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Physicochemical aspects of carbohydrate binding to some plant lectins with binding preference for *N*-acetylgalactosamine and galactose

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Abstract. This contribution illustrates the advantages of some chromophoric and fluorophoric carbohydrate derivatives such as *p*-nitrophenyl (*p*NO_2Phe) or 4-methylumbelliferyl (MeUmb) glycosides and *N*-dansylgalactosamine in studies of the binding equilibrium and kinetics with some plant lectins. The methods used involve continuous titrations of changes in ligand or protein absorption and ligand fluorescence, including substitution titrations as well as stopped-flow, temperature-jump or pressure-jump relaxation kinetics.

When monitored by temperature-jump relaxation, binding of MeUmbαGal to the bloodgroup A specific lectin GSAI-A_4 from *Griffonia simplicifolia* is a simple bimolecular association with parameters $k_+ = 9{\cdot}4 \times 10^4$ M^{-1} s^{-1} and $k_{-1} = 5{\cdot}3$ s^{-1} at 23°C, but binding to the GSAI-B_4 lectin is biphasic.

The complementarity of the peanut agglutinin binding site with Gal β1$\rightarrow$ 3GalNAc that occurs in many *O*-glycoproteins follows from enthalpic considerations and also from the value of the dissociation-rate parameter $k_{-1} = 0{\cdot}24$ s^{-1} of the MeUmbβGalβ1 $\rightarrow$ 3GalNAc·lectin complex. This value, obtained by stopped-flow kinetics is 100 times smaller than for other mono-and disaccharides investigated. The binding mechanism is simple and the derivatisation of Galβ1 $\rightarrow$ 3GalNAc does not affect the affinity to a considerable degree.

The binding preference of tetravalent *soybean agglutinin* for MeαGalNAc over MeαGal by a factor of 25 is mainly of enthalpic origin with an additional 7 kJ mol^{-1}; the NAc group causes perturbation of a tryptophanyl residue, evidenced by protein difference absorption spectrometry. In the glycosides, a large aglycon like $\beta p$$NO_2$ Phe or β MeUmb hardly affects the affinity of SBA but a large *N*-dansyl group increases the affinity by a factor 20 as compared to GalNAc. The 10-fold increase in carbohydrate-specific *N*-dansylgalactosamine fluorescence, together with a very favourable entropic contribution point at the presence of a hydrophobic region in the vicinity of the carbohydrate-binding site. The dissociation-rate parameter of the MeUmbβGalNAc SBA complex is slower than for any reported monosaccharide-lectin complex: $0{\cdot}4$ s^{-1}.

The divalent lectin from *Erythrina cristagalli* preferentially binds the Galβ1 $\rightarrow$ 4GlcNAc structure that occurs in many *N*-glycoproteins. The combining site was mapped thermodyna-

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Abbreviations used: MeUmb, 4-methylumbelliferyl (7-hydroxy coumaryl); Dns, dansyl, 5-dimethylamino-l-sulphonyl; *p*NO_2Phe, paranitrophenyl; ECA, *Erythrina cristagalli* agglutinin; GSAI-A_4, I-B_4, II, *Griffonia* (*Bandeiraea*) *simplicifolia* agglutinins; SBA, soybean (*Glycine max*) agglutinin: PNA, peanut (*Arachis hypogaea*) agglutinin.

All carbohydrates have the D-pyranoside configuration.

mically with carbohydrates ranging from mono-to pentasaccharides as derived from *N*-glycoproteins. Here, N-dansylgalactosamine was used as a fluorescent indicator ligand in substitution titrations. When Galβ1 $\rightarrow$ 4GlcNAc was linked α1 $\rightarrow$ 2 or α1 $\rightarrow$ 6 to Man, the binding enthalpy and entropy remained practically constant. Application of stopped flow kinetics and pressure-jump relaxation with *N*-dansylgalactosamine gave mono-exponential signal changes with a concentration dependence corresponding to $k_+ = 4{\cdot}8 \times 10^4$ M^{-1} s^{-1}, $k_- = 0{\cdot}4$ to 0.66 s^{-1} and a change in reaction volume of +7ml/mol.

Keywords. Stopped flow kinetics; temperature jump relaxation; pressure jump relaxation; carbohydrates; lectins.

Introduction

Physicochemical aspects of carbohydrate binding to plant lectins, including kinetics, have been studied using two main approaches. The first involves ^{13}C-NMR studies. This most powerful tool has been applied to concanavalin A (Brewer *et al*., 1973) and PNA (Neurohr *et al*., 1982a) as the most prominent examples. The second approach of such binding studies has taken advantage of the change in optical properties of the protein or of a suitable chromophoric glycoside. Only in rare cases, like those of PNA (Neurohr *et al*., 1982b) or SBA (De Boeck *et al*., 1984a), has this been exploited for detailed studies by protein difference absorption spectrometry. In most cases however there is only a small change in optical properties of a lectin due to binding of a carbohydrate and then the use of carbohydrates containing an environmentally-sensitive reporter group is recommended. When suitable controls are made, such bulky fluorophoric or chromophoric substituents are interesting ligands, particularly for rapid kinetic studies (Lewis *et al*., 1976; Williams *et al*., 1981; Clegg *et al* 1977, 1983; Loontiens, 1983; De Boeck *et al.* 1984a) and as indicator ligands (Bessler *et al*., 1974; Clegg *et al*., 1981; De Boeck *et al*., 1984b) to study the binding of glycoconjugate-derived carbohydrates. Three types of chromophoric glycosides have been introduced as ligands for lectins. These are: *p*NO_2 Phe glycosides (Hassing and Goldstein, 1970) with absorption changes at 315 nm, MeUmb glycosides (Dean and Homer, 1973; Loontiens *et al*., 1977, 1983) and GalNDns (De Boeck *et al*., 1984a,b). The use of these ligands is illustrated in this contribution that summarizes some of our recent results on lectins with GalNAc- and/or Galbinding preference.

