Probing the Topography of Lectins with Site-Specific Spin-Labeled Glycosides†

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ABSTRACT: Three new spin-labeled glycosides, spin-label I [1-[4-(β-D-galactopyranosyloxy)phenyl]-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea], spin-label II (2,2,6,6-tetramethyl-1-oxypiperidin-4-yl α-D-galactopyranoside), and spin-label III [1-(methyl 2-deoxy-α-D-galactopyranosid-2-yl)-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea], were investigated as structural probes of *Griffonia simplicifolia* I isolectins (GS I) A_4 and B_4, respectively, by electron spin resonance (ESR) and inhibition of guaran isolectin precipitation. The *p*-aminophenyl β-galactoside spin-label I was strongly immobilized by the B_4 isolectin (K_d = 0.42 mM; $2T_\parallel$ = 54.0 ± 0.3 G), while binding to the A_4 isolectin was so weak ($K_I \simeq 2$ mM) that binding was undetectable by ESR. The preference for the B_4 isolectin was indicative of a more extended hydrophobic binding locus adjacent to the carbohydrate-specific binding site. The α-galactosyl spin-label II bound slightly more strongly to the A_4 than to the B_4 isolectin, as evidenced in both K_d values and particularly by differences in the degree of immobilization ($2T_\parallel$ = 53.5 vs. 51.5 G, respectively). The 2-N-substituted methyl galactoside spin-label III was so poor an inhibitor of both isolectins ($K_I \simeq 1$–2 mM) that ESR detection of the bound complex was not feasible. In all cases above, the spin-labels were displaced by specific monosaccharide haptens.

The use of ESR[1] techniques to study protein–carbohydrate interaction is well documented. Spin-labels have been covalently linked to both simple monosaccharides (Struve & McConnell, 1972; Wien et al., 1972; Poulsen et al., 1977; Adam & Hall, 1979; Rackwitz, 1981; Plessas & Goldstein, 1981) and complex carbohydrates (Aplin et al., 1979; Suzuki et al., 1983). In some cases, their interactions with proteins were investigated.

However, there have been no studies in which the carbohydrate-specific sites of lectins have been probed with sugars containing spin-labels in strategic positions. In this paper we describe for the first time the direct interaction of spin-label-containing glycosides with the tetrameric α-D-galactosyl (GS I-B_4) and *N*-acetyl-α-D-galactosaminyl (GS I-A_4) binding isolectins from *Griffonia simplicifolia* seeds (Murphy & Goldstein, 1977, 1979).

Materials and Methods

G. simplicifolia isolectins GS I-A_4 and -B_4 were prepared by the procedure of Delmotte & Goldstein (1980). The spin-labeled derivatives I–III, containing piperidinoxy groups either as aglycons or as 2-*N*-glycosyl substituents, were synthesized as described by Plessas & Goldstein (1981); their structures are depicted in the figures and under Results and Discussion. Methyl α-D-galactoside and methyl α-D-glucoside were from Pfanstiehl Laboratories, Inc., Waukegan, IL. Lectin concentrations were estimated spectrophotometrically; $E^{1\%}_{280nm}$ = 1.41 cm^{-1} mg^{-1} mL and a *monomer* molecular weight of 30 000 (Murphy & Goldstein, 1979) were used. All concentrations and equilibrium constants reported in this work were based on monomer concentration units. ESR spectra were measured at 26 ± 2 °C on a Varian E-4 spectrometer in quartz microcapillaries for equilibrium titrations (Berliner, 1978). Dissociation constants and Scatchard plots were fit to a nonlinear regression program for the Hewlett-Packard 9835 calculator (Murakami et al., 1982). Studies using hapten inhibition of precipitation were conducted according to the procedure of Murphy & Goldstein (1979).

Results and Discussion

The subtle differences in the binding of the spin-labeled *p*-aminophenyl β-galactoside derivative I to the A_4 and B_4 isolectins were of great interest since they may be compared with results of hapten inhibition, which indicated the presence of a nonpolar binding site adjacent to the carbohydrate binding site in the GS I-B_4 isolectin (Murphy & Goldstein, 1979). The spectrum of the free spin-labeled galactoside I is shown in

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[1] Abbreviations: ESR, electron spin resonance; GS I-A_4 and GS I-B_4, *Griffonia simplicifolia* I isolectins A_4 and B_4, respectively; spin-label I, 1-[4-(β-D-galactopyranosyloxy)phenyl]-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea; spin-label II, 2,2,6,6-tetramethyl-1-oxypiperidin-4-yl α-D-galactopyranoside; spin-label III, 1-(methyl 2-deoxy-α-D-galactopyranosid-2-yl)-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea.

