Physicochemical studies of binding of 4-methylumbelliferyl β -D-galactopyranoside to cold agglutinin

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The fluorescence of 4-methylumbelliferyl β -D-galactopyranoside (MeUmbGalp) was quenched in the presence of cold agglutinin, showing that there was binding between MeUmbGalp and cold agglutinin. That binding was saccharide-specific. By using this quenching phenomenon, the association constants (K_a) of the binding of cold agglutinin at different temperatures (10 °C and 15 °C) to MeUmbGalp and also the number of binding sites were calculated. The K_a values were found to be $2.63 \times 10^3 \text{ M}^{-1}$ at 10 °C and $1.58 \times 10^3 \text{ M}^{-1}$ at 15 °C. Though there is a change in K_a values, the number of binding sites was calculated to be six at both temperatures (10 °C and 15 °C). From the K_a values the thermodynamic parameters (free energy, enthalpy and entropy) of the binding were derived, and analysis of the data indicated that the binding is spontaneous, exothermic and hydrophobic in nature.

INTRODUCTION

Gastropods are known to be a rich source of agglutinins, and these agglutinins have been primarily found in the albumin gland of snails (Gold & Balding, 1975). More than 100 species of snails have been shown to have agglutinin in this gland. Among them, one of the most widely studied agglutinins is the *Helix pomatia* albumin-gland lectin, which preferentially agglutinates human A erythrocytes (Hammarstrom & Kabat, 1969). The albumin gland of *Achatina fulica* snails was initially reported to be devoid of any agglutinin (Okotore *et al.*, 1981). This inconsistency was later resolved by us (Sarkar *et al.*, 1984), when we showed the presence of a unique cold agglutinin in the albumin gland of the snail *Achatina fulica*. The cold agglutinin was found to be a glycoprotein of native M_r 220000, it agglutinated rabbit and human umbilical-cord erythrocytes only at low temperatures (optimum 13 °C) and *N*-acetyl-lactosamine was found to be a specific inhibitor (Sarkar *et al.*, 1984; Mitra *et al.*, 1987). The carbohydrate part of the glycoprotein was found to be important for cold agglutinating behaviour (Sarkar *et al.*, 1987). The present paper describes the binding characteristics of the cold agglutinin to its specific sugar by fluorescence spectroscopy with 4-methylumbelliferyl β -D-galactopyranoside (MeUmbGalp).

MATERIALS AND METHODS

Materials

MeUmbGalp was from Sigma Chemical Co. The fluorescent sugar solution was free of 7-hydroxy-4-methylcoumarin. The MeUmbGalp concentrations were determined at 318 nm by using ϵ $1.36 \times 10^{-4} \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Loontjens *et al.*, 1977).

The cold agglutinin was prepared from the albumin gland of *Achatina fulica* snails purchased from a local market according to the procedure described previously (Mitra *et al.*, 1987). The concentration of the agglutinin was determined by the method of Lowry *et al.* (1951),

with crystalline bovine serum albumin (globulin-free; Sigma Chemical Co. lot 61F-9335) as a standard. The absorbance of the agglutinin solution ($A_{1\text{cm}}^{1\%}$) at 280 nm was measured and was found to be 13.4. This value was used as a convenient method for estimating protein concentration.

Fluorescence titrations

The binding of MeUmbGalp to cold agglutinin was monitored by fluorescence quenching in a Perkin–Elmer MPF-44B spectrofluorimeter. The excitations were done at 318 nm and the emission spectra were recorded above 330 nm. A fixed amount of MeUmbGalp solution ($3.39 \mu\text{M}$) was mixed with different amounts of $1.8 \mu\text{M}$ cold agglutinin at 10 °C and 15 °C and the fluorescence spectra were recorded. In addition, a fixed concentration of cold agglutinin ($0.9 \mu\text{M}$) was mixed with various concentrations of MeUmbGalp (0.3 – $1.5 \mu\text{M}$) at 10 °C and 15 °C and the quenching of MeUmbGalp fluorescence was recorded. A linear standard curve for MeUmbGalp in buffer only was obtained in the concentration range 0.3 – $1.5 \mu\text{M}$ at 10 °C and 15 °C. The relative fluorescence in the presence and in the absence of cold agglutinin gave the amounts of bound and free glycoside respectively, and the data were analysed according to the method of Scatchard (1949).

