khan, M.I. & Surolia, A. (1986) Thermodynamic and kinetic studies on saccharide binding to soya-bean agglutinin. *Biochem. J.* **234**, 515–522.
41. Sastry, M.V.K., Swamy, M.J. & Surolia, A. (1988) Analysis of dynamics and mechanism of ligand binding to *Artocarpus integrifolia* agglutinin. A ^{13}C and ^{19}F NMR study. *J. Biol. Chem.* **263**, 14826–14831.
42. Puri, K.D., Khan, M.I., Gupta, D. & Surolia, A. (1993) Thermodynamic and kinetic studies on the mechanism of binding of methylumbelliferyl galactosides to the basic agglutinin from winged bean (*Psophocarpus tetragonolobus*). *J. Biol. Chem.* **268**, 16378–16387.