Interaction of rice (*Oryza sativa*) lectin with *N*-acetylglucosaminides

Fluorescence studies

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The interaction of lectin isolated from rice (*Oryza sativa*) embryos with *N*-acetylglucosaminides was studied by equilibrium dialysis and fluorescence. Equilibrium dialysis with 4-methylumbelliferyl-(GlcNac)₂ showed that rice lectin (M_r 38000) contains four equivalent saccharide-binding sites. Addition of the *N*-acetylglucosaminides GlcNac, (GlcNac)₂ and (GlcNac)₃ enhanced the intrinsic fluorescence of rice lectin and this was accompanied by a 10 nm blue-shift of its maximum fluorescence with (GlcNac)₂ and (GlcNac)₃. These changes in intensity allowed determination of the association constants, which increased with the number of saccharide units: at 20°C, $K_a = (1.3 \pm 0.1) \times 10^3$, $(5.1 \pm 0.4) \times 10^4$ and $(2.6 \pm 0.1) \times 10^5 \text{ M}^{-1}$ for GlcNac, (GlcNac)₂ and (GlcNac)₃ respectively. The binding enthalpy, ΔH° , for the three glucosaminides were very low and ranged from -12.1 to $-20.6 \text{ kJ} \cdot \text{mol}^{-1}$. These results are compared with those obtained with wheat-germ agglutinin, another GlcNac-specific gramineous lectin.

Lectins specifically bind carbohydrates (Goldstein & Hayes, 1978; Lis & Sharon, 1981). As a consequence, it has been proposed that plant lectins are involved in mechanisms of recognition of, and binding to, bacteria or fungi, thus possibly participating in nitrogen fixation by plants (Dazzo, 1980; Barondes, 1981) or in their protection against pathogens (Mirelman *et al.*, 1975; Sequeira, 1978).

Among the cultivated members of the Gramineae, rice (*Oryza sativa*) plays a very important role in world nutrition. However, studies of its seed lectin are rare (Takahashi *et al.*, 1973; Tsuda, 1979; Indravathamma & Seshadri, 1980). When isolated from rice embryos, it preferentially agglutinates trypsin-treated erythrocytes (Tabary *et al.*, 1984), and *N*-acetylglucosamine (GlcNac) is a potent inhibitor of this agglutination (Tsuda, 1979).

Only one binding experiment with rice lectin has been reported (Tsuda, 1979), and no spectroscopic studies at all. Therefore, in order to specify the

physiological function of rice lectin, we thought it was interesting and important to investigate this lectin's interaction with GlcNac and its derivatives, since these saccharides are components of bacterial cell surfaces and fungal cell walls.

This work was also an attempt to compare and distinguish between the two gramineous lectins isolated from rice and wheat-germ respectively: wheat-germ lectin has been extensively studied (Loontjens *et al.*, 1983; Grivet *et al.*, 1983), and the two lectins have similar physicochemical properties, including the same specificity for glucosaminides.

Experimental

Lectin purification

Rice lectin was purified as previously described (Tabary *et al.*, 1984). The starting material was a rice-embryo defatted powder suspended in 0.05 M-HCl/0.15 M-NaCl, pH 1.3. The different purification steps were precipitation with 50%-satd. (NH₄)₂SO₄, affinity chromatography on agarose-*p*-aminophenyl- β -D-*N*-acetylglucosamine and gel filtration on Aca 54 columns. Rice lectin was shown to be homogeneous by polyacrylamide-gel electrophoresis and by immunodiffusion and immunoelectrophoresis when tested with a rabbit

Abbreviations used: GlcNac, *N*-acetylglucosamine; Me, methyl; MeUmb, methylumbelliferyl; WGA, wheat-germ agglutinin.

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antisera raised against the crude lectin fraction. The haemagglutinating activity of the purified rice lectin was verified with trypsin-treated rabbit erythrocytes: the minimum haemagglutinating dose was 50 ng/ml.

Emission spectroscopy

Emission spectra were obtained in a Jobin and Yvon model JY 3 fluorimeter coupled to a Hewlett-Packard 9815 A calculator and a 9862 A plotter. Wavelength-dependent corrections for optical and photomultiplier-tube distortions were made with rhodamine B, using the program developed for the HP 9815 A calculator by Jobin and Yvon. The fluorimeter was thermostatically maintained with a Haake F 3 K cryothermostat.