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Table I: Summary of Spin-Labeled Glycoside/GS I Isolectin Interactions[a]

spin-label	structure	GS I-A_4 ESR K_d (mM)	GS I-A_4 ESR $2T_\parallel$ (G)	GS I-A_4 precipitation reaction, K_I(app) (mM)	GS I-B_4 ESR K_d (mM)	GS I-B_4 ESR $2T_\parallel$ (G)	GS I-B_4 precipitation reaction, K_I(app) (mM)
I	1-[4-(β-D-galactopyranosyloxy)phenyl]-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea	>2.0[b]		1.3	0.42	54.0 ± 0.3	0.12
II	2,2,6,6-tetramethyl-1-oxypiperidin-4-yl α-D-galactopyranoside	0.08	53.5 ± 0.3	0.031	0.35	51.5 ± 0.3	0.05
III	1-(methyl 2-deoxy-α-D-galactopyranosid-2-yl)-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea	*b*		0.81	*b*		1.6

[a] K_d was estimated from ESR titrations; K_I(app) was obtained from inhibition of the guaran isolectin precipitation reaction after Murphy & Goldstein (1979). [b] Very weak binding, if at all.

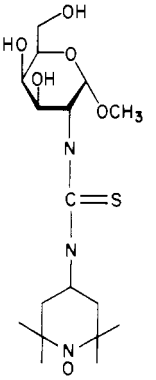
Figure 1a. In the presence of the GS I-A_4 or -B_4 isolectins, a decrease in the narrow line "free spectral" component was observed, as shown in parts b and c of Figure 1, respectively, due to the broadening of the ESR lines from the lectin-bound fraction. In order to observe the lectin/spin-label species, a "high-gain" spectrum was measured at increased modulation amplitude and at 10-fold the receiver gain. A "high-gain" ESR spectrum of spin-label I/GS I-B_4 isolectin is presented in Figure 1d. The separation between the two hyperfine extrema (arrows), $2T_\parallel$ = 54.0 ± 0.3 G, is related to the tumbling rate of the piperidinoxy moiety (Berliner, 1978). The specificity of binding of this galactoside label was demonstrated in Figure 1e by addition of the haptenic sugar methyl α-D-galactopyranoside, which completely reverted to a free label spectrum with the concomitant abolishment of the bound component. On the other hand, the GS I-A_4 bound this spin-label so weakly that the high-field extrema could not be measured.

In order to quantitate these interactions, complete ESR titrations were performed for each spin-label and isolectin studied. A Scatchard plot of the binding of spin-label I and GS I-B_4 is presented in Figure 1f. It was determined that the B_4 isolectin bound this spin-labeled galactoside stoichiometrically with a K_d of 0.42 mM. This compares well with a K_I value of 0.12 mM from inhibition of guaran precipitation. The A_4 isolectin bound the spin-label more weakly (with $K_d \simeq 2.0$ and K_I = 1.3 mM). These results are consistent with previous inhibition data (Murphy & Goldstein, 1979), where it was shown that *p*-nitrophenyl β-galactoside was bound with 5.7-fold greater affinity to GS I-B_4 than to GS I-A_4, and the present study (see Table I) in which the spin-labeled β-galactoside I was 10 times more potent an inhibitor toward the B_4 isolectin. Furthermore, spin-label I was more rigidly immobilized on the surface of the B_4 isolectin, which is indicative of a more extended hydrophobic binding locus adjacent to the carbohydrate-specific binding site. It is also interesting to note that the preferential hydrophobic binding of the GS I-B_4 isolectin to immobilized *p*-aminophenyl β-galactoside was the basis of a procedure designed for the separation of the five possible GS I isolectin tetramers (Delmotte & Goldstein, 1980).

The piperidinoxy α-D-galactoside II bound slightly more strongly to the A_4 than to the B_4 *G. simplicifolia* isolectin (parts a and b of Figure 2, respectively). It should be noted that approximately the same affinity was found for the interaction of GS I-A_4 and -B_4 with *p*-nitrophenyl α-D-galactoside from previous studies conducted by hapten inhibition of precipitation (Murphy & Goldstein, 1979; Wood et al., 1979), equilibrium dialysis (Goldstein et al., 1981), and fluorescence quenching (De Boeck et al., 1981). Additional confirmation of stronger binding to GS I-A_4 was evident in the $2T_\parallel$ values (Figure 2a,b, Table I), which suggests that the spin-labeled ligand was more immobilized on A_4 than on B_4, an observation consistent with its somewhat greater binding affinity as determined by hapten inhibition studies (see Table I).

