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Binding of Hydrophobic Ligands to Plant Lectins: Titration with Arylaminonaphthalenesulfonates[1]

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Binding of the hydrophobic ligands 1,8-anilinonaphthalenesulfonic acid (ANS) and 2,6-toluidinylnaphthalenesulfonic acid (TNS) to a variety of plant lectins was studied by lectin-induced alteration of the fluorescence spectra of the two ligands. With one exception, all legume lectins examined bound ANS, with affinity constants ranging from 10^3 to 10^4 M^{-1}. Similar ANS binding was noted for some nonlegume lectins. Titration of the five isolectins from *Phaseolus vulgaris* with ANS indicated positive cooperative binding of ANS to the two isolectins E_4 and E_3L_1. Titrations with TNS revealed high-affinity sites for this ligand in a number of lectins. Addition of haptenic sugars did not inhibit binding of ANS, suggesting that the hydrophobic binding sites of lectins are independent of the carbohydrate binding sites.

Lectins are widely used as tools for studying the nature and distribution of carbohydrate on cell surfaces and tissues. In order to interpret the results of these studies in a meaningful manner it is important to have a thorough understanding of the properties of these carbohydrate binding proteins. Binding of ligands other than carbohydrates has been observed for a few lectins. Hydrophobic binding properties of concanavalin A (Con A)[3] have been described (1-3). Indirect evidence for the involvement of hydrophobic interactions in the binding of *Phaseolus vulgaris* lectin to erythrocyte stroma has also been presented (4). Ricin and *Ricinus communis* agglutinin I have been shown to bind the fluorescent hydrophobic ligand 1,8-anilinonaphthalenesulfonic acid (ANS) (5), which is also known to bind to Con A (2). Recently, two classes of hydrophobic sites on the lima bean lectin (*Phaseolus lunatus*) have been characterized using fluorescent ligands (6).

Similarities in the properties of the hydrophobic sites in lima bean lectin and Con A prompted us to survey a number of lectins for their ability to bind hydrophobic molecules. Conservation of this property would be of interest in light of the partial sequence homology observed in legume lectins (7) and may have implications in understanding the possible physiological role of lectins in plants and the interactions of these proteins with glycoconjugates on cell surfaces.

Two ligands, ANS and 2,6-toluidinylnaphthalenesulfonic acid (TNS), were used to measure hydrophobic ligand binding. Both ligands are essentially nonfluorescent in aqueous buffers but show strong enhancement of fluorescence quantum

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[3] Abbreviations used: Con A, concanavalin A; ANS, 1,8-anilinonaphthalenesulfonate; TNS, 2,6-toluidinylnaphthalenesulfonate; CRM, cross-reactive material to antibodies against the seed lectins from *Dolichos biflorus*.

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yield upon transfer to a nonpolar solvent or binding to nonpolar sites on proteins (8, 9). A decrease in wavelength of maximum emission is also correlated with transfer to a nonpolar environment and was used to quantitatively estimate binding site polarity for a number of proteins (8).

MATERIALS AND METHODS

Lectins. A number of lectins were prepared by previously described methods: Con A (10), lima bean lectin (11), *Datura stramonium* lectin (12), and *Griffonia simplicifolia* lectins (13–15). Leucoagglutinin from *P. vulgaris* was obtained from Pharmacia (Piscataway, N. J.) and wheat germ agglutinin from Calbiochem (La Jolla, Calif). Other *P. vulgaris* isolectins were provided by Dr. R. L. Felsted (Baltimore Cancer Research Center). *Dolichos biflorus* lectin and cross-reacting material to antibodies against the seed lectin were provided by Dr. M. E. Etzler (University of California, Davis). Most other lectins were provided by Dr. E. Chu from E. Y. Laboratories (San Mateo, Calif.).

2,6-Toluidinylnaphthalenesulfonic acid (TNS) was obtained from Sigma (St. Louis, Mo.). 1,8-Anilinonaphthalenesulfonic acid (ANS) was obtained from Eastman-Kodak (Rochester, N. Y.) and recrystallized as the magnesium salt. Sugars were from Pfanstiehl Laboratory, Inc. (Waukegan, Ill.).

