# Characterization of the Adenine Binding Sites of Two *Dolichos biflorus* Lectins†

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ABSTRACT: The seed lectin and a stem and leaf lectin (DB58) from *Dolichos biflorus* have high-affinity hydrophobic sites that bind to adenine. The present study employs a centrifugal filtration assay to characterize these sites. The seed lectin contains two identical sites with $K_a$'s of $7.31 \times 10^5$ L/mol whereas DB58 has a single site with a $K_a$ of $1.07 \times 10^6$ L/mol. The relative affinities of these sites for a host of adenine analogs and derivatives were determined by competitive displacement assays. The most effective competitors for adenine were the cytokinins, a class of plant hormone, for which the lectins had apparent $K_a$'s of $1.96 \times 10^5$–$4.90 \times 10^4$ L/mol. Direct binding of the cytokinin 6-(benzylamino)purine (BAP) to both lectins showed positive cooperativity for only the seed lectin, indicating the interaction of this ligand with more than one class of hydrophobic binding site. Fluorescence enhancement assays demonstrate cooperativity between hydrophobic sites of the seed lectin and also suggest that BAP binds to more than one class of site.

Since the discovery of ricin in 1888, plant lectins have found many important applications in medicine, industry, and research (Lis & Sharon, 1986). However, despite over 100 years of active research, the function of lectins in the plant remains a mystery. Several possible roles have been suggested, including their function in seed maturation, cell wall assembly, defense mechanisms, or rhizobial nodulation of legume roots (Etzler, 1985). Nearly all of the applications and proposed functions of the plant lectins are based on their specific carbohydrate binding activities. Although sugar binding is a distinguishing feature of plant lectins, it has been difficult to identify physiologically relevant ligands.

Many of the legume lectins also contain multiple hydrophobic sites which bind the fluorescent probes ANS[1] and TNS with varying affinities, typically in the range of $10^3$–$10^4$ L/mol (Roberts & Goldstein, 1983a). Titrations of these ligands, measured by fluorescence enhancement, indicate that the lima bean lectin tetramer has a single high-affinity TNS site and four low-affinity sites which bind ANS or TNS (Roberts & Goldstein, 1982). Moreover, there appear to be binding interactions between the high- and low-affinity binding sites (Roberts & Goldstein, 1983c) but not between the hydrophobic and carbohydrate binding sites (Roberts & Goldstein, 1982). Further investigation of the lima bean lectin using direct radioligand binding and fluorescence enhancement assays has identified adenine and several of the cytokinins, a class of plant hormone, as high-affinity ligands for the hydrophobic sites (Roberts & Goldstein, 1983c).

The legume *Dolichos biflorus* contains two lectins with 87.6% homology that arise from separate genes that are differentially expressed, both spatially and temporally (Harada et al., 1990). The seed lectin is a heterotetramer (Carter & Etzler, 1975a) localized in the protein bodies of the cotyledons (Etzler et al., 1984). The related lectin DB58 is a heterodimer (Talbot & Etzler, 1978b) associated with the cell walls of stems and leaves (Etzler et al., 1984). Although both lectins recognize *N*-acetylgalactosamine, DB58 has a broader carbohydrate specificity than the seed lectin and shows other differences in carbohydrate binding activity (Etzler & Kabat, 1970; Etzler & Borrebaeck, 1980). These lectins thus constitute an excellent experimental system for exploring the relationship of lectin structure to function.

Both the seed lectin and DB58 have been found to bind adenine (Roberts & Goldstein, 1983b). In this paper we report the comparison of the affinities and specificities of the adenine binding sites of these lectins and evaluate the cytokinins as physiologically relevant ligands for these proteins.

## MATERIALS AND METHODS

*Reagents and Materials.* [$^{14}$C]Adenine (53 mCi/mmol, >98% radiochemical purity) was purchased from ICN (Irvine, CA). [$^{14}$C](Benzylamino)purine (54 mCi/mmol, 97.8% radiochemical purity) was purchased from Amersham (Arlington Heights, IL). Adenine hydrochloride, cytokinins, and other adenine derivatives were obtained from Sigma (St. Louis, MO). Adenylic cofactors were obtained from Boehringer Mannheim (Indianapolis, IN). Millipore (Bedford, MA) Ultrafree-MC filters with low-binding cellulose membranes having a nominal molecular weight cutoff of 10 kDa were used for the radioligand binding assays.