Results and discussion

4-*Methylumbelliferylglycosides and N-dansylgalactosamine as ligands for lectins*

4-Methylumbelliferyl (MeUmb) glycosides are versatile ligands for binding to lectins (Loontiens *et al*., 1983, Decastel *et al.*, 1984). Several methods are available to exploit the favourable spectroscopic properties of the MeUmb group. Examples are the almost total quenching of *e.g.* MeUmbαGal fluorescence upon binding of the GSAI-A_4 isolectin (De Boeck *et al.*, 1981) used in temperature-jump relaxation (figure 1) and the 60% increase in MeUmbβGalβ1 $\rightarrow$ 3GalNAc fluorescence with PNA (De Boeck *et al.*, 1983) used in titrations at equilibrium. Difference absorption spectrometry, especially with MeUmb glycosides measured at 322 or 334 nm, was often used. This approach is generally applicable to all the lectins that we have tested: concanavalin A, wheat germ

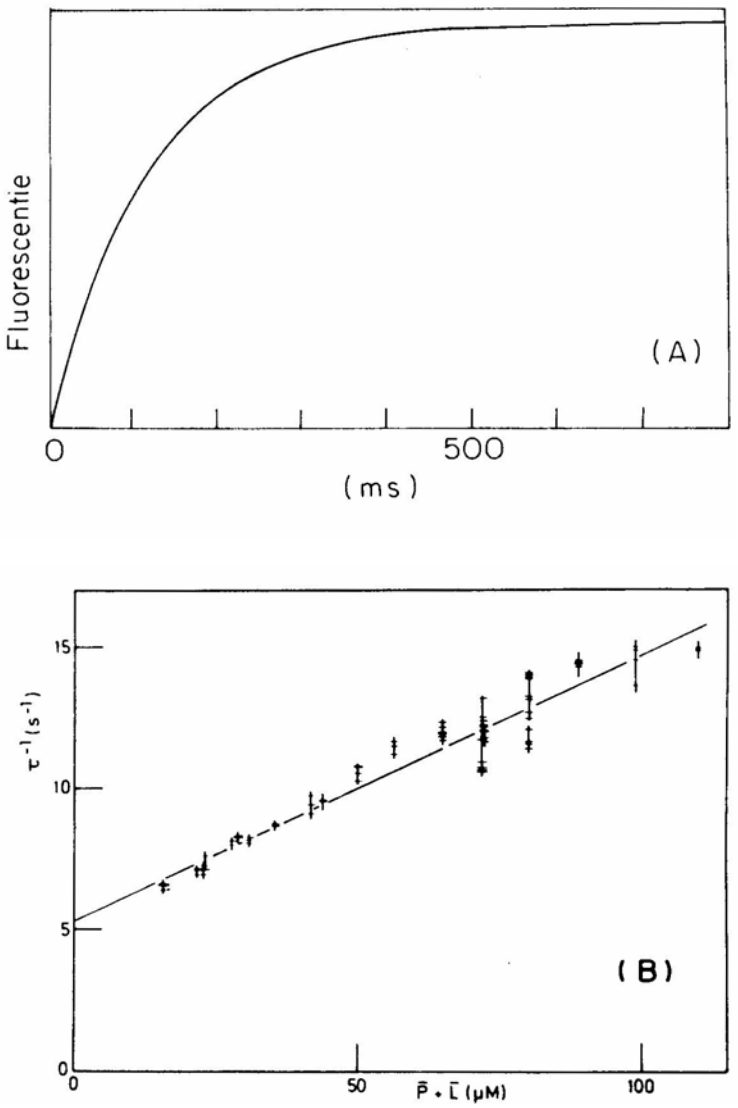

Figure 1. Determination of the reaction-rate parameters for binding of MeUmbαGal to GSAI-A_4 by temperature-jump relaxation.

A. Example of a temperature-jump relaxation caused by a 3°C jump applied to a mixture of 3·5 μM MeUmbαGal and 35 μM $GSAIA_4$ at a starting temperature of 20°C and pH 7·2. Fluorescence was excited at 313 nm and emitted light was observed at wavelengths longer than 360 nm. A smooth curve, corresponding to a mono-exponential signal change with τ = 115 ms is superposed on the data.