For the temperature-dependence experiment, the sample underwent continuous magnetic stirring and the temperature was directly measured in the sample cuvette (1 cm × 1 cm × 4.5 cm) with a YSI-727 probe (Yellow Spring Instruments) coupled to a Digitec 5810 thermometer.

The protein concentration was 1.3 μM in 0.01 M-sodium acetate/0.15 M-NaCl, pH 6.0.

Fluorimetric titrations

N-Acetylglucosaminides and their derivatives were purchased from Sigma (St. Louis, MO, U.S.A.). The titration experiments were performed at 4, 13, 20, 30 and 37°C (±0.1°C) in the buffer used to study the intrinsic fluorescence.

The excitation wavelength was 280 nm (2 nm bandwidth) and the increases in fluorescence intensity of the rice lectin (1.4 μM) on addition of GlcNac (0.1–30 mM) were measured at the wavelength of maximum emission 348 nm (20 nm bandwidth). Addition of (GlcNac)₂ (2.5 μM–1 mM) or (GlcNac)₃ (75 μM–200 μM) induced a shift of the maximum rice lectin fluorescence (see the Results section), consequently the effect of (GlcNac)₂ and (GlcNac)₃ on this fluorescence was observed through a Schott CS 7-51 filter (band-pass 290–410 nm). The same procedure was used for rice lectin titration with Me-α-D-GlcNac (0.1–3 mM). Other experimental conditions were as for the temperature-dependence experiment (see above).

All data were corrected for dilution and for a blank titration without lectin by using the corresponding ligand at each temperature. Results are expressed according to the equation:

$$\Delta F/[L] = -K_a(\Delta F - \Delta F_\infty)$$

In this equation, ΔF is the relative fluorescence enhancement of rice lectin for a concentration $[L]$ of free saccharide. ΔF_∞ is ΔF at infinite saccharide concentration (Yang & Hamaguchi, 1980). Free concentrations without approximation were calcu-

lated from analytical concentrations by iterations (De Boeck *et al.*, 1981).

All solutions used for fluorescence and equilibrium-dialysis experiments were passed through 0.45 μm-pore-size Millipore filters.

Equilibrium dialysis

In view of the small amount of extractable rice lectin (Tabary *et al.*, 1984) and of its weak apparent affinity for GlcNac, equilibrium dialysis was not performed with this ligand. Attempts at (GlcNac)₂ labelling by hydrazinolysis followed by re-acetylation with [³H]acetic anhydride proved unsuccessful, owing to the low chemical stability of this disaccharide. Equilibrium dialysis was therefore performed, as previously described (Zentz *et al.*, 1978), over a period of 48 h at 4°C with MeUmb-β-D-(GlcNac)₂ in two-compartment polymethacrylate cells with a total capacity of 200 μl per cell (Myer & Schellman, 1962), by using 0.01 M-sodium acetate/0.15 M-NaCl, pH 6.0. The M_r cut-off of the membrane (Spectra/Por 1; Spectrum Medical Industries, Los Angeles, CA, U.S.A.) was 6000–8000. The rice lectin concentration was 3.5 μM. The concentration of MeUmb-β-D-(GlcNac)₂ stock solutions was determined from $\epsilon = 1.24 \times 10^4$ M⁻¹·cm⁻¹ at 316 nm (Van Landschoot *et al.*, 1977), and the concentrations in the experiments ranged from 1 to 8 μM. This range was selected to avoid aspecific additional bindings between the hydrophobic moiety of the fluorescent saccharides and the rice lectin molecules. Such abnormal binding behaviour has been observed between this ligand and wheat-germ agglutinin (WGA) at high MeUmb-β-D-(GlcNac)₂/lectin ratios (F. G. Loontjens, personal communication). The degree of lectin binding was calculated from the concentration of MeUmb-β-D-(GlcNac)₂, determined fluorimetrically in the lectin-free compartment to avoid correction for ligand fluorescence changes on binding, and from a blank determination in the absence of lectin (De Boeck *et al.*, 1981).

Data were plotted as described by Scatchard (1949).