*Isolation of Lectins.* The *D. biflorus* seed lectin was isolated from a crude seed extract by affinity chromatography on polyleucyl-hog blood group A+H substance as previously described (Etzler & Kabat, 1970; Etzler, 1972). DB58 was extracted from stems and leaves (Talbot & Etzler, 1978b) and isolated by chromatography on hog blood group A+H Sepharose as previously described (Etzler & Borrebaeck, 1980). Further purification of DB58 was achieved by Cibracon blue Sepharose chromatography.

*Equilibrium Binding.* A centrifugal ultrafiltration assay with Ultrafree-MC filters was used to determine concentra-

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[1] Abbreviations: PBS, 10 mM $NaPO_4$, pH 7.2, 150 mM NaCl, 0.02% $NaN_3$; 50 mM PBS, 50 mM $NaPO_4$, pH 7.2, 150 mM NaCl, 0.02% $NaN_3$; GalNAc, *N*-acetyl-D-galactosamine; BAP, 6-(benzylamino)purine; BTH, *N*-benzyl-9-(2-tetrahydropyranyl)adenine; TNS, 2,6-toluidinylnaphthalenesulfonic acid; ANS, 1,8-anilinonaphthalenesulfonic acid.

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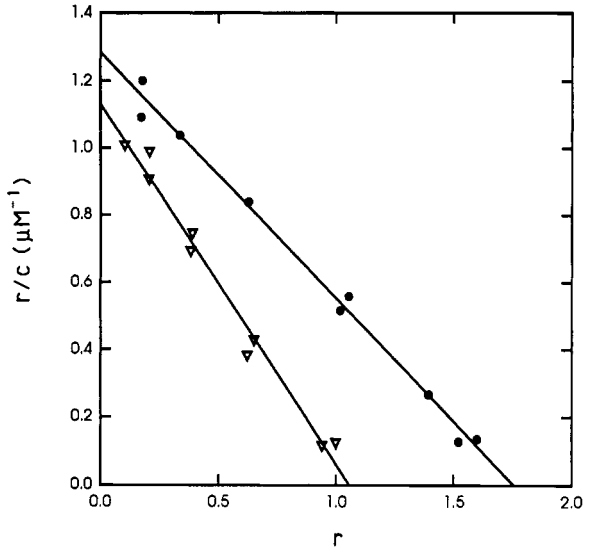


FIGURE 1: Scatchard plot of [$^{14}$C]adenine binding to the *D. biflorus* lectins. Centrifugal ultrafiltration assays conduced in a total volume of 200 μL of PBS and 2 μM seed lectin (●) titrated with 0.5–15 μM [$^{14}$C]adenine or 3.79 μM DB58 (▽) titrated with 0.5–12 μM [$^{14}$C] adenine. The concentration of bound adenine divided by the total concentration of lectin is defined as *r*, and this value divided by the concentration of free adenine is defined as $r/c$ ($\mu M^{-1}$).

tions of unbound radioligand as described by Martin et al. (1989). All assays were performed with either 2 or 4.55 μM seed lectin or 3.79 μM DB58, which was equilibrated with ligands in PBS for 1 h at room temperature. Centrifugation was done in an Eppendorf microfuge at 1310*g* for 6–9 min. Unbound radioligand was measured by scintillation counting aliquots of the filtrate in BCS cocktail from Amersham (Arlington Heights, IL).

*Competitive Displacement.* Binding of unlabeled ligands was measured by displacement of [$^{14}$C]adenine from the seed lectin or DB58. Ligand candidates were evaluated at 10, 20, and 50 μM in PBS for binding to 2 μM seed lectin or 3.79 μM DB58 in the presence of 2 μM [$^{14}$C]adenine as a reporter. The determined concentrations of displaced [$^{14}$C]adenine were used to calculate relative affinity constants for the competitive ligands from the known affinity and binding stoichiometry of adenine with each lectin by the method of Martin et al. (1991).