We demonstrated specific binding of the piperidinoxy α-D-galactoside II by its displacement by the haptenic glycoside, methyl α-D-galactoside (Figure 2d), whereas the nonhaptenic glycoside, methyl α-D-glucoside, did not displace the spin-labeled galactoside II (Figure 2c). A Scatchard plot of the binding of spin-label II with GS I-A_4 is shown in Figure 2e, which compared well with its inhibition behavior (Table I).

Spin-label III, a methyl 2-amino-2-deoxy-α-D-galactoside, which contains a piperidinoxy substituent joined via a thiourea linkage to the C-2 amino group, bound to both isolectins too



III

weakly to obtain reliable K_d values from the spectra (not shown). Although it was expected to bind very poorly to the GS I-B_4 isolectin, from hapten inhibition studies (Murphy & Goldstein, 1979; Wood et al., 1979), it was thought that this spin-label III might bind to GS I-A_4 moderately well. However, both the hapten inhibition (Table I) and spin-label results suggest that spin-label III also binds poorly to the A_4 isolectin, probably due to steric hindrance from the bulky piperidinoxy group.

In summary, we have shown the utility of investigating lectin binding sites using specific spin-labeled glycosides as probes. Measurement of the hyperfine extrema gives information about interactions between the protein and hapten at the molecular level. Competitive displacement studies and biological activity measurements aid in confirming specificity of binding. Future studies with these and other plant lectins are planned to investigate aspects of depth of binding sites, site–site interactions, secondary hapten sites, and distances between carbohydrate and cation binding loci.