RESULTS

The fluorescence of the MeUmbGalp was quenched (Fig. 2a) on the addition of cold agglutinin. When the same experiment was done with 4-methylumbelliferyl β -D-glucopyranoside no quenching was observed (results not shown), which excludes the possibility of a contribution of the 4-methylumbelliferyl moiety to the binding site. The fluorescence quenching of MeUmbGalp was reversed by the addition of lactose (results not shown), which confirmed that the binding was solely due to the presence of sugar moiety.

The fluorescence quantum yield of MeUmbGalp bound

Abbreviation used: MeUmbGalp, 4-methylumbelliferyl β -D-galactopyranoside.

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to cold agglutinin was obtained graphically by the relationship:

$$F_0/(F_0 - F) = q/(q - q')K_a[P] + q/(q - q')$$

where F_0 and F are the measured fluorescence of sugar alone and in the presence of the lectin at concentration

Fig. 1. Plot of the fluorescence intensity changes $F_0/(F_0 - F)$ of MeUmbGalp upon addition of cold agglutinin at 10 °C

Portions of a solution of cold agglutinin ($1.8 \mu\text{M}$) were added to 1 ml of a solution of MeUmbGalp ($3.39 \mu\text{M}$). After volume correction, the data were plotted. The experiment was carried out in phosphate-buffered saline (10 mM-sodium phosphate buffer, pH 7.2, containing 150 mM-NaCl).

[P] respectively, and q and q' are the quantum yields of MeUmbGalp and its complex with the lectin (Dean & Homer, 1973). The quantum yield of MeUmbGalp bound to the agglutinin was obtained by extrapolating a plot of $F_0/(F_0 - F)$ versus $1/[P]$ to $1/[P] = 0$. The plot gave a straight line with an intercept on the ordinate $F_0/(F_0 - F) = 0$, demonstrating that the fluorescence quantum yield of MeUmbGalp bound to cold agglutinin was zero (Fig. 1), and this indicates that the fluorescence quantum yield could be used as a measure of ligand binding to cold agglutinin in titration.

From the quenching of MeUmbGalp fluorescence by various concentrations of cold agglutinin (Fig. 2a), the value of the association constant K_a for the agglutinin binding to MeUmbGalp was calculated according to the relationship (Chipman *et al.*, 1967):

$$\log \left(\frac{F_0 - F}{F - F_\infty} \right) = \log K + \log \left[[P]_t - [M]_t \cdot \left(\frac{F_0 - F}{F_0} \right) \right]$$

in which F_0 , F and F_∞ are the values of the fluorescence maximum in the absence of protein, in the presence of protein and at infinite protein concentration respectively. F_∞ was obtained from the intercept on the ordinate of a plot of $1/F$ versus $1/[P]$. $[P]_t$ is the concentration of cold agglutinin added and $[M]_t$ is the concentration of MeUmbGalp added. The term $\log \{ [P]_t - [M]_t \cdot [(F_0 - F)/F_0] \}$ represents the free protein concentration, abbreviated in the plot to $\log [P]_f$. A plot of $\log [(F_0 - F)/(F - F_\infty)]$ against $\log [P]_f$ assuming 1:1 stoichiometry for the binding with a protein of 220000 M_r gave a straight line, as represented in Fig. 2(b). The value of the association constant obtained from the intercept on the abscissa was $K_a = 2.63 \times 10^3 \text{ M}^{-1}$ at

Fig. 2. Quenching of the fluorescence spectra of MeUmbGalp by cold agglutinin

(a) Experiments were carried out in phosphate-buffered saline at 10 °C. The fluorescence spectra were recorded in the absence (spectrum a) and after addition of several portions of $1.8 \mu\text{M}$ cold agglutinin to 1 ml of $3.39 \mu\text{M}$ -MeUmbGalp: spectrum b, $25 \mu\text{l}$; spectrum c, $50 \mu\text{l}$; spectrum d, $100 \mu\text{l}$; spectrum e, $200 \mu\text{l}$; spectrum f, $500 \mu\text{l}$. After each addition of the agglutinin, part of the spectrum was recorded and the total spectrum is given for the final addition. (b) After correction for dilution, the value of the association constant was determined graphically.