*Fluorescence Enhancement.* The effect of adenine analogs on binding of ANS or TNS to seed lectin was examined using a fluorescence enhancement assay as previously described for lima bean lectin (Roberts & Goldstein, 1983c).

## RESULTS

*Adenine Binding.* The centrifugal ultrafiltration assay was evaluated over a 24–30-fold concentration range for its efficacy in determining the equilibrium binding of adenine to the seed lectin or DB58. Scatchard analyses (Scatchard, 1949) showed affinity constants of $7.31 \times 10^5$ and $1.07 \times 10^6$ L/mol for the seed lectin and DB58, respectively (Figure 1). These values are in agreement with the affinity constants previously determined by equilibrium dialysis (Roberts & Goldstein, 1983b). Extrapolation of the data to determine binding stoichiometries gave 1.76 sites for the seed lectin tetramer and 1.06 sites for the DB58 dimer.

*Competitive Displacement.* A panel of ligand candidates was examined with competitive displacement assays for binding to both the seed lectin and DB58 using [$^{14}$C]adenine as a reporter ligand (Table I). Among the most effective competitors were several cytokinins, which comprise a class of

Table I: Relative Affinities of Ligands for the *D. biflorus* Seed Lectin and DB58 As Determined by Competitive Displacement of Adenine[a]

| competitor | $K_a$ (L/mol) seed lectin | DB58 |
|---|---|---|
| adenine | $(7.03 \pm 0.64) \times 10^5$ | $(1.56 \pm 0.20) \times 10^6$ |
| kinetin | $(1.81 \pm 0.10) \times 10^5$ | $(1.96 \pm 0.45) \times 10^5$ |
| isopentenyladenine | $(1.15 \pm 0.12) \times 10^5$ | $(2.03 \pm 0.46) \times 10^5$ |
| benzyltetrahydropyranyladenine | $(8.38 \pm 0.52) \times 10^4$ | ND |
| zeatin | $(7.36 \pm 0.46) \times 10^4$ | $(1.11 \pm 0.10) \times 10^5$ |
| benzyladenine | $(7.09 \pm 0.49) \times 10^4$ | ND |
| dihydrozeatin | $(4.90 \pm 1.22) \times 10^4$ | ND |
| benzoyladenine | $(2.94 \pm 0.64) \times 10^4$ | ND |
| adenosine | $(8.65 \pm 1.89) \times 10^3$ | $(2.25 \pm 1.63) \times 10^4$ |
| xanthine | $(4.51 \pm 3.27) \times 10^3$ | $(9.62 \pm 0.64) \times 10^3$ |
| hypoxanthine | $(2.51 \pm 1.79) \times 10^3$ | ND |
| ferulic acid | $(1.80 \pm 1.03) \times 10^3$ | ND |
| diphenylurea | $(1.53 \pm 0.11) \times 10^3$ | ND |
| guanine | $(1.29 \pm 0.22) \times 10^3$ | $<10^3$ |
| uracil | $<10^3$ | $<10^3$ |
| cytosine | $<10^3$ | $<10^3$ |
| thymine | $<10^3$ | $<10^3$ |
| ATP | $<10^3$ | $<10^3$ |
| ADP | $<10^3$ | ND |
| AMP | $<10^3$ | $<10^3$ |
| cAMP | $<10^3$ | ND |
| FAD | $<10^3$ | ND |
| NAD | $<10^3$ | ND |
| NADH | $<10^3$ | ND |
| ribose | $<10^3$ | $<10^3$ |
| GalNAc | $<10^3$ | $<10^3$ |

[a] ND, not determined.

