

# Temperature-induced ultraviolet difference absorption spectrometry for determination of enthalpy changes

## Binding of 4-methylumbelliferyl glycosides to four lectins

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Raising the temperature in a single mixture of a lectin and a chromophoric glycoside allows determination of the binding enthalpy. This is made possible by continuously monitoring the displacement of the complex from its equilibrium concentration with a sensitive difference absorption spectrophotometer. The method is illustrated with the following lectins: concanavalin A, soybean agglutinin, peanut agglutinin and *Erythrina cristagalli* agglutinin. The ligands are 4-methylumbelliferyl glycosides. The binding enthalpies found range from  $-60 \text{ kJ} \cdot \text{mol}^{-1}$  for the Gal $\beta$ 1 $\rightarrow$ 3GalNAc- $\beta$ glycoside and peanut agglutinin to  $-30 \text{ kJ} \cdot \text{mol}^{-1}$  for a monosaccharide glycoside and the other lectins.

Lectin      Carbohydrate      Binding enthalpy      Difference absorption spectrometry

### 1. INTRODUCTION

In studies on the carbohydrate-binding specificity and on the restricted or extended nature of the combining sites of lectins such as Con A and SBA or PNA and ECA, current interest includes the slow dissociation rate [1–3] of carbohydrate-lectin complexes as well as the change in enthalpy,  $\Delta H$ , due to binding [4–8]. Direct determination of  $\Delta H$  by calorimetry has been performed for the binding of a simple carbohydrate to Con A [9]. Usually, however,  $\Delta H$  is calculated indirectly from the values of the association constants at several temperatures or, rarely, from the differences in activation enthalpies [1,5] or from amplitude analysis of temperature-jump relaxations [10–12]. Here, we take advantage of the highly sensitive double beam spectrophotometers that are available nowadays and suggest a very easy method. It ap-

plies the principle of temperature-jump relaxation to a single, equimolar mixture of lectin sites and a chromophoric ligand, that is continuously shifted from its chemical equilibrium by a gradually changing temperature. The displacement is continuously followed as the change in concentration of the complex measured by UV difference absorption spectrometry. A blank experiment, with the same concentration of chromophore alone, affords a combined correction for broadening of the ligand spectrum and for volume expansion with temperature. As such, a reliable value of  $\Delta H$  is obtained, independent of the sign of the difference spectra for complex formation. The method works well with MeUmb glycosides and the 4 lectins tested, Con A, SBA, PNA and ECA.

### 2. MATERIALS AND METHODS

Con A was prepared according to [13]; SBA, purified by affinity chromatography on acid-treated Sepharose [14], PNA purified according to [15] and ECA purified according to [16] were gifts

*Abbreviations:* Con A, concanavalin A; ECA, *Erythrina cristagalli* agglutinin; PNA, peanut agglutinin; SBA, soybean agglutinin; MeUmb-, 4-methylumbelliferyl-; all sugars mentioned are of the D-pyranoside configuration

from Halina Lis and Nathan Sharon (Rehovot, Israel). MeUmb $\beta$ GalNAc was obtained from Koch-Light, MeUmb $\alpha$ Man was prepared as indicated [17] and MeUmb $\beta$ Gal $\beta$ 1  $\rightarrow$  3GalNAc was a gift from Khushi L. Matta (Buffalo, NY).

Experimental details are the same as in previous work and are referenced in the legend to table 1.

A Uvikon 810 double beam spectrophotometer was used with a 2-nm spectral band width. It contained two independently thermostatted cuvette holders connected to two kryostats. Differences in UV absorption were induced by changing the temperature in the sample cuvette, equipped with a temperature probe, while keeping the temperature of the reference cuvette constant. Both cuvettes were filled with the same solution containing the chromophoric ligand and an equimolar concentration of lectin sites. In the blank series, the lectin was omitted. For mixtures of MeUmbglycosides and lectins like PNA [3] or SBA [7], with slow binding kinetics, the maximal heating rate was 0.2°C/min. With the other lectins or for a ligand alone, the rate did not exceed 0.4°C/min. These rates were obtained by adjusting the intensity of the heating current with a fixed maximal temperature. 5-digit UV difference absorbance readings were obtained by integrating the undamped signal, for 100–40% transmittance of incident light, over a period of 5–10 s. Outprints at intervals of 0.5–2 min contained absorbance at 322 nm and, as a control, at 370 nm, together with the temperature in the sample cell. In order to obtain statistically significant absorbance differences as a function of temperature, up to 100 data points were collected over a temperature range of 20°C. Any shift of the baseline over a 2–4-h period was negligible ( $\pm 0.0001$  A at 370 nm) and a prolonged change in temperature never deformed the baseline at wavelengths longer than 300 nm, even if protein was present in the samples.