Fluorescence spectra and titrations were done as described previously (6) using a modified Aminco Bowman spectrofluorometer. Titrations were done by addition of small volumes of ligand (10 mM ANS or 1 mM TNS) to a 1.00-ml sample of lectin (0.2–1.0 mg/ml) in phosphate buffer (0.1 M sodium phosphate, 0.15 M NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM $MnCl_2$, pH 6.8) at 25°C. Lectin solutions were clarified by filtration through a 0.45-μm filter prior to assay. Net fluorescence enhancement was obtained by correcting for dilution due to added ligand and for fluorescence of free ligand determined in a parallel titration without protein.

RESULTS

The fluorescence emission spectra of ANS and TNS were altered both in yield and wavelength of maximum emission by a majority of the lectins tested. Typical fluorescence emission spectra for ANS and ANS in the presence of several lectins are presented in Fig. 1. Enhancement of ANS fluorescence was observed for a majority of the lectins tested. The degree of enhancement of fluorescence and decrease in emission maximum varied, however, indicating variation in hydrophobicity of the lectin binding sites. Of the legume lectins tested only the *G. simplicifolia* I-B_4 isolectin failed to bind ANS based on alteration in ligand fluorescence. Several nonlegume lectins were also found to bind ANS and TNS. Binding of ANS to *R. communis* agglutinin I was similar to that observed previously (5). Wheat germ agglutinin also enhanced fluorescence of both probes. Enhancement varied, however, between different preparations of wheat germ agglutinins. This variation may be related to the presence of a contaminating chromophore in the lectin.

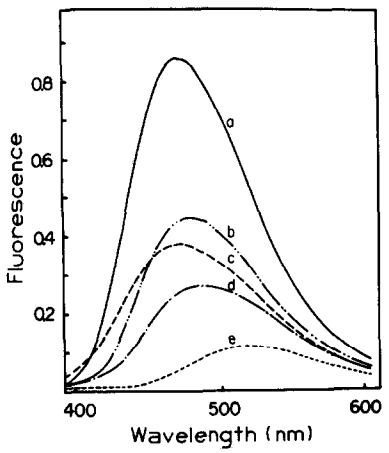

FIG. 1. Fluorescence emission spectra of ANS and ANS in the presence of lectins. Spectra were recorded in ratio mode for the indicated lectins in the presence of 100 μM ANS in 0.1 M sodium phosphate, 0.15 M NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM $MnCl_2$, and 0.01% NaN_3, pH 6.8, at 25°C with a 2.5-nm excitation and 5-nm emission band width. Excitation wavelength was 350 nm for all spectra. Spectra were corrected for nonlinearity of the monochromators. (a) 0.35 mg ml^{-1} lima bean lectin, (b) 0.5 mg ml^{-1} *Dolichos biflorus* lectin, (c) 0.5 mg ml^{-1} wheat germ agglutinin, (d) 1.0 mg ml^{-1} Con A, and (e) 100 μM ANS blank.

The lectin-induced fluorescence enhancement of ANS and TNS provided a signal with which to following binding of these ligands to a number of lectins. Previous studies with Con A (2) and lima bean lectin (6) have established excellent correlation between binding constants for ANS and TNS, determined by ligand fluorescence enhancement, and binding con-