phytohormones (Moore, 1989), with relative affinities for the lectins of $1.96 \times 10^5$–$4.90 \times 10^4$ L/mol in the order of kinetin > isopentenyladenine > 6-benzyl-9-(2-tetrahydropyranyl)-adenine > zeatin > 6-(benzylamino)purine > dihydrozeatin. The lectins had little affinity for other purine or pyrimidine bases or for the adenylic cofactors FAD, NAD, or NADH. Although the lectins did bind slightly to adenosine, xanthine, and hypoxanthine, the affinities for the nucleoside and the adenine catabolites were nearly 100-fold less than for the free base. Moreover, adenine binding sites of the lectins showed no affinity for AMP, cAMP, or the nucleotide phosphates ADP and ATP. The sugar ligand for these lectins, GalNAc, had no effect on adenine binding, indicating the independence of the adenine binding site from the carbohydrate binding site.

Because of its high relative affinity [$(1.81–1.96) \times 10^5$ L/mol] for the seed lectin and DB58, kinetin was selected to verify the competitive nature of adenine displacement. The seed lectin and DB58 were each titrated with a 24-fold range of [$^{14}$C]adenine concentrations in the presence of varying kinetin concentrations. Adenine binding to the seed lectin or DB58 was analyzed by double-reciprocal plot and shown to be competitive with respect to kinetin (Figure 2A,B). The straight lines plotted in Figure 2 are based on the assumption that the concentrations of the unbound competitor ligand, kinetin, did not significantly change over the range of adenine concentrations used in this experiment. Although not always a valid assumption, in this case it was found that, with the seed lectin (Figure 2A), unbound kinetin concentrations determined by the method of Martin et al. (1991) varied from 36.4 to 38.2 μM or 4.5% when 40 μM kinetin was used and from 7.0 to 8.7 μM or 17.3% when only 10 μM kinetin was added. With DB58 (Figure 2B), the calculated unbound kinetin concentrations ranged from 46.8 to 48.3 μM or 3.7%. The resulting plots show close abscissa intercepts with changes

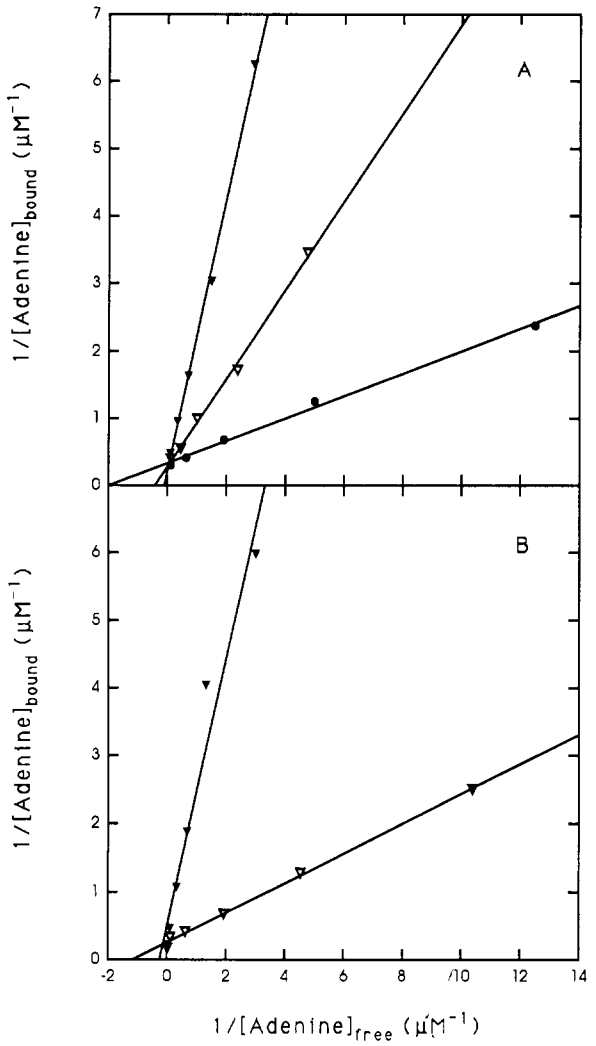


FIGURE 2: Competitive displacement of [$^{14}$C]adenine from the *D. biflorus* lectins by kinetin. (A) Double-reciprocal plot of 0.5–12 μM [$^{14}$C]adenine binding to 2 μM seed lectin in the presence of 0 (●), 10 (▽), or 40 μM (▼) kinetin. (B) Double-reciprocal plot of 0.5–12 μM [$^{14}$C]adenine binding to 3.79 μM DB58 in the presence of 0 (▽) or 50 μM (▼) kinetin.

in slope indicative of competitive binding of kinetin to both lectins with respect to the adenine ligand.