B. The inverse relaxation time is plotted against the sum of the concentration of the free reaction partners. The data were obtained from several independent series of experiments, consisting of kinetic titration series with the lectin in excess. The unaveraged data result from individual temperature jumps. For a simple bimolecular association, $\tau^{-1} = k_+ (P + L) + k_{-1}$, linear regression yields $k_+ = (9{\cdot}4 \pm 0{\cdot}2)10^4$ M^{-1} s^{-1} and $k_{-1} = (5{\cdot}3 \pm 0{\cdot}2)$ s^{-1}. The buffer used is 0·05 M Hepes, 0·15M NaCl, 1 mM$CaCl_2$ (pH7·2).

agglutinin, *Ricinus communis* toxin (M_r 60,000) and agglutinin (M_r 120,000), GSAI-A_4, GSAI-B_4, GSAII, PNA, SBA and EGA. In this respect we have developed a continuous titration of difference absorption (De Boeck *et al.*, 1982), with an excess of either chromophoric ligand or of protein, and as an indicator system for displacement titrations with nonchromophoric carbohydrates (De Boeck *et al.*, 1983). Further applications of MeUmbglycoside difference absorption spectrometry include: stopped-flow kinetics with PNA (Loontiens, 1983) or with SBA (De Boeck *et al.*, 1984a) and determination of $\Delta H°$ from a single mixture of a MeUmbglycoside and a lectin subjected to temperature-induced difference absorption spectrometry as applied to concanavalin A, PNA (figure 4). SBA and ECA (Loontiens and Dhollander, 1984).

N-Dansylgalactosamine (GalNDns) binds to *N*-acetylgalactosamine-specific lectins and is an interesting ligand in view of the intense change in dansyl fluorescence. Typically we have observed a 5- to 10-fold increase when it binds with SBA (De Boeck *et al.*, 1984a), ECA (De Boeck *et al.*, 1984b), GSI-A_4 (figure 2) and the lectin from *Dolichos biflorus* (figure 3) or from *Vicia cracca.* We have used this ligand as a carbohydrate-specific fluorescent indicator in substitution titrations with ECA to study the binding of *N*-glycoprotein-derived oligosaccharides. In some cases, such as the lectin from Lima bean, there may be some partially unspecific binding

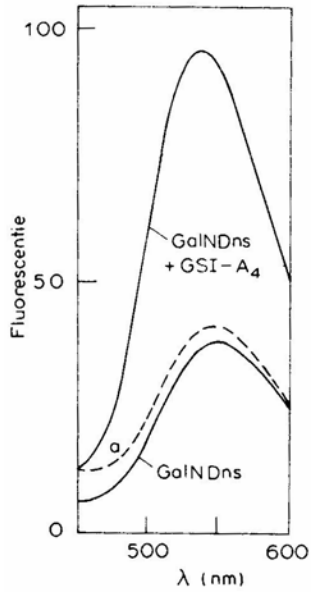

Figure 2. Fluorescence emission spectra of unbound *N*-dansylgalactosamine and in the presence of near-saturating amounts of GSAI-A_4. Excitation of the samples in a 1 × 0·4cm cuvette at 13°C is at 330 nm; the spectral band widths for excitation and emission are 2 nm and 20 nm respectively. The spectra represented as full lines are valid for 10μm *N*-dansylgalactosamine as such or in the presence of 56 μM GSAI-A_4 sites. The spectrum with the dashed line was obtained after dissolving GalNAc in the mixture of 10μM *N*-dansylgalactosamine and 54 μM GSAI-A_4 sites. Buffer: 0·05 M Hepes, 0·15 M NaCl, 1 mM $CaCl_2$ (pH 7·2).

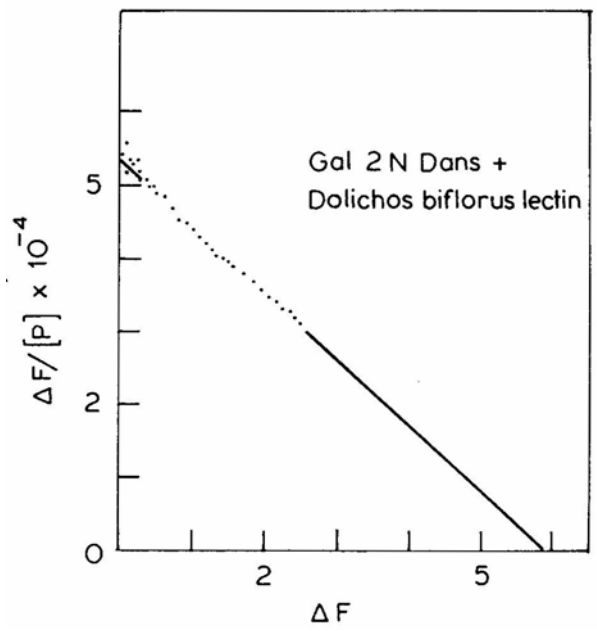

Figure 3. Linearized titration of *N*-dansylgalactosamine (7·7 μM, 0·025 M sodium phosphate, 1 M sodium chloride, pH 6·9, 7·2°C, 1·14 ml in a 0·7 ×0·7-cm cuvette) fluorescence upon addition of 0 to 142 μl of 727 μM *Dolichos biflorus* agglutinin sites (20 mg/ml, Carter and Etzler, 1975). Excitation is at 325 nm (spectral band width 2 nm); emission is measured at 525 nm. The data are corrected for dilution and for a protein blank (maximally 9% of the fluorescence). ΔF is the increase in fluorescence as corrected for dilution and [P] is the concentration of free protein sites as obtained by iteration. The slope corresponds to the association constant $K = (9{\cdot}1 \pm 0{\cdot}1)\ 10^3\ M^{-1}$.