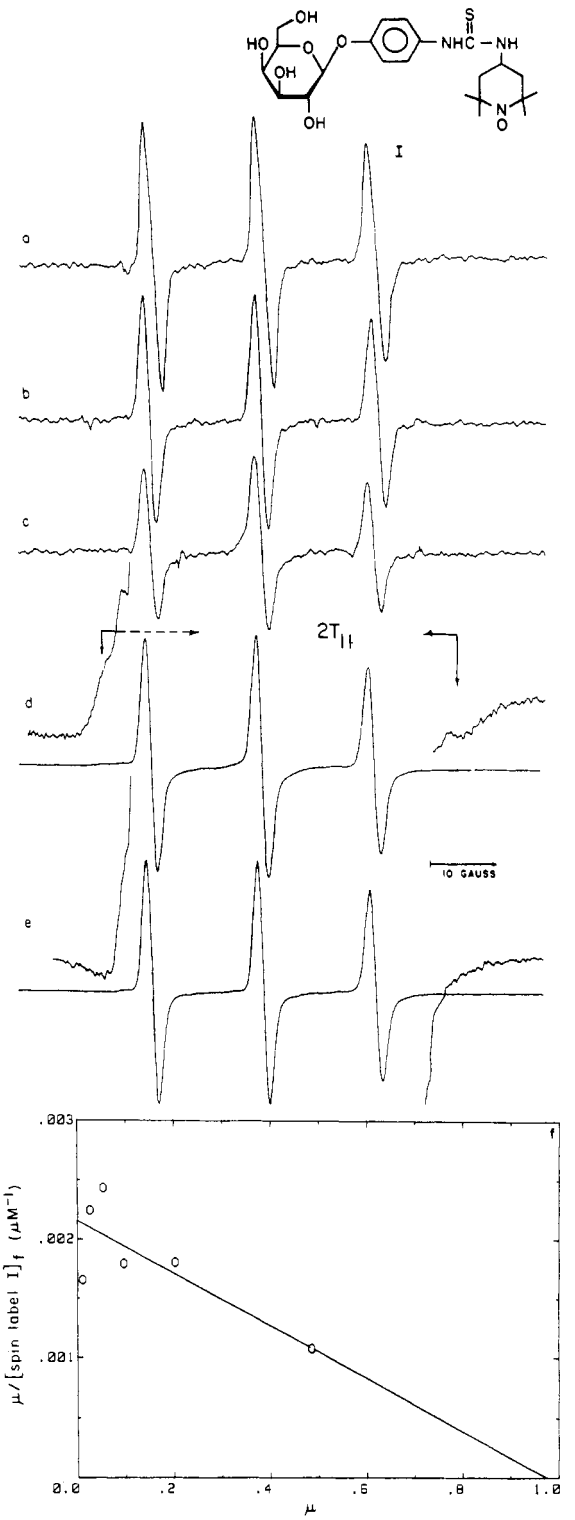


FIGURE 1: ESR titration of spin-label I with the GS I isolectin noted. (a) Free spin-label I (18.6 μM). (b) Spin-label I (18.6 μM) plus 284 μM GS I-A_4. (c) Spin-label I (18.6 μM) plus 279 μM GS I-B_4. (d) High-gain ESR spectrum of a spin-label I/GS I-B_4 mixture (570 μM/248 μM). The superimposed "high-gain" spectrum was measured typically at 10 times the receiver gain and 2–4 times the modulation amplitude of the full-scale three-line spectrum. The hyperfine separation measured between the two extrema in the "high-gain" spectrum (arrows) was 54.0 ± 0.3 G. (e) Sample d plus 1.0 M methyl α-D-galactoside. The complete displacement of saccharide spin-label was further confirmed by the absence of bound label hyperfine extrema in the "high-gain" spectrum. Conditions were 0.1 M phosphate, pH 7.0, 0.15 M NaCl, and 0.1 M $CaCl_2$. Instrument parameters typically were 20-mW microwave power, 9.54 GHz, 1.0- or 2.0-G modulation amplitude, 3300-G applied field, 100-G scan range, 4-min scan time, and 0.5-s time constant. (f) Scatchard plot of the binding of the phenyl β-galactosyl spin-label I (9.4–570 μM) with the GS I isolectin B_4 (270 μM). The data were fit to a single binding constant, K_d = 0.42 mM.