Fig. 3. Scatchard plots for the binding of MeUmbGalp to cold agglutinin from the data obtained by fluorescence titration at 10 °C and at 15 °C in phosphate-buffered saline

A constant concentration of the cold agglutinin (0.9 μM) was mixed with various concentrations of the glycoside (0.3–1.5 μM). Experiments were carried out at 10 °C and at 15 °C. r is the ratio of mol of MeUmbGalp bound per mol of cold agglutinin, and c is the concentration of free MeUmbGalp.

Table 1. Association constants and the thermodynamic parameters for the binding of MeUmbGalp to cold agglutinin

Temperature (°C)	K_a (M^{-1})	ΔF ($\text{kJ} \cdot \text{mol}^{-1}$)	ΔH ($\text{kJ} \cdot \text{mol}^{-1}$)	ΔS_u ($\text{kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$)
10	2.63×10^3	-18.5		-121.4
15	1.58×10^3	-17.6	-52.3	-122.0
25*	54.5	-9.9		-142.6

* Back-calculated from ΔH value.

10 °C. The same experiment was repeated at 15 °C and the K_a was then found to be $1.58 \times 10^3 \text{ M}^{-1}$.

The Scatchard plots obtained from the results of the quenching of the fluorescence of various concentrations of MeUmbGalp at a fixed concentration of the cold agglutinin at 10 °C and at 15 °C are shown in Fig. 3. The number of binding sites obtained at both the temperatures was the same, six per molecule. The association constants obtained from these plots were $2.5 \times 10^3 \text{ M}^{-1}$ at 10 °C and $1.5 \times 10^3 \text{ M}^{-1}$ at 15 °C, which are close to the values obtained from the fluorescence-quenching experiments. It should be noted that the K_a decreased with increase in temperature. From the K_a values from the fluorescence-quenching experiments for MeUmbGalp binding to the agglutinin the free energy (ΔF), enthalpy (ΔH) and entropy (ΔS) values of this interaction were calculated according to eqns. (1), (2) and (3) respectively. These values are summarized in Table 1. The free energy (ΔF) of association follows from the relationship:

$$\Delta F = -RT \cdot \ln K_a \quad (1)$$

where R is the gas constant and T the absolute temperature. The enthalpy (ΔH) of the association can be calculated by measuring K_a at two temperatures according to the van't Hoff equation:

$$\Delta H = \frac{4.576(\log K_2 - \log K_1)}{\frac{1}{T_1} - \frac{1}{T_2}} \quad (2)$$

where K_1 and K_2 are values for the association constant of the binding at two different temperatures T_1 and T_2 respectively. The entropy (ΔS) can be computed from the equation:

$$\Delta F = \Delta H - T \cdot \Delta S \quad (3)$$

Entropy values were converted into the unitary entropy change by the equation $\Delta S_u = \Delta S + 7.98$ (Kauzmann, 1959).

