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Use of a biosensor to determine the binding kinetics of five lectins for Galactosyl-*N*-acetylgalactosamine

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The dietary lectins, edible mushroom (ABL) and Jacalin (JAC) inhibit the proliferation of colonic cancer cells, whereas Amaranth (ACL) and peanut (PNA) stimulate their proliferation. All these lectins share as their preferred ligand the Thomsen-Friedenreich (TF) antigen galactosyl β1,3 *N*-Acetylgalactosamine (Galβ1,3GalNAc), but differ in their finer specificities for modifications of this determinant and in their specificities for cancerous epithelia. We have investigated, using a resonant mirror biosensor, the kinetics of binding of these lectins, and *Maclura pomifera* lectin (MPL), which is similar to JAC, to two different Gal-GalNac bearing glycoproteins, antarctic fish antifreeze glycoprotein (AFG) and asialofetuin. JAC had the highest affinity for AFG [K_d 0.027 μM] due to a fast association rate constant [k_{ass} 610,000 (Ms)$^{-1}$]. The other lectins had considerably lower affinities, with K_d ranging from 0.16 μM (ABL) to 5.7 μM (PNA), largely due to slower k_{ass} [ABL 74,000 (Ms)$^{-1}$ to PNA 2700 (Ms)$^{-1}$]. Similarly, JAC had a much higher affinity for asialofetuin [K_d 0.083 μM] than the other lectins [K_d 1.0 μM–4.5 μM]. Affinities were also calculated from the extent of binding at equlibrium and were generally similar to those calculated from the kinetic parameters indicating the true nature of these values.

Keywords: **peanut lectin, mushroom lectin, jacalin, resonant mirror biosensor, Thomsen-Friedenreich antigen**

Abbreviations: **ABL, *Agaricus bisporus* (edible mushroom) lectin; ACL, *Amaranthus caudatus* lectin; JAC, *Artocarpus integrifolia* lectin (Jacalin); MPL, *Maclura pomifera* lectin; PNA, *Arachis hypogaea* (peanut) lectin; TF (Thomsen-Friedenreich Antigen), Galβ1,3GalNAc, galactosylβ1,3*N*-Acetylgalactosamine; AFG, antarctic fish antifreeze glycoprotein; PBST, phosphate buffered saline-Tween20; EGF, epidermal growth factor.**

Introduction

The Thomsen-Friedenreich (TF) antigen, Galactosyl ß1,3 *N*-Acetylgalactosamine (Galβ1,3GalNAc), is a carbohydrate antigen commonly expressed by malignant intestinal epithelia. We have previously shown that of four edible Gal-GalNAc binding lectins two, edible mushroom, *Agaricus bisporus*, (ABL) and jacalin (JAC), inhibit the proliferation of HT29 colonic cancer cells, whereas two, peanut agglutinin (PNA) and *Amaranthus caudatus* lectin (ACL), stimulate the proliferation of such cells [1–3].

Although these four lectins all bind to Galβ1,3GalNAc, they differ in the finer specificity of their binding, particularly regarding sialylation of either the Gal or the GalNAc component [4–10]. Also of likely importance in the interaction of these lectins with cellular binding sites is their valency; ACL is a dimer and has two binding sites [6] whereas ABL, PNA and JAC, and also *Maclura pomifera* lectin (MPL), which is similar to Jacalin both in its structure and binding specificity, are all tetramers and have four binding sites, as established by X-ray crystallographic studies [8,9,11–13].

Since these different TF-binding lectins have opposing effects on cellular proliferation, we have measured, using an optical biosensor, the kinetics of the interactions between ABL, PNA, JAC, ACL and MPL with two rather different TF-antigen containing glycoproteins, the Antarctic fish antifreeze glycoprotein (AFG) and asialofetuin. The Antarctic fish antifreeze glycoprotein [14] is a simple polymer of (alanine-[Galβ1,3GalNAc]-threonine-alanine)-, where all the carbohydrate chains should be equivalent. Asialo-fetuin, in which the normally sialylated side chains of fetuin have been chemically desialylated, has three Galβ1,3GalNAc determinants per polypeptide chain, two of which are close to each other and the third remote from the other two [15].

It is shown that whereas the kinetics of binding of each lectin to the two different ligands are broadly similar, JAC is markedly

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different from the other four lectins, possessing much faster association rate constants, and commensurately higher affinities.