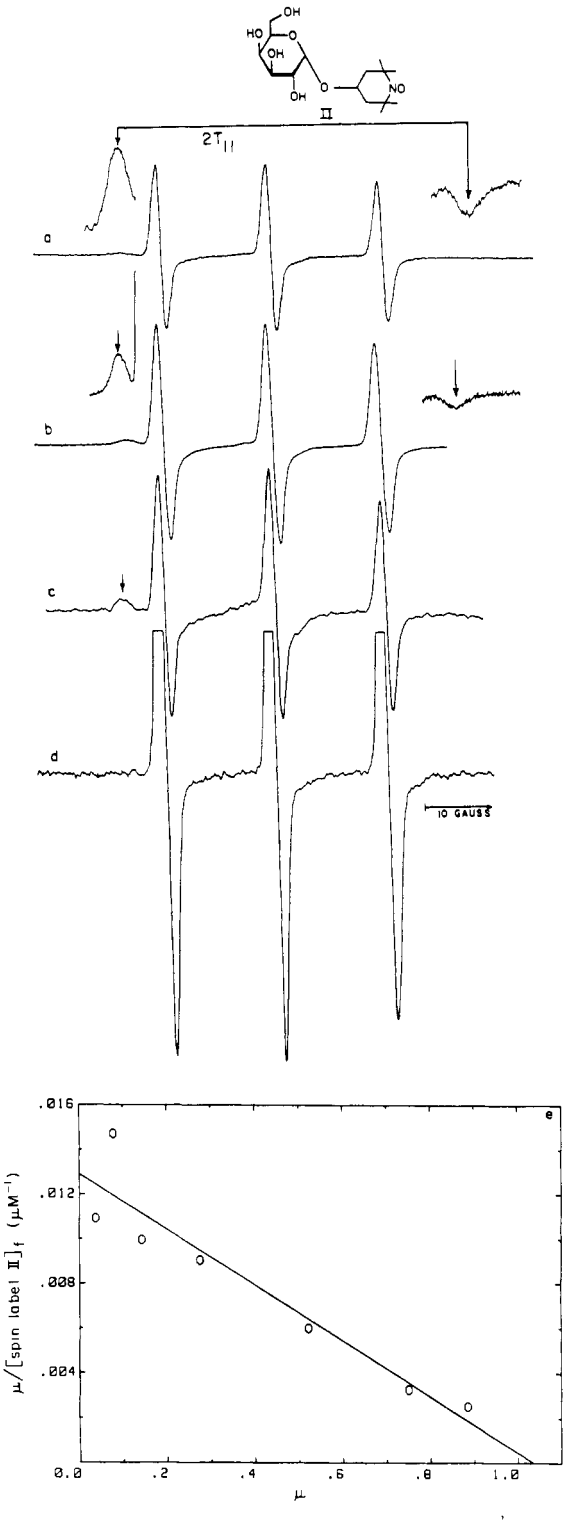


FIGURE 2: ESR titration of the α-galactosyl spin-label II with the GS I isolectin noted. (a) Spin-label II (317 μM) plus 224 μM GS I-A_4. (b) Spin-label II (245 μM) plus 235 μM GS I-B_4. (c) Spin-label II (118 μM) plus 264 μM GS I-A_4 plus 1.2 M glucose. (d) Sample c plus 1.0 M methyl α-D-galactoside. Conditions were 0.1 M phosphate, pH 7.0, 0.15 M NaCl, and 0.1 M $CaCl_2$. (e) Scatchard plot of the binding of spin-label II (14–618 μM) with the GS I isolectin A_4 (280 μM). The data were fit to a single binding constant, K_d = 0.08 mM.

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Articles

Wheat Germ Agglutinin Dimers Bind Sialyloligosaccharides at Four Sites in Solution: Proton Nuclear Magnetic Resonance Temperature Studies at 360 MHz†

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ABSTRACT: Equilibrium binding studies have been performed over a range of temperatures from 25.4 to 47.3 °C between wheat germ agglutinin isolectin I (WGA I) and the α2-3 isomer of (*N*-acetylneuraminyl)lactose (NeuNAcα2-3Galβ1-4Glc). Proton nuclear magnetic resonance spectroscopy at 360 MHz has been used to monitor titrations in this system under conditions where the fraction of total ligand which is bound is small, yet the fractional occupation of sites covers a wide range. Several of the ligand resonances, including the *N*-acetyl methyl and the axial and equatorial hydrogens at carbon 3 of the NeuNAc residue, are shifted and broadened in the presence of WGA due to chemical exchange between the free and bound environments. The lifetime broadening of the *N*-acetyl resonance at room temperature of a series of related sialyloligosaccharides has been previously used by us to measure binding affinities to two WGA isolectins [Kronis, K. A., & Carver, J. P. (1982) *Biochemistry 21*, 3050-3057]. In this paper we report the temperature dependence of the apparent bound shifts and the apparent bound line widths of the *N*-acetyl, H3a, and H3e peaks. The true bound shifts for the three resonances have been obtained from these data by using the equations derived by Swift and Connick [Swift, T. J., & Connick, R. E. (1962) *J. Chem. Phys. 37*, 307-320]. The total bound shifts, per monomer, were found to be -1.98, -4.0, and -0.8 ppm for the *N*-acetyl, the H3a, and the H3e resonances, respectively. The *N*-acetyl data are consistent with there being four NeuNAc binding sites on the WGA I dimer, each with a bound shift for the *N*-acetyl resonance of -0.99 ± 0.03 ppm. This is in contrast to the X-ray diffraction studies that revealed only two NeuNAc sites per dimer. The sources of the ring current shifts for the *N*-acetyl resonance have been assigned to two equivalent sets of tyrosine aromatic side chains per monomer (Tyr-73 and Tyr-159). When the ^{1}H NMR and the X-ray diffraction data are compared, it is apparent that the bound NeuNAc residue occupies a position in each binding site that is analogous to that occupied by the terminal GlcNAc of WGA-bound oligomers of GlcNAc. The *N*-acetyl of the bound NeuNAc or GlcNAc residue is oriented in the same way over the face of a Tyr ring in each binding site.

Wheat germ agglutinin (WGA)[1] is one of the more widely used of the plant lectins in probing the carbohydrate moieties of cell surfaces and mammalian tissues. The sugar specificity of a lectin such as WGA is generally defined on the basis of

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[1] Abbreviations: WGA, wheat germ agglutinin; NeuNAc, *N*-acetyl-D-neuraminic acid; N3L, NeuNAcα2-3Galβ1-4Glc; ^{1}H NMR, proton nuclear magnetic resonance; GlcNAc, *N*-acetyl-D-glucosamine; $(GlcNAc)_2$, GlcNAcβ1-4GlcNAc; $(GlcNAc)_3$, GlcNAcβ1-4GlcNAcβ1-4GlcNAc; $(GlcNAc)_2\beta$1-Me, GlcNAcβ1-4GlcNAcβ1-CH_3; N3Ln, NeuNAcα2-3Galβ1-4GlcNAc; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; NAc, *N*-acetyl; Me, methyl; CD, circular dichroism.

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