of *N*-dansylgalactosamine (David Roberts, unpublished results). Precautions must be taken if the equilibrium is reached very slowly as with SBA (De Boeck *et al*., 1984a). With some *N*-acetylglucosamine-specific lectins, such as wheat germ agglutinin and GSA-II lectin, no change in the fluorescence of the corresponding *N*-dansylglucosamine could be observed.

Binding of 4-*methylumbelliferyl galactoside to the bloodgroup-A specific homo-isolectin GSAI-A*$_4$ *from Griffonia* (*Bandeiraea*) *simplicifolia*

MeUmbαGal was used as a fluorescent ligand to study the binding of some of the Group I isolectins from *Griffonia simplicifolia* (Murphy and Goldstein, 1979; Goldstein, 1983) by temperature-jump relaxation kinetics. This ligand has a comparable affinity for the homo-isolectin GSAI-A_4 or GSAI-B_4, with binding preferences respectively for GalNAc or for Gal (De Boeck *et al*., 1981). It has been reported that stopped-flow traces for binding of MeUmbαGal with a mixture of the five GSAI isolectins show "slight biphasic characteristics" (Harrington *et al*., 1981). Here, we report that the binding of

MeUmbαGal with the A_4 lectin is monophasic, which contrasts to the more complex binding process with the B_4 or A_2B_2 isolectins which we have also measured.

For the GSAI-A_4-MeUmbαGal system, a simple bimolecular one-step association is in agreement with our kinetic data (figure 1). In the 14-110 μM range all relaxations are perfectly represented by a mono-exponential progress curve with relaxation times in the range τ = 165–65 ms. The corresponding τ^{-1} values increase linearly with the concentration of reactants and correspond to a bimolecular association rate constant of k_{+1} = 9·4 × $10^4 M^{-1}s^{-1}$ and a dissociation rate constant of k_{-1} =5·3s^{-1}. In contrast, the binding of MeUmbαGal to GSAI-B_4 is more complex. In a comparable concentration range of GSAI-B_4 (25–100 μM), the average kinetic process is about three times slower than with the A_4 isolectin. However, with the B_4 isolectin there is a small but systematic deviation between a progress curve and a "best fit" mono-exponential simulation. If the overall process for B_4 is simulated by a mono-exponential progress curve, the concentration dependence of τ^{-1}, corresponding to this overall process, is linear and appears to be τ^{-1} = 4·2 × 10^4 (P + L) + 1.7 with a surprisingly good correlation coefficient of 0·991. However, the relaxation curves for GSAI-B4 can be perfectly simulated by a bi-exponential analysis. Then there are two close relaxation time constants varying from 170 to 70 ms and from 500 to 200 ms throughout the concentration range; the latter always has the larger amplitude. The following two controls indicate that the two relaxations observed with the GSAI-B4 preparation originate from binding of MeUmbαGal to the B site. First, no trace of a lower-M_r subunit could be detected by Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and subsequent staining with Coomassie blue which would be indicative of the A subunit as an impurity. Secondly, a large excess of GalNAc, which binds nearly 1,000 times better to the A subunit than to the B subunit (Goldstein *et al*., 1981), did not displace fluorescent MeUmbαGal from its quenched complex with GSAI-B_4 at equilibrium (GalNAc can completely dissociate the MeUmbαGal GSAI-A_4 complex). The two relaxation times are separated only by a factor of 3 throughout the concentration range and show a considerable scatter so that precise determinations are difficult. Because of this, we have not tried to differentiate any kinetic mechanisms of binding of MeUmbαGal with GSAI-B_4. Additional kinetic experiments with MeUmbαGal and excess of GSAIA_2B_2 isolectin (14–100 μM sites) yielded apparently bi-exponential relaxation curves with two sets of τ values ranging from 125 to 50 ms and 340 to 130 ms. However, in view of the complex kinetics already observed with the GSAI-B_4 isolectin alone, the relaxation curves for GSAI-A_2B_2 may well be expected to consist of more than two separate processes, which we are unable to ascertain. In this sense it may be misleading to interpret the perfectly linear concentration dependence of both sets of τ^{-1} values for A_2B_2 in terms of a detailed model. Binding of carbohydrates to the two homo-isolectins GSAI-A_4 and GSAI-B_4, with unique sequences of the A and B subunit (Lamb and Goldstein, 1984), is characterized by differences involving the carbohydrate-binding specificity (Wood *et al*., 1979; Murphy and Goldstein, 1979; Goldstein *et al.*, 1981), the interactions of tryptophanyl residues with a ligand (Goldstein *et al*., 1981; De Boeck *et al.*, 1981), the interaction of the MeUmb group in MeUmbαGal with the protein (De Boeck *et al.*, 1981) and, as outlined here, the kinetics of MeUmbαGal binding.