Results

Fluorescence of rice lectin: temperature dependence

With excitation at 280 or 295 nm, the corrected emission spectrum of rice lectin was maximal at 348 nm (Fig. 1), a result that is typical for tryptophan residues exposed to solvent. The absence of any emission in the 300–310 nm region would indicate that tyrosine emission is completely quenched by non-radiative collision or transferred to neighbouring emitting tryptophan residue(s) in the native rice lectin.

Fig. 1. Fluorescence spectrum of rice lectin in the absence (I) and presence (II) of saturating concentrations of *N*-acetylglucosaminides

(a) GlcNac; (b) (GlcNac)₂; (c) (GlcNac)₃. The rice lectin concentration was 1.3 μ M in 0.01 M-sodium acetate/0.15 M NaCl, pH 6.0, at 20°C. The excitation wavelength was 280 nm.

From 4 to 50°C, the fluorescence intensity of this lectin decreased linearly (Fig. 2). In this temperature range, the shape of the emission spectrum was almost unaffected and only a progressive 1–2 nm red-shift of the maximum was observed.

Effect of GlcNac and its derivatives on rice lectin fluorescence: influence of temperature

When rice lectin was saturated with GlcNac, a 12% enhancement of maximum fluorescence intensity was observed at 20°C (Fig. 1a). This enhancement was accompanied by a weak (1–2 nm) blue-shift of the maximum.

Addition of the oligosaccharides (GlcNac)₂ and (GlcNac)₃ to rice lectin produced larger enhancements of fluorescence intensity than addition of the corresponding monosaccharide (Fig. 1). When the lectin was saturated with (GlcNac)₂, the enhancement was 23% at 20°C, and saturation with (GlcNac)₃ at the same temperature raised this enhancement to 28%. These changes in intensity were accompanied by a 10 nm blue-shift of the maximum.

Binding of Me- α -D-GlcNac to rice lectin induced a 5 nm blue-shift and an increase of 9% in fluorescence intensity. On the other hand, addition of Me- β -D-GlcNac produced a blue-shift of only 2 nm, without any change in fluorescence intensity.

The effect of ligands on the intrinsic fluorescence of rice lectin was temperature-dependent. Thus, when the temperature was raised, the fluorescence intensity of this lectin decreased more slowly in the presence of an excess of ligand than did that of free lectin (Fig. 2).

These results indicate the presence of (a) tryptophan residue(s) in or near the saccharide-binding site of rice lectin. This property enabled us

Fig. 2. Effect of temperature on the fluorescence intensity of the rice lectin in the absence (○) and presence of saturating concentrations of GlcNac (■), (GlcNac)₂ (●) or (GlcNac)₃ (▲)

In the presence of *N*-acetylglucosaminides, data were calculated from the relative fluorescence enhancements at infinite ligand concentrations, ΔF_{∞} , obtained by fluorescence titration (Fig. 4). Results were expressed as the percentage of fluorescence intensity of free rice lectin at 20°C (F_0).

to use unsubstituted saccharides to investigate the thermodynamic parameters of the interaction between rice lectin and its ligands.

Binding studies

To determine the stoichiometry of the interaction between rice lectin and *N*-acetylglucosaminides, equilibrium dialysis of this lectin was performed with MeUmb- β -D-(GlcNac)₂.

Fig. 3 shows the linear Scatchard plot obtained for this binding at 4°C (pH 6.0). At this temperature, rice lectin was found to contain four equivalent saccharide-binding sites (4.12 ± 0.21) per oligomer (M_r 38000; F. Tabary, unpublished work) with an association constant $K_a = (5.5 \pm 0.6) \times 10^5 \text{ M}^{-1}$. This is in agreement with the report by Tsuda (1979), who observed that "... about 1.8 mol of GlcNac binds to 19,000 g of rice lectin."

The changes in the fluorescence intensity of rice lectin when complexed with GlcNac, (GlcNac)₂ or (GlcNac)₃ were analysed to obtain the binding constants of the system. Association constants increased with the carbohydrate chain length. At 20°C, K_a values of $(1.3 \pm 0.1) \times 10^3$, $(5.1 \pm 0.4) \times 10^4$ and $(2.6 \pm 0.1) \times 10^5 \text{ M}^{-1}$ were determined for GlcNac, (GlcNac)₂ and (GlcNac)₃ respectively (Fig. 4). An association constant, K_a , of $(3.3 \pm 0.2) \times 10^3 \text{ M}^{-1}$ was measured at 20°C for Me- α -D-GlcNac.