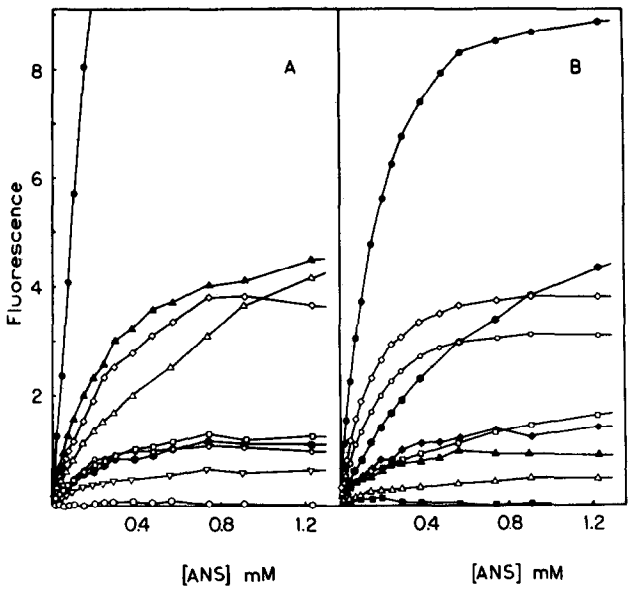

FIG. 2. Fluorescence enhancement titration of ANS binding to plant lectins. Titrations were done by addition of small volumes of 10 mM ANS to a 1.00 ml volume of lectin maintained at 25°C, excitation at 420 nm, emission at 480 nm. Net fluorescence enhancement was calculated by subtraction of fluorescence of free ANS determined by parallel titrations in the absence of protein and corrected for dilution due to ligand addition. Fluorescence intensities were normalized using a reference standard. All lectins were used at a concentration of 1 mg ml^{-1} except as noted. Panel A: *Phaseolus vulgaris* E_4 isolectin (●), soybean agglutinin (▲), concanavalin A (△), pea lectin (◇), *P. vulgaris* L_4 isolectin (0.3 mg ml^{-1}, □), *Griffonia simplicifolia* II lectin (●), peanut lectin (○), *Lotus tetragonolobus* lectin (▽), and *G. simplicifolia* I-B_4 isolectin (○). Panel B: lima bean lectin (0.7 mg ml^{-1}, ●), *Ricinus communis* agglutinin I (●), *Dolichos biflorus* lectin (◇), wheat germ agglutinin (○), potato lectin (□), lentil lectin (◆), *G. simplicifolia* I-A_4 isolectin (0.7 mg ml^{-1}, ▲), *G. simplicifolia* IV lectin (0.5 mg ml^{-1}, △), and *Datura stramonium* lectin (■).

stants obtained from equilibrium dialysis. Titration curves for ANS binding to a variety of lectins are presented in Figs. 2A and B. Conversion of these titration curves to the Scatchard form as described in (16) allowed determination of affinity constants for binding of these probes to lectins. These are summarized in Table I. In some cases the Scatchard plots were nonlinear, indicating site heterogeneity or site-site interaction. Except where indicated, the calculated association constants and standard errors were determined by linear regression analysis from linear Scatchard plots. Heterogeneous binding of TNS to lima bean lectin has been analyzed previously (6). Stoichiometries measured by equilibrium dialysis and fluorescence enhancement indicated that heterogeneity is due to the binding of TNS to one high-affinity site per lectin tetramer, and low-affinity binding to each of four sites per tetramer. Binding of TNS to Con A has been shown to be due to a single high-affinity site per lectin tetramer (2). Binding heterogeneity of ANS and TNS to other lectins has not been interpreted further, but is likely to reflect site heterogeneity as was found for lima bean lectin.

The hydrophobic binding properties of the *P. vulgaris* isolectins were exceptional and could not be explained by site heterogeneity. Binding of TNS to the L_4 and E_4 isolectins produced linear Scatchard plots, although limited heterogeneity may be undetected due to the low enhancement of fluorescence. Binding of ANS to the L_4 isolectin also gave a linear Scatchard plot. However, the E_4 isolectin gave a concave downward Scatchard plot, indicative of