Materials and methods

Biotinylation of glycoproteins

Asialofetuin (Sigma, Poole, UK) and antifreeze glycoprotein (a gift from Dr. A.L. deVries, University of Illinois) were biotinylated on the free amino groups of the protein. Five hundred μg of the glycoprotein in 50 μl 150 mM NaCl were incubated with 25 μl *N*-hydroxysuccinimide amino caproate (LC) biotin (Pierce-Warriner, Chester, UK) 50 mM in dimethyl sulphoxide, over 72 h. The reaction was quenched by the addition of 25 μl 1 M Tris-HCl, pH 8.9, for 1 h at 4°C. After the addition of 150 μl 1 mg/ml bovine serum albumin (Sigma) as protein carrier, unreacted biotin was removed by fractionation on a PD10 column (Pharmacia, St Albans, UK).

Binding assays

Binding reactions were carried out in an IAsys two channel resonant mirror biosensor (Affinity Sensors, Saxon Hill, Cambridge, UK), as previously described [16] with minor modifications. Planar aminosilane surfaces, with which a signal of 600 arc s (1 arc s = 1/3600 degrees) corresponds to 1 ng/mm^2 of bound protein, were derivatized with streptavidin according to the manufacturer's instructions. Controls showed that the lectins did not bind to streptavidin-derivatized surfaces (result not shown). Biotinylated glycoproteins were immobilized on streptavidin-derivatized surfaces, which were then washed with 20 mM HCl and phosphate buffered saline-Tween (PBST) (140 mM NaCl, 10 mM NaH_2PO_4, 0.02% v/v Tween 20, pH 7.2); the amount of AFG bound was 0.125 ng/mm^2 and of asialofetuin 0.033 ng/mm^2. Binding assays were conducted in a final volume of 50 μl PBST at 20 $\pm$ 0.1°C, with the stirrer set at maximum. The association phase of the binding reaction was initiated by the addition of lectin at a known concentration in 1–5 μl PBST to PBST in the cuvette. After 2–5 min, the dissociation reaction was initiated by quickly washing the cuvette 3 times with 50 μl PBST. Two distinguishable responses were observed. Rapid bulk shifts occurred within 5 s of the solution in the cuvette being changed, due to the different refractive indices of the solutions, whilst sustained responses followed due to the association and dissociation of the ligate from the immobilized ligand. To remove residual bound ligate after the dissociation phase, and thus regenerate the immobilized glycoprotein, the cuvette was washed with 50 μl 20 mM HCl. This procedure removed 98%–100% of the bound lectin.

Data analysis

A single binding assay yielded four binding parameters calculated with the non-linear curve fitting FastFit software (Affinity Sensors) provided with the instrument: the slope of the initial rate of binding, the on-rate (k_{on}) and the extent of binding at equilibrium, calculated from the association phase, and the off-rate (k_{off}, equivalent to the dissociation rate constant, k_{diss}), calculated from the dissociation phase. The equilibrium dissociation constant (K_d) was calculated from the kinetic parameters and from the extent of binding at equilibrium, which is a measurement independent of the kinetic parameters.

Analysis of binding kinetics in optical biosensors may generate artefactual second phase binding sites [17,18], which have three principal sources. The first occurs when the rate of diffusion of the soluble ligate from the bulk, stirred solution, through the boundary layer of immobile solution, which exists next to the surface of the sensor, is equivalent to or slower than k_{on}. In this case, after the initial rapid depletion of soluble ligate from the solution near the surface, the observed association kinetics reflect diffusion rates rather than association rates [18]. The second affects the measurement of k_{off} and arises when the density of immobilised ligand is high and low levels of soluble ligate are bound. Under these circumstances dissociated ligand may rebind before it diffuses into the bulk stirred solution, thus producing an artifactually slow k_{off}. The third results from the steric hindrance of binding sites that arises when the immobilized ligand is at high density and/or randomly oriented, though this is most prominent on 3-dimensional carboxymethyldextran surfaces [17]. Steps were taken to avoid these possible artefacts. First, the two Galβ1,3GalNAc containing glycoproteins, AFG and ASF, possess very different densities of Galβ1,3GalNAc. If the artefact of binding site density were making a major contribution to the observed binding kinetics, the observed binding kinetics of the lectins to AFG and to ASF would be significantly different. Second, since the glycoproteins were immobilized through their peptide moiety, immobilization was oriented with respect to TF-antigen, rather than random, thus reducing steric hindrance between the surface and the Galβ1,3GalNAc. Third, since k_{on} depends directly on concentration, concentrations of lectin were kept as low as possible during measurements of k_{on}, to reduce the possibility of the rate of diffusion controlling the binding reaction. Fourth, k_{off} was always measured at high ligate concentration.