*Binding of MeUmbβGalβ*1 → 3*GalNAc to peanut agglutinin* (De Boeck et al., 1983; Loontiens, 1983)

It has been suggested that peanut agglutinin (PNA) possesses an extended binding site, complementary to the disaccharide Galβ1 → 3GalNAc, which binds 36 times better than MeβGal and 14 times better than Meβ-lactoside (Pereira *et al.*, 1976; Neurohr *et al.*, 1982b). This conclusion is consistent with the values of $-\Delta H°$ for binding that are much larger for the disaccharide like MeβGalβ1 → 4Glc (65 kJ mol^{-1}) or Galβ1 → 3GalNAc (78 kJ mol^{-1}) than for MeaGal (42 kJ mol^{-1}) or MeβGal (43 kJ mol^{-1}) (Neurohr *et al*., 1982a, b). This trend for the $\Delta H°$ values is maintained for MeUmbαGal and MeUmbβGalβ1 → 3GalNAc (Decastel *et al.*, 1982). A direct determination of. $\Delta H°$ for binding of MeUmbβGalβ1 → 3GalNAc with PNA is possible as illustrated in figure 4. This method (Loontiens and Dhollander, 1984) uses a single mixture of MeUmbβGalβ1 → 3GalNAc and PNA in comparable concentrations. It combines the sensitive detection of difference absorption spectrometry with the principle of temperature-jump relaxation (Jovin, 1975; Bernasconi, 1976). The extent of reaction is displaced by a continuous temperature change which is slow enough to retain equilibrium conditions. According to figure 4, $\Delta H° = -63$ kJ mol^{-1} which is in good agreement with the classical van 't Hoff plot for binding of MeUmbβGalβ1 → 3GalNAc to PNA yielding, $\Delta H° = -58{\cdot}4 \pm 2$ kJ mol^{-1} (De Boeck *et al*., 1983).

The dissociation-rate parameter of carbohydrate complexes provides strong supporting evidence, in addition to the value of $-\Delta H°$, for the complementarity of the Galβ1 → 3GalNAc structure and the PNA combining site. For MeαGal., MeβGal and MeβGalβ1 → 4Glc, these k_{-1} values are 27 to 29 s^{-1} (Neurohr, 1982a, b). However, for the disaccharide derivative MeUmbβGalβ1 → 3GalNAc the dissociation rate is 0·24 s^{-1}, that is at least 100 times less. This is very suggestive of site-ligand complementarity. In addition, the binding mechanism proposed from stopped-flow studies (Loontiens, 1983) is a simple one-step process without any indication of multiple possibilities for the ligand to attach to the protein binding site. The kinetic process most likely corresponds to the major carbohydrate-binding process since the kinetically determined K value for MeUmbβGalβ1 → 3GalNAc ($2{\cdot}9 \times 10^4$ M^{-1} at 25°C) agrees with the value determined by equilibrium measurements for the unsubstituted Galβ1 → 3GalNAc ($K = 2{\cdot}8 \times 10^4$ M^{-1} at 25°C). This suggests that it is the Galβ1 → 3GalNAc structure itself rather than the MeUmb group that causes the 100 fold decrease in dissociation rate.

Binding of simple carbohydrates to soybean agglutinin (De Boeck *et al.*, 1984a)

Soybean agglutinin (SBA), the tetrameric lectin from seeds of *Glycine max* was shown by equilibrium dialysis with MeUmbβGalNAc to be tetravalent and not divalent as was reported originally with *N*-acetylgalactosamine as a ligand (Lotan *et al.*, 1974). This discrepancy is unclear since we have shown by competitive equilibrium dialysis that *N*-acetylgalactosamine totally displaces MeUmbβGalNAc from its complex with SBA, consistent with a simple displacement mechanism.

SBA binds MeαGalNAc 25 times better than Meα*Gal*. This binding preference, known from inhibition of hemagglutination involves a supplementary interaction of