TABLE I

ANS AND TNS BINDING TO LECTINS

Lectin	ANS K_a (M^{-1})	TNS K_a (M^{-1})
Legumes		
Concanavalin A	$2.8 \pm 0.4 \times 10^3$ [b]	1.9×10^4 [d]
Dolichos biflorus	$4.5 \pm 0.2 \times 10^3$	$1.9 \pm 0.2 \times 10^4$
D. biflorus CRM	$1.1 \pm 0.2 \times 10^3$	$2.8 \pm 0.8 \times 10^4$
Griffonia simplicifolia I-A_4	$7.4 \pm 0.6 \times 10^3$	$6.7 \pm 1.5 \times 10^4$
G. simplicifolia I-B_4	No enhancement	—[a]
G. simplicifolia II	$5.1 \pm 0.3 \times 10^3$	—[a]
G. simplicifolia III	$1.4 \pm 0.1 \times 10^3$	—[c]
G. simplicifolia IV	$6.1 \pm 1.2 \times 10^3$	No enhancement
Lentil	$3.9 \pm 0.2 \times 10^3$	$1.2 \pm 0.3 \times 10^5$
Lima bean	$4.5 \pm 0.1 \times 10^3$	$7.9 \pm 1.2 \times 10^4$ $2.2 \pm 0.8 \times 10^5$
Lotus tetragonolobus	$5.5 \pm 0.5 \times 10^3$	—[a]
Pea	$3.1 \pm 0.2 \times 10^3$ [b]	$2.3 \pm 0.7 \times 10^4$
Peanut	$7.7 \pm 0.2 \times 10^3$	—[a]
Phaseolus vulgaris E_4	+cooperative	$2.2 \pm 0.6 \times 10^4$
P. vulgaris E_3L_1	+cooperative	—[c]
P. vulgaris E_2L_2	$4.6 \pm 0.1 \times 10^3$	—[c]
P. vulgaris E_1L_3	$5.9 \pm 0.1 \times 10^3$	—[c]
P. vulgaris L_4	$5.2 \pm 0.1 \times 10^3$	$8.5 \pm 2.3 \times 10^4$
Soybean	$4.5 \pm 0.1 \times 10^3$	$4.7 \pm 0.4 \times 10^4$ [b]
Nonlegumes		
Datura stramonium	No enhancement	No enhancement
Potato	$4.3 \pm 0.6 \times 10^3$ [b]	—[c]
Ricinus communis agglutinin I	$1.6 \pm 0.1 \times 10^3$	$7.0 \pm 0.5 \times 10^4$
Wheat Germ agglutinin	$5.2 \pm 0.2 \times 10^3$	$7.0 \pm 0.3 \times 10^4$

[a] Binding data insufficient to determine K_a.

[b] Nonlinear Scatchard plot was obtained. Reported values are apparent K_a's from linear portion of curve ± SE.

[c] Not done.

[d] Data from Ref. (2).

positive cooperative binding of ANS by this isolectin (Fig. 3). A Hill plot (17) for E_4 binding of ANS gave a Hill coefficient (n_H) of 1.59. To further examine the difference in binding of ANS to E and L subunits, the three intermediate isolectins (18) were titrated with ANS. Scatchard plots for ANS binding to the four isolectins are shown in Fig. 3. Apparent cooperative binding is seen only for the isolectins E_4 and E_3L. The E_2L_2, E_1L_3, and L_4 isolectins gave linear Scatchard plots. The stoichiometry of ANS binding to the *P. vulgaris* isolectins was estimated by fluorescence titration of the E_1L_3 isolectin at several lectin concentrations (19). This method gave an apparent stoichiometry of 4.16 sites/tetramer assuming a subunit molecular weight of 31,500.

Hydrophobic and carbohydrate binding sites have been found to be distinct and noninteracting for the lima bean lectin (6) and Con A (2, 3). The effect of haptenic sugars on ANS fluorescence enhancement by several of the lectins used in the present work was studied in order to determine if this is a general property of the hydrophobic binding sites of lectins. The indicated sugars were added to the following lectins in the presence of 0.1 mM ANS: *D. biflorus* (20 mM GalNAc), lentil (100 mM mannose), pea (100 mM mannose), peanut

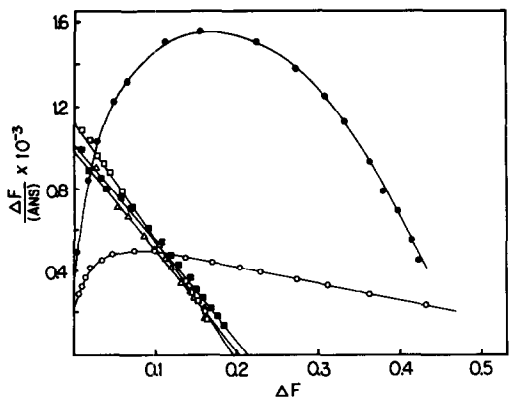

FIG. 3. Fluorescence enhancement titration of ANS binding to *Phaseolus vulgaris* isolectins. Titrations were performed as described in Fig. 2. Binding data are presented as Scatchard plots according to Glaudemans and Jolley (16). Protein concentrations were 1.0 mg ml^{-1} for titration of E_4 (●), E_3L_1 (○), E_2L_2 (■), E_1L_3 (□), and L_4 (△) isolectins.