Results and discussion

The first important consideration in analysis of binding data from optical biosensors is whether the binding kinetics are monophasic or biphasic with respect to the association phase of the binding reaction. In the case of PNA the plot of k_{on} against concentration is linear over a wide concentration range (Figure 1A), indicating that the interaction between PNA and Galβ1,3GalNAc is best described by a one site model and this is confirmed by examination of the individual binding curves for each determination of k_{on}. Moreover, when the slope of the initial rate was plotted against concentration for PNA the points describe a straight line (Figure 1B) However, with the other lectins, whereas the initial part of the plot of k_{on} against concentration is linear, at higher concentrations it is curved, suggestive of biphasic kinetics (results not shown). Indeed the

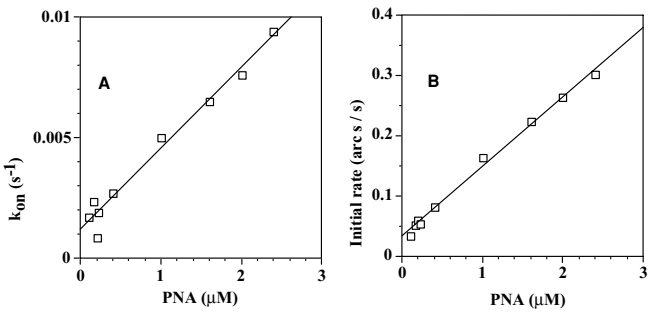

Figure 1. (A) k_{on} of PNA binding to AFG measured at different concentrations of PNA. Since at all concentrations of PNA the binding curves were best described by a one site binding model, k_{on} was calculated using a single site model as described in materials and methods. The SE for each data point is smaller than the symbol. (B) Analysis of the initial rate of binding (arc s per s) against lectin concentration (μM) of PNA binding to AFG.

errors of fit for the individual association curves, from which each value is calculated, clearly indicate a monophasic model only at low concentrations of lectin. Consequently, only k_{on} values obtained at low concentrations of ligand were used to calculate k_{ass} for these lectins. With respect to the dissociation phase, the individual dissociation curves were best described by a monophasic curve.

PNA lectin binding to AFG showed a K_d of 5.7 μM (Table 1) and to asialofetuin a K_d of 4.5 μM. Analysis of the extent of binding at equilibrium to AFG and asialofetuin gave K_d values of 1.6 μM and 1.8 μM respectively, 3.5 and 2.4 fold lower than the corresponding K_d calculated from the kinetic data. The kinetics of the interaction of ACL with AFG and asialofetuin were similar to those observed with PNA (Table 1), K_d 3.2 μM and 2.2 μM, respectively.

ABL bound to AFG with a considerably faster association rate constant than PNA, so with a higher affinity (K_d 0.16 μM) (Table 1). However, the association rate constant of ABL for asialofetuin was considerably slower, giving a K_d of 1.2 μM. The K_d calculated from the extent of binding at equilibrium was similar for AFG binding, but for binding to asialofetuin was lower (3.7 fold) than that calculated from the kinetic data.

In contrast to the other lectins JAC possessed much faster association kinetics, for AFG, K_d 15 nM and asialofetuin, K_d 83 nM. The values for K_d calculated from the extent of binding at equilibrium were similar to the kinetic derived data for both AFG and asialofetuin binding. The association of MPL to AFG and asialofetuin was much slower than observed with JAC (K_d 0.49 μM and 1.0 μM, respectively).

This investigation encompasses two main areas, the determination of the best parameters for investigating lectin binding in an optical biosensor and the comparison of the binding kinetics of a group of lectins of differring molecular structure, but that possess the same ligand binding activity. Two rather different TF-expressing proteins, AFG and asialofetuin, were used to assess whether the observed binding rates were independent of the immobilised ligand.

It is important to be aware that although these five lectins all bind Galβ1,3GalNAc there are differences in their finer binding specificity. For example ABL, ACL and JAC bind NeuAcα2,3Galβ1,3GalNAc- [4,6,7] whereas PNA does not. However, PNA will bind Galβ1,3(NeuAcα2,6)GalNAc whereas ABL will not [4]. JAC is less dependent for its binding on the GalNAc component of the disaccharide [8] and can tolerate varied substitutions thereon.