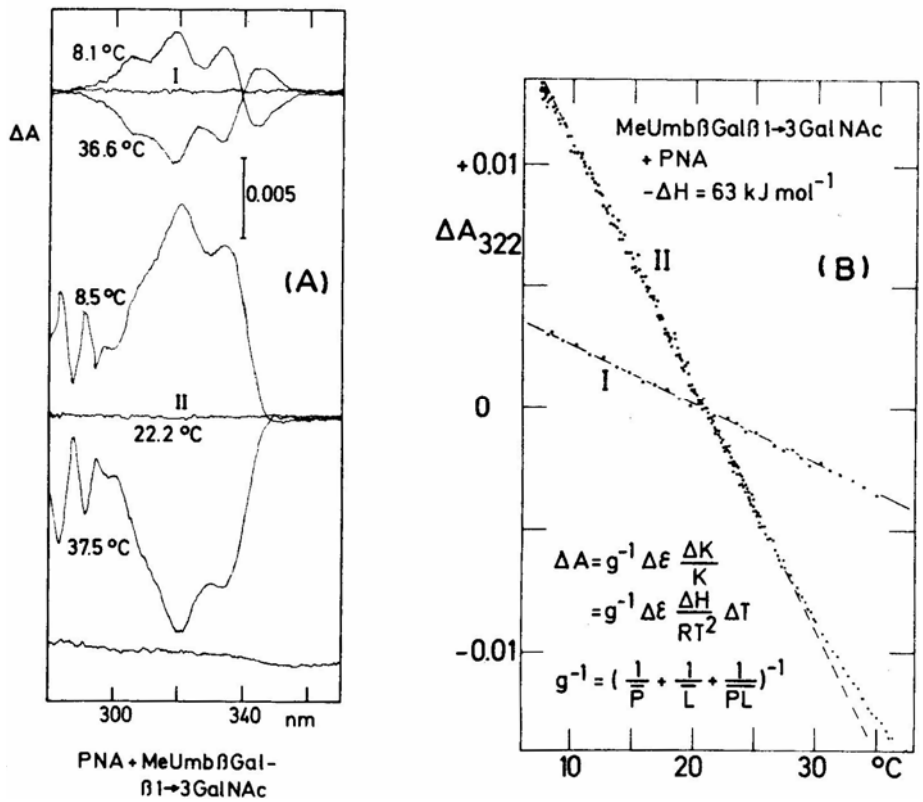

Figure 4. Determination of $\Delta H°$ for binding of MeUmbβGalβ1 → 3GalNAc to PNA by temperature-induced difference absorption spectrometry using a single mixture of ligand and lectin.

A. The sample and reference cuvette were filled with an identical solution: either 21·2 μM MeUmβGalβ1 → 3GalNAc as (I) or a mixture of 21·2 μM MeUmbβGal1 → 3GalNAc and 20·0 μM PNA (II). In each case, any spectral difference between the two chromophore containing cuvettes at 22·2°C was recorded as the baseline and was set to zero (bisecting horizontal trace). In each case, the temperature in the reference cuvette was kept constant at 22·2°C and the temperature in the sample cuvette was changed. For the ligand · PNA mixture in II, the difference spectrum obtained with the sample cuvette at 8·5°C closely resembles the conventionally obtained and positive difference spectrum for binding of MeUmbβGalβ1 → 3GalNAc with PNA, (De Boeck *et al*., 1983) which is exothermic. Here at 8·5°C, the positive difference in MeUmb absorption mainly corresponds to an increase in concentration of the MeUmbβGalβ1 → 3GalNAc·PNA complex as the temperature is lowered in the sample cuvette. The corresponding symmetrical difference spectrum for a higher temperature in the sample cuvette is also shown. The bottom trace indicates that after changing the temperature in the sample cuvette over 4 h, there is only a slight deviation in the baseline due to slightly affected protein stray light. In I, the difference spectra for ligand alone mainly result from broadening of the absorption spectrum with temperature, the difference due to the increase in volume with temperature being smaller.

B The decrease in absorption at 322 nm of 21·2 μM MeUmbβGalβ1 → 3GalNAc in the absence (I) or the presence (II) of 20·0μM PNA, measured every 0·5 to 2 min when heating the sample cuvette (maximally 0·2°/min while keeping the reference at 22·2°C, is plotted as a function of temperature. In the 16–26°C region the difference between the slopes II and I

the protein with the *N*-acetyl group as observed by a distinct change of the tryptophanyl absorption. This change is maximally 1·5 % when the lectin is titrated with ligands like *N*-acetylgalactosamine or Me*α*GalNAc. Saturating amounts of Me*α*Gal or lactose produce an absorption change at least six times smaller. This supplementary interaction by the *N*-acetyl group in Me*α*GalNAc is almost exclusively enthalpically determined; this contribution amounts to 7 kJ mol^{-1} as compared to Me*α*Gal (table 1) and could involve at least one additional hydrogen bond between the sugar and the protein. It was necessary to obtain the parameters for binding of Me*α*Gal indirectly and this was done by displacement titrations, using the change in *p*NO_2Phe*β*GalNAc absorption as the indicator reaction. The agreement of the association constant determined by different methods was excellent.

Table 1. Association constants and the thermodynamic parameters for the binding of simple carbohydrates to SBA at 25°C. The majority of the data was obtained from titrimetric procedures of protein difference absorbance, ligand difference absorbance, or ligand fluorescence. For MeUmb*β*GalNAc, the data include the results of equilibrium dialysis and stopped flow kinetics.