(100 mM Me-α-Gal), soybean (100 mM Me-α-Gal), and wheat germ agglutinin (20 mM GlcNAc). In all cases the sugar had no effect on the degree of ANS fluorescence enhancement.

DISCUSSION

An increase in quantum yield and a decrease in fluorescence emission maximum have been correlated with binding of ANS and TNS to hydrophobic sites on a number of proteins (8, 9). For a majority of the lectins studied, alteration in ANS fluorescence provides evidence for hydrophobic binding sites. This site appears to be highly conserved in legume lectins with only the B_4 isolectin of *G. simplicifolia* I lacking an ANS binding site. Binding of ANS to the cross-reacting material from *D. biflorus* indicates that hydrophobic binding has been maintained in this material although carbohydrate binding properties have been altered relative to the seed lectin (20). It is of interest that several nonlegume lectins also have hydrophobic binding sites. However, with the limited number of nonlegume lectins used, it is not possible to draw conclusions concerning the distribution of hydrophobic binding sites in these lectins. Of the two lectins from the family Solanaceae examined, the potato lectin binds ANS whereas the *Datura stramonium* lectin did not interact with either probe used in these studies.

Two general conclusions can be drawn from a comparison of the affinity of hydrophobic ligands for the legume lectins tested. First, although there is variation in the measured K_a, the affinity constants for ANS binding all fall within one order of magnitude. Thus, despite large differences in alteration of ANS spectral properties between lectins, the free energies of binding fall within a rather narrow range. Secondly, where sufficient data have been obtained to determine the affinity of TNS binding, this affinity is always greater than that for ANS. The affinity of TNS binding to Con A was determined previously ($K_a = 1.92 \times 10^4\ M^{-1}$) (2). This binding constant is similar to the TNS constants measured here for other legume lectins and is also greater than the affinity of Con A for ANS.

The differences in binding of ANS to the *P. vulgaris* isolectins were unexpected in view of the similar properties of the two subunits (18, 21). Due to the limitations of fluorescence titration it cannot be determined whether the cooperativity of ANS binding is due to alterations in K_a, quantum yield of bound ANS, or both as a function of occupancy of the hydrophobic sites. In either case, however, site-site interaction is indicated. The observation that cooperativity is seen only with the E_4 and E_3L isolectins suggests that these two isolectins may be capable of undergoing conformation changes upon binding ANS, changes which are not possible in the E_2L_2, E_1L_3, and L_4 isolectins. At high ANS concentrations the apparent affinity of ANS binding to E_4 approaches that of L_4 (limiting slope of the E_4 Scatchard plot is $6 \times 10^3\ M^{-1}$ at high ANS). Thus, ANS binding to one site on E_4 may induce conversion of E_4 hydrophobic sites to L_4-like sites. This hypothetical conversion may also be induced by interaction of E with L subunits in the E_2L_2 and EL_3 isolectins. Only minor differences have been noted in the CD spectra of E_4 and L_4 isolectins (22, 23), suggesting similar secondary structure for the E and L subunits. The two isolectins E_4

and L_4 differ greatly, however, in their sensitivity to unfolding at alkaline pH, as measured by perturbation of their CD spectra (22, 24). Further examination of these differences and study of the effect of ANS on the CD spectrum of the isolectins would be helpful in determining the molecular basis for apparent positive cooperative binding of ANS.

Evidence has accumulated for conservation of a number of features of legume lectins. All are multimeric (glyco)proteins which possess multiple carbohydrate binding sites. Most also share the property of divalent cation binding sites which must be occupied for carbohydrate binding to occur. Comparison of primary sequence data of legume lectins indicates considerable NH_2-terminal sequence homology, with some residues being highly conserved (7). The common hydrophobic binding properties of the legume lectins suggest that the residues involved in hydrophobic interactions may be conserved and that this feature of the three-dimensional structure of legume lectins has been maintained during their evolution.

The physiological role of the hydrophobic site is unknown at present. However, present studies in our laboratory suggest the possibility that plant growth regulators, e.g., cytokinins could bind to these sites.

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