The displacement of [$^{14}$C]adenine from the seed lectin in response to increasing concentrations of kinetin or unlabeled adenine is demonstrated in Figure 3. Affinity constants for each ligand were calculated by the method of Martin et al. (1991) at each point in the displacement curve. Increasingly high degrees of error were observed when the total competitor concentration was <10 μM. Therefore, only displacement values for total competitor concentrations of 10–125 μM were used to calculate affinity constants for unlabeled competitors (Table I and Figure 3, inset). The average $K_a$ value calculated from Figure 3 for kinetin was (1.7 ± 0.3) × $10^5$ L/mol and for adenine (6.0 ± 1.7) × $10^5$ L/mol. The latter value is in good agreement with the affinity constant determined by Scatchard analysis (Figure 1).

*Fluorescence Enhancement.* Both adenine and 6-(benzylamino)purine enhance ANS fluorescence in the presence of the seed lectin (Figure 4). The concentration of adenine required for half-saturation in the fluorescence assay (1.5 × $10^{-6}$ M) was lower than that for 6-(benzylamino)purine (5 × $10^{-6}$ M). The former constant is consistent with the affinity of adenine determined by radioligand binding and suggests that both ligands bind to a site distinct from the ANS site.

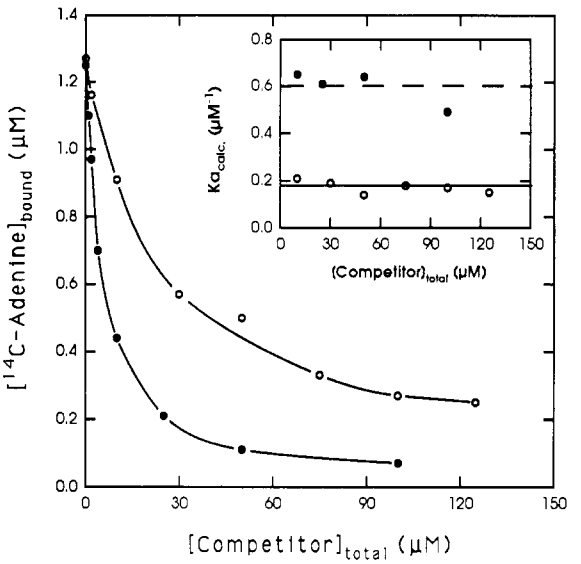


FIGURE 3: Displacement of 2 μM [$^{14}$C]adenine from 2 μM seed lectin in 50 mM PBS in response to titration with 0–125 μM kinetin (○) or 0–100 μM unlabeled adenine (●). $K_a$ values calculated from the displacement curve for both kinetin (○) and unlabeled adenine (●) are plotted in the inset as a function of competitor concentration. The solid and dashed lines represent the average $K_a$ values for kinetin and adenine, respectively.

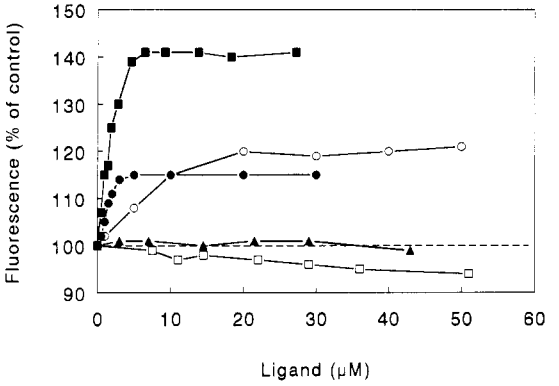


FIGURE 4: Effect of adenine analogs on 1,8-anilinonaphthalenesulfonate binding to *D. biflorus* seed lectin. A solution containing 2 μM seed lectin and 100 μM ANS, pH 6.8 at 25 °C, was titrated with adenine (●), 6-(benzylamino)purine (○), 2-methyladenine (■), 1-methyl-6-(methylamino)purine (□), or purine (▲). Fluorescence was measured at 480 nm with excitation at 420 nm. Fluorescence of bound ANS in the presence of added ligand is presented as a percent of that determined without additions and is corrected for dilution due to addition of ligand.