The three holosides of GlcNac were subjected to a series of titrations in the temperature range 4–37°C. The association constant decreased slightly with temperature, as illustrated by the van't Hoff plots in Fig. 5. For all three ligands, the plots are linear over the temperature range studied. The calculated thermodynamic parameters are re-

ported in Table 1. The ΔH^0 values for the three glucosaminides were very low, within the range of -12.1 to $-20.6 \text{ kJ} \cdot \text{mol}^{-1}$.

Discussion

The fluorescence spectrum observed in the present work for rice lectin resembles that found for WGA (Privat *et al.*, 1974a). In both cases, emission maxima were at 348 nm, indicating that the tryptophan residue(s) were exposed to the solvent. Addition of GlcNac, (GlcNac)₂ or (GlcNac)₃ induced the same spectroscopic events for both lectins. Similar blue-shifts were observed on binding of (GlcNac)₂ and (GlcNac)₃, but intensity changes were temperature-dependent for rice lectin and seemed smaller than those observed for WGA. Four glucosaminide-binding sites per lectin molecule were found for both rice lectin and WGA (Nagata & Burger, 1974; Privat *et al.*, 1974b), which have M_r values of 38 000 and 36 000 respectively.

As for WGA, the association constant of rice lectin and (GlcNac)_n increased with n . Nevertheless, the affinity of this lectin for these glucosaminides was from 2 to 13 times higher than that of WGA for the same saccharides when measured by the same method (Lotan & Sharon, 1973; Privat *et al.*, 1974a).

At 4°C, the association constant of rice lectin and MeUmb- β -D-(GlcNac)₂ was ten times higher than the association constant for rice lectin and (GlcNac)₂. In addition to the problematical role of the anomeric linkage (see below), this was probably due to a contribution of the MeUmb moiety of the substituted disaccharide, as similarly high

affinities have already been observed between WGA and MeUmb-glucosaminides (Privat *et al.*, 1974c; Van Landschoot *et al.*, 1977).

The fluorescence spectrum of rice lectin was diversely affected by Me- α -D-GlcNac and Me- β -D-

Fig. 3. Scatchard (1949) plot for MeUmb- β -D-(GlcNac)₂ binding to rice lectin, determined by equilibrium dialysis

Experiments were performed at 4°C, pH 6.0, and at a final lectin concentration of 3.5 μM . The binding was monitored by the fluorescence at 378 nm of MeUmb- β -D-(GlcNac)₂ in the absence of lectin, with r_1 as the ratio of bound ligand to total lectin (M_r 38 000) and [L] as the free MeUmb- β -D-(GlcNac)₂ concentration at equilibrium.

Fig. 4. Linearized titration curves for rice lectin fluorescence enhancements by an excess of GlcNac (■), (GlcNac)₂ (●), (GlcNac)₃ (▲) or Me- α -D-GlcNac (□) at 20°C

ΔF is the relative fluorescence enhancement and [L] is the concentration of free ligand. The lectin concentration was 1.4 μM .

Table 1. *Thermodynamic parameters for rice lectin binding to three glucosaminides at 25°C*

These parameters are calculated from the regression equations corresponding to the van't Hoff plots in Fig. 5. ΔG^0 , ΔH^0 and ΔS^0 are standard (Gibbs) free energy, enthalpy and entropy of binding (25°C) respectively, and $\Delta G = -RT \log K_a$.

Glucosaminide	ΔH^0 (kJ·mol ⁻¹)	ΔG^0 (kJ·mol ⁻¹)	ΔS^0 (J·mol ⁻¹ ·K ⁻¹)
GlcNac	-12.1 ± 1.1	-17.9 ± 0.1	19.4 ± 4.0
(GlcNac) ₂	-17.6 ± 3.4	-26.9 ± 0.2	31.4 ± 12.1
(GlcNac) ₃	-20.6 ± 0.8	-31.0 ± 0.1	34.9 ± 3.0

Fig. 5. Van't Hoff plots for the association constants, K_a , of *N*-acetylglucosaminides and rice lectin

Data were obtained by continuous titration of fluorescence enhancement (Fig. 4) on addition of GlcNac (■), (GlcNac)₂ (●) or (GlcNac)₃ (▲). The association constant for rice lectin–GlcNac interaction was not measurable at 4°C, owing to the very small fluorescence changes at this temperature (see extrapolation in Fig. 2).