DISCUSSION

4-Methylumbelliferyl sugars have been found to be useful ligands for the study of lectin–sugar interactions. The fluorescence of MeUmbGalp is totally quenched for wheat-germ agglutinin (Privat *et al.*, 1974; Van Landschoot *et al.*, 1977) or partially quenched for concanavalin A (Dean & Homer, 1973; Loontjens *et al.*, 1977), or enhancement of ligand fluorescence may occur (Delmotte *et al.*, 1975). The fluorescence quantum yield of MeUmbGalp bound to the agglutinin is zero. Thus it is possible to measure the K_a values and number of binding sites at different temperatures by mixing a fixed concentration of agglutinin with various concentrations of MeUmbGalp. That the fluorescence quenching of MeUmbGalp bound to the agglutinin is solely due to saccharide-specific binding to the agglutinin was further substantiated by the absence of any quenching effect when 4-methylumbelliferyl β -D-glucopyranoside is added to cold agglutinin. The K_a values for the binding of cold agglutinin to MeUmbGalp were found to be $2.63 \times 10^3 \text{ M}^{-1}$ at 10 °C and $1.58 \times 10^3 \text{ M}^{-1}$ at 15 °C. No binding was observed at 25 °C. The number of binding sites was calculated to be six per molecule.

From the thermodynamic values the free energy of the binding is negative at both temperatures, indicating the spontaneous binding of the MeUmbGalp to cold agglutinin. The negative enthalpy value indicates that the binding of MeUmbGalp to cold agglutinin is exothermic in nature, and hence it is expected that a decrease in temperature will strengthen the binding. The significance in the negative entropy change as shown in Table 1 can be understood from the following considerations. When a ligand binds to a protein, usually the process of binding occurs in two steps: firstly the removal of ligand from the solution and secondly the ‘attachment’ of this ligand to the protein. The total change in the entropy (ΔS_u) of the bulk system during the process of binding can be separated into three components:

$$\Delta S_u = \Delta S_L + \Delta S_{WL} + \Delta S_{WP}$$

ΔS_L is the contribution of the ligand molecule to the entropy change, and ΔS_{WL} and ΔS_{WP} are the entropy changes due to stripping of water molecules from the ligand and from the protein molecule respectively. As a ligand molecule is removed from the solution and gets attached to the protein, its movement becomes more

restricted than that of one in solution. In other words, the process of binding decreases the 'randomness' of the motion of a ligand molecule, resulting in a decrease in the entropy of the system. Hence ΔS_L must have a negative value. But this negative entropy effect may be neutralized by the positive values of ΔS_{WL} and ΔS_{WP} during the process of binding. When a water molecule that was initially attached to a ligand or a protein molecule gets stripped off and goes back to the bulk solution, it becomes more mobile, and this enhancement in mobility causes an increase in the entropy of the system, which is reflected in the positive values of both ΔS_{WL} and ΔS_{WP} . The sign of the net change in entropy (ΔS_u), therefore, depends on the strength of the binding. For a weak binding process, not all the binding sites get saturated, and there may remain some residual water molecules around the protein even after the binding takes place. In such cases ΔS_{WP} will not attain a sufficiently high positive value as to cancel the negative effect of ΔS_L , and as a result ΔS_u will have a negative value. The stronger is the binding, the more and more water molecules will get stripped off the protein and ligand molecule, and their return to the solution will raise the ΔS_{WP} and ΔS_{WL} values. As soon as $|\Delta S_{WP}| + |\Delta S_{WL}| > |\Delta S_L|$, ΔS_u becomes positive. When all the binding sites are saturated, ΔS_{WP} and hence ΔS_u reach maxima.

In the case of the binding of the MeUmbGalp to cold agglutinin, ΔS_u is negative, which means that ΔS_{WP} and ΔS_{WL} are not sufficiently high, or, in other words, $|\Delta S_{WP}| + |\Delta S_{WL}| < |\Delta S_L|$. This indicates that the binding is not strong enough and the effect of immobilization of the MeUmbGalp is dominant over the process of binding of MeUmbGalp to cold agglutinin.

The increase of the negative entropy effect with increase in temperature suggests that the binding becomes weaker at higher temperature. As mentioned above, it was expected from the exothermic nature of the binding process. This it can be concluded that the overall binding of the protein is low, in which it resembles cold agglutinin of human origin, having low K_a values $< 10^3$ (Tsai *et al.*, 1976), and also the binding constant decreases to zero with increase in temperature.

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