### 3. RESULTS AND DISCUSSION

The absorption spectra of a MeUmbglycoside as such and bound to a suitable lectin show a different temperature dependence; these differences are small but are characteristic and informative. Two examples of temperature-induced difference absorption spectra obtained with a single mixture

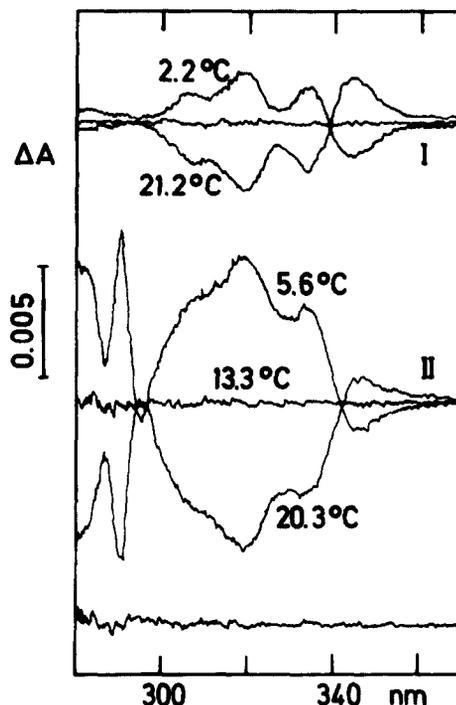


Fig. 1. Temperature-induced difference absorption spectra for identical solutions in the sample and reference cuvettes: either (I) 25  $\mu$ M MeUmb $\beta$ GalNAc or (II) a mixture of 25  $\mu$ M MeUmb $\beta$ GalNAc and 25.5  $\mu$ M SBA. In I and II, the horizontal traces are the baselines for equal temperatures (10.6°C in I and 13.3°C in II) in both cuvettes. In each case, the temperature in the reference cuvette was kept constant and a first difference spectrum was recorded after decreasing the temperature to a constant value in the sample cuvette. Then in this cuvette the temperature was slowly increased, while recording the decrease in absorbance at 322 nm as in fig. 2, and a symmetrical difference spectrum was recorded at the highest temperature. Finally, the temperature of both cuvettes was set equal again to verify that even in the presence of protein the baseline (bottom trace, for II) was virtually unchanged after 2–4 h.

of SBA and MeUmb $\beta$ GalNAc are given in fig. 1 (II). The more intense spectrum, with a maximum near 320 nm, closely resembles the conventionally obtained and positive difference absorption spectrum for binding of MeUmb $\beta$ GalNAc to SBA, that is, exothermic[7]. Here, the positive difference in 4-methylumbelliferyl absorption results mainly from a shift in the chemical equilibrium towards a higher concentration of the MeUmb $\beta$ GalNAc·

SBA complex in the sample cuvette with a temperature that is lower than in the reference cuvette. Reversing this temperature difference, as in fig. 1, reverses this shift as well as the sign of the difference spectrum. 3 additional factors contribute to the difference spectra in fig. 1 (II): (i) small temperature-induced differences, given as the spectra in part I of fig. 1 and mainly due to broadening of the absorption spectrum of MeUmb $\beta$ GalNAc itself; (ii) a similar but intense effect on protein absorption in the 280–295 nm region (iii) a very small difference due to a shift in the carbohydrate-induced protein difference absorption [7]. For binding of MeUmbglycosides by Con A [17], PNA [21], SBA [7] and ECA [8] the difference in

MeUmb absorption is maximal at 321–322 nm.