GlcNac. For both anomers, the effect observed was different from that obtained with unsubstituted GlcNac. At 20°C, the affinity of Me- α -D-GlcNac for rice lectin was three times that of GlcNac, which seems to indicate a contribution of the methyl group to the binding. Although the slight alteration in the lectin spectrum by Me- β -D-GlcNac did not allow measurement of the corresponding association constant and consequently comparison of the constants for the α - and β -anomers, these constants are probably similar, since the two Me substituted GlcNac used exhibited the same potency as inhibitors of trypsin-treated-rabbit-erythrocyte agglutination by rice lectin (F. Tabary, unpublished work).

The distinct effect of temperature on the fluorescence intensity of rice lectin when complexed with its saccharidic ligands (Fig. 2) has not been reported for WGA. Furthermore, the results of Privat *et al.* (1974a) showed that temperature had virtually no effect on the association constant

of WGA and (GlcNac)₃ between 6 and 50°C, whereas the affinity of rice lectin for the same ligand decreased by about half in the range 4–37°C. The thermodynamic parameters estimated from the van't Hoff plots in Fig. 5 and reported in Table 1 indicate ΔH^0 values of -12.1 to -20.6 kJ·mol⁻¹ for the binding between rice lectin and the three glucosaminides.

The ΔH^0 values reported for other lectin–saccharide interactions were of -33 to -38 kJ·mol⁻¹ for concanavalin A and mannosides (Van Landschoot *et al.*, 1980), -44 kJ·mol⁻¹ for ricin and galactosides (Zentz *et al.*, 1977), and -46 to -51 kJ·mol⁻¹ for *Ricinus communis* (castor bean) agglutinin and galactosides (Zentz *et al.*, 1979). All these interactions were enthalpically driven.

ΔH^0 values of -25.9, -47.7 and -59.8 kJ·mol⁻¹ have been reported for the interaction between lysozyme and GlcNac, (GlcNac)₂ and (GlcNac)₃ (Banerjee & Rupley, 1973). ΔH^0 , which was -42 kJ·mol⁻¹ for the interaction between peanut (*Arachis hypogea*) agglutinin and methyl α -D-galactopyranoside rose to -78 kJ·mol⁻¹ with Gal- β (1-3)-GalNac (Neurohr *et al.*, 1982). For these two saccharide-binding proteins, this increase in ΔH with the size of the ligand molecule has been related to the presence of an extended binding site.

ΔH^0 values for the binding of GlcNac, (GlcNac)₂ and (GlcNac)₃ to WGA have not been reported, but values between -34.3 and -41.8 kJ·mol⁻¹ were found for the binding to this lectin of MeUmb derivatives of these saccharides (Van Landschoot *et al.*, 1977); this range of ΔH does not seem related to the size of the extended sugar-binding site of WGA. From the thermodynamic balance of the interaction between rice lectin and the three glucosaminides forms reported in Table 1, it can be inferred that both enthalpic and entropic driving forces are favourable to the binding of all three saccharides, with a certain preponderance of the enthalpic contribution. The observation that ΔH^0 increased with the size of the ligand suggests the presence of extended saccharide-binding sites on the rice lectin molecule too.

However, it seems difficult to relate ΔH^0 solely to the number of GlcNac units bound at these sites, since the ΔH^0 values observed here were markedly lower than those generally obtained with other protein-saccharide complexes (see above), indicating that binding cannot be essentially attributed to hydrogen bonds, as suggested by Banerjee & Rupley (1973) for lysozyme binding to the same three ligands as those used here.

In conclusion, the present paper gives the first results to be reported for the interaction between rice lectin and its specific saccharide ligands. Although rice lectin displays many spectroscopic similarities to WGA, its affinities for glucosaminides are distinctly higher. It is possible that the smaller amount of lectin extractable from rice embryos than from wheat-germ might be functionally compensated for by the higher affinity of rice lectin for its ligands.

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