The specificity for binding to this site is similar to that of lima bean lectin (Roberts et al., 1986), as 2-methyladenine also bound strongly ($K_{act}$ – 1.5 × $10^{-6}$ M) but the 1-substituted analog and purine did not (Figure 4).

Adenine also enhanced TNS fluorescence in the presence of the seed lectin (Figure 5). The enhancement was saturable with half-maximal increase obtained at 1.5 × $10^{-6}$ M adenine. 6-(Benzylamino)purine, however, inhibited seed lectin-induced fluorescence of TNS with an apparent inhibition constant of 2.3 × $10^{-5}$ M.

*6-(Benzylamino)purine Binding.* To explore the unexpected differences in adenine and cytokinin binding to the seed lectin, $^{14}$C-labeled 6-(benzylamino)purine (BAP) was used in direct, noncompetitive binding studies with the seed lectin and DB58. Both lectins were titrated with a 16–20-fold range of [$^{14}$C]BAP and binding was analyzed (Figure 6). For reasons of solubility and availability it was not possible to titrate the comparatively low affinity [$^{14}$C]BAP ligand over the complete

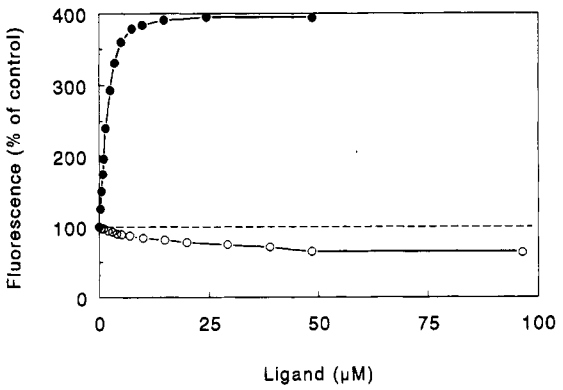


FIGURE 5: Effect of adenine or 6-(benzylamino)purine on 2,6-toluidinylnaphthalenesulfonate binding to *D. biflorus* seed lectin. A solution containing 2 μM seed lectin and 1.8 μM TNS was titrated with adenine (●) or 6-(benzylamino)purine (○). Fluorescence was measured at 436 nm with excitation at 350 nm.

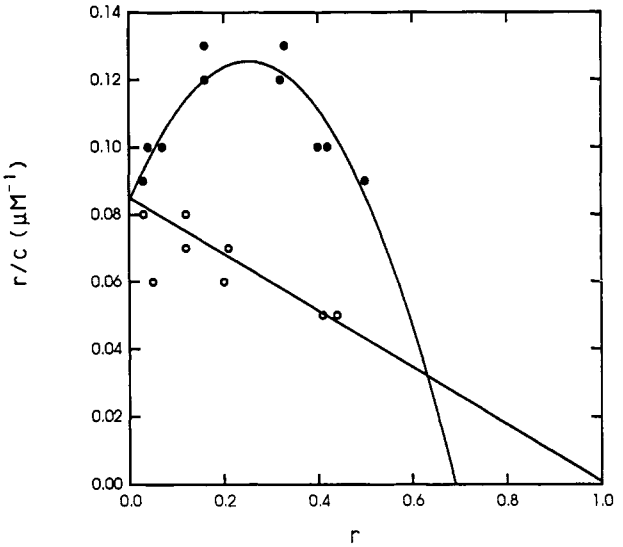


FIGURE 6: Scatchard plot of [$^{14}$C]benzyladenine binding to the *D. biflorus* lectins. Centrifugal ultrafiltration assays conducted in a 200-μL total volume of 50 mM PBS and 4.55 μM seed lectin (●) titrated with 0.5–8 μM [$^{14}$C]BAP or 3.79 μM DB58 (○) titrated with 0.5–10 μM [$^{14}$C]BAP. The concentration of bound BAP divided by the total concentration of lectin is defined as $r$, and this value divided by the concentration of free BAP is defined as $r/c$ (μM$^{-1}$).