The temperature-induced difference in MeUmbglycoside absorption at 322 nm, in absence or presence of an appropriate lectin, changes linearly over a limited range of 10–20°C. This was observed with the 4 lectins and 3 MeUmbglycosides in table 1 and is shown for MeUmb $\beta$ GalNAc and SBA in fig. 2. With the concentrations used here, all observed absorption differences are small (maximally 0.02 for a 20°C difference with a mixture of 21  $\mu$ M MeUmb $\beta$ Gal $\beta$ 1  $\rightarrow$  3GalNAc and 20  $\mu$ M PNA) and are measured as a function of temperature with a regression error of only 0.3–1%. The difference in the slopes  $\Delta A/\Delta T$ , for the linear plots II and I in fig. 2 with identical concentrations

Table 1  
Determination of  $\Delta H$  values for binding of carbohydrates to four lectins

	Conc. ( $\mu$ M)	$10^3 \times \Delta A_{322}/\Delta T$	$-\Delta H$ (kJ $\cdot$ mol $^{-1}$ )		
			Experimental	Literature	
<sup>a</sup> SBA	25.5	–0.58	31 $\pm$ 2	33 $\pm$ 4	[7]
MeUmb $\beta$ GalNAc	25	$\pm$ 0.01			
<sup>b</sup> Con A	32	+0.694	38 <sup>c</sup>	34.7 $\pm$ 0.4	[17]
MeUmb $\alpha$ Man	31	$\pm$ 0.001			
<sup>c</sup> PNA	20	–0.760	63 <sup>c</sup>	58 $\pm$ 2	[21]
MeUmb $\beta$ Gal $\beta$ 1 $\rightarrow$ 3GalNAc	21	$\pm$ 0.007			
<sup>d</sup> ECA	26	–0.284	31 <sup>c</sup>		
MeUmb $\beta$ GalNAc	27	$\pm$ 0.004			

In most cases the temperature range was 20°C except for the mixture of SBA and MeUmb $\beta$ GalNAc where it was 12°C. The error of  $\Delta A/\Delta T$  is for the difference between the two regression lines (as in fig. 2) obtained with the chromophoric ligand in the presence and absence of lectin. The small error for Con A or PNA is due to a large number of data points (100) in the regression

<sup>a</sup>  $M_r$  of SBA protomer = 30 000 [23],  $\epsilon_{280} = 1.28$  mg $^{-1}$   $\cdot$  cm $^2$  [23], 0.025 Na-K phosphate, 1 M NaCl (pH 6.9);  $\Delta\epsilon_{321} = (3.0 \pm 0.2) \times 10^3$  M $^{-1}$   $\cdot$  cm $^{-1}$  and  $K = 5.7 \times 10^4$  M $^{-1}$  at 13°C [7] in the reference cuvette

<sup>b</sup>  $M_r = 25$  500 [24],  $\epsilon_{280} = 1.14$  mg $^{-1}$   $\cdot$  cm $^2$  [17], 0.05 M NaOAc-HOAc, 1 M NaCl, 1 mM NiCl $_2$ , 1 mM CaCl $_2$  (pH 5.5);  $\Delta\epsilon_{322} = -2.37 \times 10^3$  M $^{-1}$   $\cdot$  cm $^{-1}$  and  $K = 4.3 \times 10^4$  M $^{-1}$  at 20°C [17] in the reference cuvette

<sup>c</sup>  $M_r = 27$  500 [25],  $\epsilon_{280} = 0.96$  mg $^{-1}$   $\cdot$  cm $^2$  [25]; 0.05 M Hepes;  $\Delta\epsilon_{322} = 2.46 \times 10^3$  M $^{-1}$   $\cdot$  cm $^{-1}$  [22] used here ( $2.2 \times 10^3$  M $^{-1}$   $\cdot$  cm $^{-1}$  according to [21]) together with  $K = 5.6 \times 10^4$  M $^{-1}$  at 22°C [21] in the reference cuvette