	$\Delta\varepsilon^a$	$K_{25°C}$	$-\Delta G°$	$-\Delta H°$	$\Delta S°$
	$M^{-1}\,cm^{-1}$	M^{-1}	$kJ\,mol^{-1}$	$kJ\,mol^{-1}$	$J.K^{-1}\,mol^{-1}$
Me*α*Gal[b]	80	$(0{\cdot}98 \pm 0{\cdot}10) \cdot 10^3$	$17{\cdot}1 \pm 0{\cdot}3$	38 ± 1	$-(69 \pm 1)$
Me*α*GalNAc[c]	435 ± 35	$(2{\cdot}43 \pm 0{\cdot}10) \cdot 10^4$	$25{\cdot}0 \pm 0{\cdot}2$	45 ± 2	$-(67 \pm 6)$
pNO_2Phe*β*GalNAc[c]	$(2{\cdot}7 \pm 0{\cdot}2) \cdot 10^3$	$(4{\cdot}93 \pm 0{\cdot}21) \cdot 10^4$	$26{\cdot}8 \pm 0.1$	50 ± 1	$-(78 \pm 4)$
MeUmb*β*GalNAc[c]	$(3{\cdot}0 \pm 0{\cdot}2) \cdot 10^3$	$(3{\cdot}24 \pm 0{\cdot}17) \cdot 10^4$	$25{\cdot}7 \pm 0{\cdot}2$	33 ± 4	$-(24 \pm 12)$
GalNDns[d]		$(5{\cdot}9 \pm 0{\cdot}4) \cdot 10^5$	$32{\cdot}9 \pm 0{\cdot}2$	15 ± 3	$+(60 \pm 10)$

[a] Δε is estimated from the SBA difference absorption spectra induced by Me*α*Gal or is calculated from titration of SBA difference absorption for Me*β*GalNAc (ΔA at291·6 nm minus at 288·8 nm) or as the average of 4 to 7 titrations with the chromophoric ligands pNO_2Phe*β*GalNAc and MeUmb*β*GalNAc.
[b] Thermodynamic parameters obtained from two substitution titrations at 5°C and 20°C with the difference absorbance of pNO_2Phe GalNAc at 315 nm as an indicator system.
[c] Thermodynamic parameters calculated from 6 or 7 values of K in the 4–25°C range.
[d] Thermodynamic parameters calculated from the results of two discontinuous titrations at 13°C and 25°C, allowing prolonged equilibration of individual mixtures.

Large aromatic *β*-aglycons such as the *p*NO_2Phe- or MeUmb groups, used here for their change in absorption to monitor binding with SBA, only marginally increase the affinity for the protein when compared to Me*α*GalNAc (a factor of 2). In contrast, a large *N*-acyl group such as the *N*-dansyl substituent in *N*-dansylgalactosamine increases the affinity by a factor of 20. Unlike the ΔS° values for the other ligands investigated, the $\Delta S°$ for binding to *N*-dansylgalactosamine to SBA is positive and

(equal to 1·0001 ±0·0006 and 0241 ± 0·001) is a simple function of $\Delta H°$. This can be defined by expressions used in temperature-jump relaxation kinetics (Jovin, 1975; Bernasconi, 1976) as indicated. $\Delta\varepsilon$ is the increase in molar extinction coefficient and equals 2·46 × 10_3 M^{-1} cm^{-1} (De Boeck *et al*., 1982) and g, equal to the sum of all inverse equilibrium concentrations was calculated with $K = 5{\cdot}6 \times 10^4$ M^{-1} (De Boeck *et al*., 1983) at 22°C. This yields=$\Delta H° = -63$ kJ mol^{-1}.

favourable. This property, together with the ten-fold increase in *N*-dansylgalactosamine fluorescence upon binding to SBA most strongly suggest that there is a hydrophobic region in the immediate vicinity of the carbohydrate-binding site.

The carbohydrate-binding kinetics of SBA were studied with the stopped-flow technique using MeUmbβGalNAc as a ligand and monitored by the change in its absorption at 334 nm. The observed pseudo-first order association rate increased linearly with excess concentration of SBA; the association-rate constant equals $1{\cdot}1 \times 10^4$ M^{-1} s^{-1}. The dissociation-rate constant, obtained either indirectly by extrapolating the first-order apparent reaction rate to zero concentration or directly by displacement kinetics, is very small ($0{\cdot}4$ s^{-1} at 25°C). This is slower than for any monosaccharide·lectin complex reported to date and it can reflect the pronounced GalNAc-binding preference of this lectin.

Binding of simple carbohydrates and N-glycoprotein-derived oligosaccharides to Erythrina cristagalli agglutinin (De Boeck *et al.*, 1984b)