The analysis of the kinetics of the lectin-TF-antigen interactions demonstrates some similarities and major differences in these binding interactions. The dissociation rate constants vary just over twofold. In contrast, the association rate constants varied by a much greater extent, from 2,700 $(Ms)^{-1}$ (PNA binding to AFG) to 610,000 $(Ms)^{-1}$ (JAC binding to AFG) a 225-fold difference. The equilibrium disociation values determined from the kinetic parameters or from the extent of binding at equilibrium are either similar or differ by no more than two or three fold, suggesting that the binding parameters observed are close

Table 1. Binding of lectins to Galβ1,3Galnac ligands

Lectin	*Ligand (no. of expts)*	k_{ass} $(Ms)^{-1} \pm$ s.e.	k_{diss} $(s)^{-1}$ ($\times$ 100 $\pm$ s.e.)	K_d (M) $\pm$ s.e. (k_{diss}/k_{ass})	K_d (M) $\pm$ s.e. (equilibrium)
PNA	AFG(3)	2700 ± 460	1.60 ± 0.06	$5.72 \pm 0.99 \times 10^{-6}$	$1.61 \pm 0.49 \times 10^{-6}$
	ASiFet(3)	3700 ± 410	1.70 ± 0.12	$4.45 \pm 0.59 \times 10^{-6}$	$1.84 \pm 0.63 \times 10^{-6}$
ABL	AFG(4)	74000 ± 5600	1.20 ± 0.04	$0.162 \pm 0.014 \times 10^{-6}$	$0.11 \pm 0.02 \times 10^{-6}$
	ASiFet(5)	18000 ± 5000	2.10 ± 0.29	$1.13 \pm 0.35 \times 10^{-6}$	$0.31 \pm 0.11 \times 10^{-6}$
ACL	AFG(4)	3800 ± 400	1.20 ± 0.08	$3.17 \pm 0.39 \times 10^{-6}$	$0.35 \pm 0.12 \times 10^{-6}$
	ASiFet(3)	12000 ± 4700	2.70 ± 0.79	$2.21 \pm 1.08 \times 10^{-6}$	$0.73 \pm 0.44 \times 10^{-6}$
MPL	AFG(3)	46000 ± 11000	2.20 ± 0.38	$0.491 \pm 0.148 \times 10^{-6}$	$0.16 \pm 0.04 \times 10^{-6}$
	ASiFet(7)	17000 ± 5600	1.80 ± 0.10	$1.04 \pm 0.32 \times 10^{-6}$	$1.01 \pm 0.41 \times 10^{-6}$
JAC	AFG(4)	610000 ± 49000	0.94 ± 0.22	$1.53 \pm 0.38 \times 10^{-8}$	$2.67 \pm 0.49 \times 10^{-8}$
	ASiFet(5)	200000 ± 55000	1.70 ± 0.23	$8.28 \pm 2.50 \times 10^{-8}$	$4.01 \pm 1.76 \times 10^{-8}$

Dissociation constants (K_d) of five lectins for the TF-antigen bearing glycoproteins, antarctic fish glycoprotein (AFG) and asialofetuin (AsiFet) calculated from k_{diss}/k_{ass} and from the extent of binding at equilibrium. Data are expressed as mean $\pm$ s.e.

to the intrinsic values. Three of the lectins, MPL, ABL and JAC, but not PNA and ACL, possess faster association rate constants for AFG than asialofetuin, which may reflect differences in the environment of the TF provided by the surrounding protein chain.

While several methods for measuring dissociation equilibrium constants of lectin-saccharide interactions have been used, of the TF-binding lectins studied here only JAC [10] and PNA [19] have been studied previously. Surface plasmon resonance investigation of PNA binding to Galβ1,3GalNAc attached to polyacrylamide produced a K_d 5.1 $\times$ 10^{-6} M, similar to our values of 4.7 and 5.5 $\times$ 10^{-6} M [19]. This result using a synthetic carbohydrate also validates our approach of using natural substrates.

The association rate constant reflects the probability of a collision between the lectin and the TF-antigen leading to a binding event. Variations in association rate between different lectins all binding the same carbohydrate determinant could be due to the different shapes of the carbohydrate binding pockets of the lectins and the involvement of water in the binding site. Crystallographic studies of four of the lectins investigated here PNA [11,19], ACL [12], MPL [9] and JAC [8] have revealed considerable differences in the shape of the carbohydrate binding sites, the number of points of attachment therein and the role of water as a linking molecule. For example ACL binding to Galβ1.3GalNAcα-*O*-benzyl involves 16 H-bonds and four water molecules, but the site differs in shape from the PNA site which involves 9 H-bonds and two water molecules; the position of the disaccharide in the site is such that substitution of the 3 position of Gal with sialic acid does not interfere with binding to the ACL site [12] but does to the PNA site [11,19]. MPL binding to Galβ1,3GalNAc also involves H-bonds and water molecules and is predominantly through the GalNAc component, whereas JAC, which though it has a similar structure, is more dependent on the Gal component for binding as indicated by its ability to bind Methyl-Galactose. The nature of the sites of these two lectins is entirely different from that of PNA.

In conclusion the results suggest that the binding mechanisms for glycoproteins of these dietary TF-antigen binding lectins differ markedly. More detailed dynamic analysis of the lectin-TF-antigen interaction will require an approach such as single molecular force spectroscopy [20].

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