<sup>d</sup>  $M_r = 28$  000 [16],  $\epsilon_{280} = 1.53$  mg $^{-1}$   $\cdot$  cm $^2$  N. Sharon, personal communication; 0.05 M Na-K phosphate, 0.15 M NaCl (pH 7.3);  $\Delta\epsilon_{322} \times 10^3$  M $^{-1}$   $\cdot$  cm $^{-1}$  and  $K = 4.4 \times 10^4$  M $^{-1}$  at 5°C (H. De Boeck, personal communication) with the reference cuvette at 20°C

<sup>e</sup> These values are somewhat too large due to underestimated values of  $\Delta\epsilon$ , used in eq. (4), formerly determined as referenced with less sensitive spectrophotometric equipment and a larger spectral band width (3.3 nm instead of 2 nm here)

of MeUmb $\beta$ GalNAc in the presence or absence of an equimolar concentration of SBA, is proportional to a temperature-induced decrease in concentration of the complex PL, between protein  $P$  and ligand  $L$ , showing a change in molar extinction coefficient with respect to  $L$ . This extent of displacement from equilibrium contains information about the molar enthalpy change  $\Delta H$  [18–20] and for a small displacement it can be expressed with the amplitude factor  $\Gamma$  [18] and the relative change in association constant  $K$

$$\Delta PL = \frac{\Delta A}{\Delta \varepsilon} = \Gamma \frac{\Delta K}{K} = \Gamma \frac{\Delta H}{RT^2} \Delta T \quad (1)$$

using the absolute temperature  $T$ . The factor is

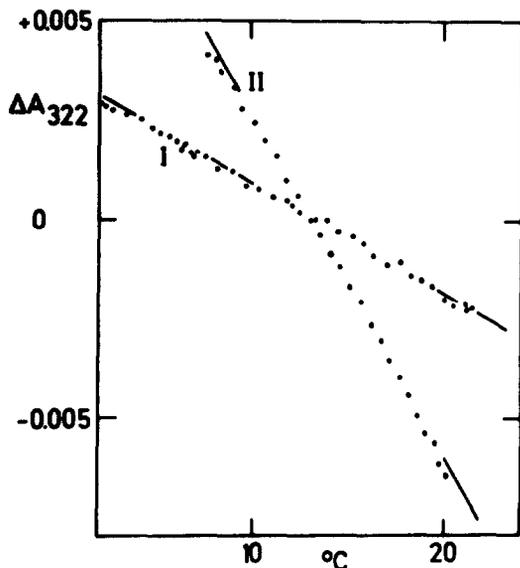


Fig. 2. Determination of  $\Delta H$  for MeUmb $\beta$ GalNAc and SBA. The decrease in absorption at 322 nm for (I) 25  $\mu$ M MeUmb $\beta$ GalNAc and (II) a mixture of 25  $\mu$ M MeUmb $\beta$ GalNAc and 25.5  $\mu$ M SBA were obtained by heating the sample cuvette (0.2°C/min) while keeping the reference cuvette at 13.2°C. The slopes, in  $10^3 \times \Delta A / \Delta T$ , are (I)  $-0.273 \pm 0.002$  and (II)  $-0.856 \pm 0.008$  with the difference  $-0.583 \times 10^{-3} = (\Gamma \cdot \Delta \varepsilon \cdot \Delta H) / (RT^2)$ . The required values  $\Gamma = 4.34 \mu$ M and  $\Delta \varepsilon = (3.0 \pm 0.2) 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$  were obtained from a continuous titration of the difference in absorption yielding  $K = 5.7 \times 10^4 \text{ M}^{-1}$  at 13°C [7] and from eqs (2) and (3) with  $PL = 0.5 \{ (P_0 + L_0 + K^{-1}) - [(P_0 + L_0 + K^{-1})^2 - 4L_0P_0]^{0.5} \}$ . This yields  $\Delta H = -(30.5 \pm 2) \text{ kJ} \cdot \text{mol}^{-1}$ .

concentration dependent and is characteristic for each mechanism [18–20]. Here it is given by

$$\Gamma = (1/P + 1/L + 1/PL)^{-1} \quad (2)$$

with concentrations at equilibrium that are calculated from the analytical concentrations  $P_0$  and  $L_0$  using the known value of  $K$  (see legend to fig. 2), defined for a simple bimolecular association  $P + L \rightleftharpoons PL$  with

$$K = \frac{PL}{(P_0 - PL)(L_0 - PL)} = \frac{PL}{P \cdot L} \quad (3)$$

Whenever investigated for the systems under study such as Con A [10], PNA [3] and SBA [7], the binding of the corresponding MeUmbglycoside has been found to be simple, so that eqs (1–3) apply.