The lectin from *Erythrina cristagalli* (Iglesias *et al.*, 1982; Kaladas *et al.,* 1982) preferentially binds to the Galβ1 4GlcNAc structure that frequently occurs in glyconjugates and especially in the branches of *N*-glycoproteins. The lectin is a dimeric glycoprotein with M_r = 56,000 and was shown by equilibrium dialysis to be bivalent for MeUmbβGal. Upon binding to the lectin, MeUmbβGal shows a difference absorption spectrum with two maxima (at 322 and 336 nm) of equal intensity ($\Delta\varepsilon$: $1{\cdot}2 \times 10^3$ M^{-1} cm^{-1}). A similar spectrum with a comparable value of $\Delta\varepsilon$ was obtained with MeUmbβGalNAc. A smaller protein difference spectrum is exhibited by the binding of MeαGal, lactose and *N*-acetyllactosamine which all produce similar maxima ($\Delta\varepsilon = 2{\cdot}8 \times 10^2$ M^{-1} cm^{-1}) at 291·6 nm. Although these changes allowed titrimetric evaluation on the association constant, it was easier to obtain these values from substitution titrations with *N*-dansylgalactosamine as a fluorescent indicator ligand. Upon binding of *N*-dansylgalactosamine to the lectin, there is a 5-fold increase in fluorescence intensity of this ligand. The high association constant for *N*-dansylgalactosamine (table 2) is due to a very favourable ΔS° of the dansyl group which is responsible for the 60-fold increase in affinity of *N*-dansylgalactosamine over *N*-acetylgalactosamine while retaining the strictly carbohydrate-specific character of binding. *N*-Dansylgalactosamine was used as an indicator in substitution titrations to determine the equilibrium association constants of simple carbohydrates, *N*-acetyllactosamine and *N*-acetyllactosamine-containing oligosaccharides which occur in the carbohydrate moiety of *N*-glycoproteins; the latter are Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 2Man, Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 6Man and Gaβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 6[Galβ1 $\rightarrow$ 4GlcNAcβ1 $\rightarrow$ 2]Man. These fluorescence titrations were performed at two temperatures to determine the thermodynamic parameters (table 2). In the series *N*-acetylgalactosamine, MeαGal and lactose, ΔH°-increases from 24 to 41 kJ mol^{-1} it further increases for *N*-acetylgalactosamine and then remains unchanged for the *N*-acetyllactosamine-containing oligosaccharides: 55 ± 1 kJ mol^{-1}. This indicates that the site preferentially accomodates *N*-acetyllactosamine with an important contribution of the 2-acetamido group in the penultimate sugar. Beyond, no additional

Table 2. Association constants K and thermodynamic parameters for binding of somecarbohydrates to *Erythrina cristagalli* agglutinin at 25°C.

	$10^{-4} \times K_{25°C}$ (M^{-1})	$-\Delta G°$ (kJ mol^{-1})	$-\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J mol^{-1} K^{-1})
GalNDns	7·79 ± 0·03	27·92 ± 0·01	23·8 ± 0·2	+ (14 ± 1)
GalNAc	0·125 ± 0·001	17·68 ± 0·02	24·5 ± 0·4	− (23 ± 1)
MeαGal	0·159 ± 0·001	18·27 ± 0·02	27·8 ± 0·3	− (32 ± 1)
Galβ1 → 4Glc	0·303 ± 0·001	19·87 ± 0·01	41·2 ± 0·2	− (71 ± 1)
Galβ1 → 4GlcNAc	1·73 ± 0·02	24·19 ± 0·03	54·5 ± 0·4	− (102 ± 2)
[a]TRI 6	2·27 ± 0·01	24·87 ± 0·01	53·8 ± 0·2	− (97 ± 1)
[a]TRI 2	2·86 ± 0·02	25·43 ± 0·02	57·0 ± 0·3	− (106 ± 1)
[a]PENTA 2,6	3·30 ± 0·03	25·79 ± 0·02	55·0 ± 0·4	− (98 ± 1)

[a] TRI 6: Galβ1 → 4GlcNAcβ1 → 6Man; TRI 2: Galβ1 → 4GlcNAcβ1 → 2Man; PENT*A* 2,6: Galβ1 → 4GlcNAcl → 6 [Galβ1 → 4GlcNAcβ1 → 2] Man.
All data are obtained from titrimetric evaluation of GalNDns fluorescence, either in direct titrations (GalNDns) or in substitution titrations for all other carbohydrates. All titrations were performed at two temperatures (10·7 and 22·1°C). In view of the weak binding of MeβGal., the following are tentative values: $K = 7{\cdot}2 \times 10^2$ M^{-1} at 22·1 °C; $-\Delta G° = 16{\cdot}2$ kJ mol^{-1}; $-\Delta H° = 13{\cdot}6$ kjmol^{-1} and $\Delta S° = +9$ J mol^{-1} K^{-1}. The errors for K and ΔG refer to regression of a linearized titration.

contacts are evident. This conclusion also follows from considerations of $\Delta S°$ values which reach a limiting negative value of $\Delta S°$ (– 101 ± 4J mol^{-1} K^{-1}) for *N*-acetyllactosamine and the three *N*-acetyllactosamine-containing-oligosaccharides.

The binding kinetics of *N*-dansylgalactosamine and the *Erythrina cristagalli* lectin were followed by stopped-flow kinetics and the pressure-jump relaxation technique. Upon standing of the lectin solutions at pH 7·3 there is formation of soluble aggregates and these can complicate the kinetics due to artifacts. However, at pH 4·7, and in fresh solutions at pH 7·3, where such aggregates are absent, binding occurs as a simple bimolecular association with $k_+ = 4{\cdot}8 \times 10^4$ M^{-1} s^{-1}, $k_- = 0{\cdot}4$ to 0·66 s^{-1} depending on the method used and on the experimental conditions. The change in reaction volume (+ 7 ml/mol of subunit) is small (De Boeck, H., Macgregor, R. B. Jr., Clegg, R. M., Sharon, N. and Loontiens, F. G., unpublished results) like for binding of 4-methylumbelliferyl α-D-mannopyranoside to concanavalin A (Thompson and Lakowicz, 1984).

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