range of saturation. Nevertheless, extrapolation of [$^{14}$C]BAP binding to DB58 (Figure 6) suggests a single binding site with an apparent affinity of $9 \times 10^4$ L/mol, whereas [$^{14}$C]BAP binding to seed lectin appears to be cooperative, suggesting two classes of binding sites for BAP on the seed lectin. This result supports the above fluorescence enhancement data and indicates multiple interactive sites.

## DISCUSSION

The difference in localization of the seed lectin and DB58 in the *D. biflorus* plant has raised the possibility that these lectins may be adapted for different physiological functions in their respective tissues (Etzler, 1985). The adenine binding sites of these lectins are of particular interest in considering lectin function because of their interactions with cytokinins, a group of compounds that constitute a class of plant hormones.

The filter centrifugation assay used in this study to determine equilibrium binding of adenine with the *D. biflorus* seed lectin and DB58 gave affinity constants similar to the constants previously determined by equilibrium dialysis (Roberts & Goldstein, 1983b). As found in the previous study, one adenine binding site per DB58 dimer was observed; however, a binding valence of 1.76 sites obtained for the seed lectin now suggests two identical sites per seed lectin tetramer, rather than the single site reported (Roberts & Goldstein, 1983b), which was based on determining protein concentration by Lowry assay with a bovine serum albumin standard. Recalculation of these data based on protein concentration determined by absorbance at 280 nm and $E_{1\%} = 13.8$ agrees with the present result of 1.7 sites (D. Roberts, unpublished data).

The seed lectin tetramer is composed of two related subunits, I and II (Carter & Etzler, 1975a,b). Biosynthetic and structural studies indicate that subunit II arises from subunit I by posttranslational proteolytic cleavage at the carboxyl terminal end (Carter & Etzler, 1975b; Roberts & Etzler, 1982; Schnell & Etzler, 1987; Quinn & Etzler, 1989). Data supporting a similar relationship have been obtained for the two subunits that constitute DB58 (Schnell & Etzler, 1988). The seed lectin has two carbohydrate binding sites per tetramer, and these sites appear to be due to the presence of subunit I (Etzler et al., 1981). The inability of DB58 to agglutinate glycoconjugates indicates that this lectin may be monovalent with respect to carbohydrate binding (Talbot & Etzler, 1978b; Etzler & Borrebaeck, 1980). These valences of the lectins for carbohydrate binding match the valences of the lectins for adenine determined in this study. The finding that the sugar ligand GalNAc has no apparent effect on adenine binding indicates that there is no interaction between the carbohydrate binding and adenine binding sites of these lectins.

In contrast to differences found in specificity of the carbohydrate binding sites between these two lectins (Etzler & Kabat, 1970; Etzler & Borrebaeck, 1980), no difference in specificity was found between the adenine binding sites of the lectins. Both the seed lectin and DB58 bound the cytokinins with comparatively high affinities in the order of adenine > kinetin > isopentenyladenine > zeatin. The less active cytokinins, dihydrozeatin, and assorted cytokinin ribosides, as well as the adenylic nucleoside and nucleotides, all bound poorly to this site on the lectins. This finding supports the assumption that the site is hydrophobic in nature. Another interesting feature of this site is the ability to accommodate fairly bulky substitutions to the purine ring at the N6 position (i.e., the cytokinins) and the N9 position [i.e., 6-benzyl-9-(2-tetrahydropyranyl)adenine] providing that these substitutions are hydrophobic in both positions and planar at N6. The fluorescence enhancement results indicate that the specificity of the adenine site on the seed lectin is similar to that of the lima bean lectin. Both tolerate 2-methyl but not 1-methyl substitution and require the 6-amino group. Based on apparent affinities, this site is identical to that measured by radioligand binding using [$^{14}$C]adenine. It is distinct from the ANS binding site(s). Further evidence of a requirement for a delocalized π-orbital in the purine ring was obtained with lima bean lectin, where methylation of the N1, N3, or N7 position of adenine substantially reduced binding, while substitutions at C2 and C8 were tolerated (Roberts et al., 1986). This requirement may also be a factor in the failure of guanine to bind the site with a high affinity, as the energetically favored tautomer of guanine is the amino–oxo form, which lacks the π-orbital resonance structure of the most stable amino tautomer of adenine (Katritzky & Karelson, 1991).