The data for binding of MeUmb $\beta$ GalNAc and SBA in fig. 2 are calculated from eqs (1) and (2), rearranged into

$$\Delta H = \frac{\Delta A}{\Delta T} \cdot \frac{RT^2}{\Gamma \Delta \varepsilon} \quad (4)$$

and yield  $-\Delta H = 30.5 \pm 2 \text{ kJ} \cdot \text{mol}^{-1}$ , in perfect agreement with  $33 \pm 4 \text{ kJ} \cdot \text{mol}^{-1}$  obtained from a van 't Hoff plot in the 5–25°C range [7]. The present value of  $\Delta H$  is reliable, since the value of  $\Delta \varepsilon = (3.0 \pm 0.2) \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$  used in eq. (4) was obtained [7] under identical spectrophotometric conditions. The values of  $\Delta H$  for MeUmb $\alpha$ Man and Con A and for MeUmb $\beta$ Gal $\beta$ 1  $\rightarrow$  3GalNAc and PNA obtained here are also in fair agreement with those determined previously (table 1), although here the  $-\Delta H$  values tend to be somewhat higher; the probable reason for this is too low a value of  $\Delta \varepsilon$  previously determined for Con A [17], PNA [21] and also ECA [8] with a larger spectral band width (3.3 nm) required on a less sensitive instrument. With ECA,  $-\Delta H$  for binding of MeUmb $\beta$ GalNAc calculated here as  $31 \text{ kJ} \cdot \text{mol}^{-1}$  is comparable to the value  $24.5 \text{ kJ} \cdot \text{mol}^{-1}$  for binding of GalNAc, indicating that the MeUmb group contributes much less to the  $-\Delta H$  than does an additional monosaccharide like in Gal $\beta$ 1  $\rightarrow$  4Glc ( $41 \text{ kJ} \cdot \text{mol}^{-1}$ ) or in the preferred ligand Gal $\beta$ 1  $\rightarrow$  4GlcNAc ( $54 \text{ kJ} \cdot \text{mol}^{-1}$ ) [8].

## 4. CONCLUDING REMARKS

We suggest a simple determination of all thermodynamic binding parameters by (i) a single continuous titration [22] of ligand absorption difference, preferably with the nonchromophoric component (lectin) as the titrant to determine  $\Delta\epsilon$ ,  $K$  and  $\Delta G$  at e.g., 25°C; (ii) temperature-induced difference absorption spectrometry to determine  $\Delta H$ , as described here on a single mixture with a necessary blank of the chromophoric ligand to correct for its spectral broadening and for any volume changes.

According to eq. (4), the following factors are advantageous for the application of this method: a sensitive double beam spectrophotometer, an appreciable binding enthalpy, a large change in extinction coefficient due to formation of the complex. Additional factors are comparable concentrations of the reactants and a large association constant. With respect to the latter two requirements, it can be shown [20] that for a simple binding process, the normalized expression for  $\Gamma$  is

$$\Gamma K = 0.5 \left[ \frac{1}{\sqrt{1 - \frac{4P_0L_0}{(P_0 + L_0 + K^{-1})^2}}} - 1 \right] \quad (5)$$

Considering the ratio of the reaction partners  $Q = P_0/L_0$  and the ratio of their sum with respect to the dissociation constant,  $S = (P_0 + L_0)/K^{-1}$ , then according to [19], expression (5) is rewritten as

$$\Gamma K = 0.5 \left\{ \left[ 1 - 4 \left( \frac{S}{1+S} \right)^2 \frac{Q}{(1+Q)^2} \right]^{-0.5} - 1 \right\} \quad (6)$$

and shows the following properties: for any value of  $S$ , the displacement from equilibrium is maximal for  $Q=1$  and for any value of  $P_0$  it is maximal for  $L_0 = P_0 + K^{-1}$ .

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