Further examination of the interaction of 6-(benzylamino)-purine with the seed lectin indicates a more complex mechanism of binding than had been supposed from the binding

of adenine (Figure 1) and its displacement by kinetin (Figures 2 and 3). Fluorescence results indicate that 6-(benzylamino)-purine probably binds to the adenine site with an apparent $K_a$ of $2 \times 10^5$ L/mol. However, the ability of 6-(benzylamino)-purine but not adenine to inhibit TNS binding and the approximately 5-fold higher inhibition constant for BAP measured using TNS instead of ANS suggest that 6-(benzylamino)purine also binds to a second site. These results were supported by [$^{14}$C]BAP binding measured directly and shown to be cooperative. The multiplicity of different sites may simply reflect BAP binding to both the TNS and the ANS sites, as suggested for the lima bean lectin (Roberts & Goldstein, 1983c), whereas adenine presumably binds only the high-affinity TNS site. Maliarik and Goldstein (1988) have proposed that a single adenine binding site occurs at the subunit interface of the lima bean lectin. Should this situation exist with the *D. biflorus* lectins, the less than ideal (<2) stoichiometry of adenine and BAP binding to the seed lectin could be explained by multiple, subunit-derived isoforms of the lectin, only a subset of which is competent to bind BAP. In the case of seed lectin, the competent assembly would have two sites per tetramer which may bind BAP with positive cooperativity. Alternatively, a single seed lectin site may accommodate two BAP molecules as a stacked dimer, the formation of which is concentration dependent so that the convex curve actually reflects ligand–ligand cooperativity. The fluorescence results are consistent with both the two-site and ligand–ligand cooperativity models of BAP binding.

As previously indicated, the seed lectin is found in the protein bodies of *D. biflorus* cotyledons. Proteinase activities in these tissues from germinating squash seeds have been found to be altered by the cytokinins, kinetin, and BAP (Penner & Ashton, 1967). Moreover, the cytokinins are known to be active in concert with auxins in the meristematic tissues and elongating internodes of many plant species, where they modulate cell differentiation and growth (Moore, 1989). In this context it is of interest that DB58 levels are most abundant in young stems and leaves and in the most rapidly elongating stem internodes of the plant (Roberts & Etzler, 1984). It is clear that both *D. biflorus* lectins are present at a time and place at which cytokinins exert an effect on the plant. The ability of these lectins to interact with these hormones raises the possibility that their physiological function may be related to their ability to serve as hormone receptors. The low affinities of the adenine binding sites for xanthine, hypoxanthine, uracil, cytosine, thymine, and other assorted adenylic containing cofactors indicate that the lectins do not play a direct role in purine or pyrimidine metabolism.

Despite the differences in quaternary structure, carbohydrate binding (Etzler & Kabat, 1970; Talbot & Etzler, 1978b; Etzler & Borrebaeck, 1980), and tissue and subcellular localization (Talbot & Etzler, 1978a; Etzler et al., 1984) of these two lectins, the adenine binding properties of these proteins are quite similar. This similarity in adenine binding properties extends to lectins with different carbohydrate specificities isolated from seeds of a variety of legume species (Roberts & Goldstein, 1983c; Roberts et al., 1986). This interaction with adenine is thus a unifying feature that may be a key to the function of these lectins in the plant. Experiments are in progress to determine the localization of these adenine binding sites on the lectins.

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