Photoaffinity Labeling of the Adenine Binding Sites of Two Dolichos biflorus Lectins

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Colin V. Gegg and Marilyn E. Etzler
From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616

Two differentially expressed lectins from the legume Dolichos biflorus, the seed lectin and a stem and leaf lectin (DB58), were photoaffinity-labeled at their adenine binding sites using the probe [2-3H]8-azidoadenine. Both heteromeric subunits I and II of the seed lectin and α and β of DB58 were specifically labeled. This result, combined with the adenine binding site stoichiometries of two identical sites/seed lectin tetramer or one site/DB58 dimer, indicates that the adenine binding site resides at a heterologous subunit interface. Three radiolabeled peaks from seed lectin and one from DB58 were isolated from chymotryptic digests of the labeled lectins by reverse phase chromatography at pH 7.0. From these four peaks, six unique peptide sequences were determined. When aligned with the concanavalin A sequence, four of these peptides map to three loops in the metal binding domain of concanavalin A. The remaining two sequences represent carboxyl-terminal peptides unique to the D. biflorus lectins which may extend to the putative binding site from adjacent, heterologous subunits. It thus appears that the adenine binding sites of these D. biflorus lectins are within the metal binding domain and adjacent to the carbohydrate binding site.

The carbohydrate binding properties of plant lectins have made them important tools in medicine, research, and industry (Lis and Sharon, 1986). In addition to binding carbohydrate, plant lectins have been shown to contain high affinity hydrophobic binding sites (Roberts and Goldstein, 1983a). The legume lectins from lima bean (Phaseolus lunatus), soybean (Glycine max), kidney bean (Phaseolus vulgaris), and Dolichos biflorus bind adenine at these hydrophobic sites, with affinities as much as 200-fold greater than the affinities for their respective carbohydrate ligands (Roberts and Goldstein, 1983b; Gegg et al., 1992).

The adenine binding sites of the lima bean (P. lunatus) and D. biflorus lectins also have high affinity for active forms of cytokinin (Roberts and Goldstein, 1983b; Gegg et al., 1992), an adenine-derived class of phytohormone (Moore, 1989). However, the adenylc cofactors, nucleotides, or nucleosides are poor ligands for the adenine binding sites of these lectins. In addition, no binding interactions are observed between the adenine and carbohydrate binding sites of these lectins. The physiological roles for the carbohydrate and adenine binding sites of the legume lectins in the plant are still unknown and cause for considerable speculation (Roberts and Goldstein, 1983b; Etzler, 1992; Gegg et al., 1992). Other cytokinin binding proteins have been isolated from wheat germ (Fox and Erion, 1975), cucumber cotyledons (Jayabaskaran, 1990), etiolated mung bean seedlings (Akbari and Kamei, 1992), and tobacco leaves (Momotani and Tsuji, 1992). In contrast to the legume lectins, these proteins bind cytokinins with affinities 10–50-fold higher yet bind adenine poorly.

The two best characterized lectins of D. biflorus, the seed lectin and DB58, derive from separate, differentially expressed genes (Harada et al., 1990), yet share 87% homology in their primary structures (Schnell and Etzler, 1988). The seed lectin is a heterotetramer (Carter and Etzler, 1975) localized in the protein bodies of the seed cotyledons (Etzler et al., 1984), whereas DB58 is a heterodimer (Talbot and Etzler, 1978) localized in the vacuoles of young stems and leaves (Bunker and Etzler, 1983). The heteromeric subunits I and II of the seed lectin appear to differ only by a truncation at their carboxyl termini, whereas subunit II arises by the proteolytic cleavage of subunit I (Quinn and Etzler, 1989). Similarly, the only difference between the α and β subunits of DB58 is also at the carboxyl terminus. The seed lectin binds two molecules of GalNAc per tetramer (Etzler et al., 1981). DB58 also binds GalNAc (Etzler and Borrebaek, 1980), but does not agglutinate erythrocytes or precipitate glycoconjugates (Talbot and Etzler, 1978) and is presumed to be monovalent for carbohydrate binding. Recent studies have shown that adenine is also bound to two identical sites/seed lectin tetramer or one site/DB58 dimer (Gegg et al., 1992), and that both these lectins bind adenine with similar affinity and specificity.

Because of the many uses for plant lectins as research tools (Lis and Sharon, 1986), the presence of high affinity hydrophobic binding sites should be a consideration in their application. Perhaps more intriguing is what the physiological significance of this activity might be in the plant. The finding that these sites bind the most active forms of cytokinin (Roberts and Goldstein, 1983b; Gegg et al., 1992) and, in at least one case, appear to bind cytokinin cooperatively (Gegg et al., 1992), is suggestive that this class of phytohormone ligand may be associated with the function of the lectin in the plant. Localization of the adenine binding sites on the legume lectins may provide insights into the role of this activity. Photoaffinity labeling of the lima bean and kidney bean lectins with 8-azidoadenine has identified peptides which correspond to an asymmetric subunit interface of ConA2 (Maliarik and Goldstein, 1988). The present

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† To whom correspondence and reprint requests should be addressed: Dept. of Biochemistry & Biophysics, University of California, Davis, CA 95616. Tel.: 916-752-3628; Fax: 916-752-3085.

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2 The abbreviations used are: ConA, concanavalin A; GalNAc, N-acetylgalactosamine; ÑAsd, 8-azidoadenine; ÑPHAds, 2-3H8-azidoadenine; PAGE, polyacrylamide gel electrophoresis; ANS, 1,8-anilino- naphthalenesulfonic acid; TEAP, triethylammonium-phosphate buffer; FPLC, fast protein liquid chromatography; TLCK, Ñ-p-tosyl-L-lysine chloromethyl ketone.
photoaffinity labeling study was undertaken to better understand the structure/function relationship between the seed lectin and DB58 from *D. biflorus* with respect to their adenine binding sites.

**EXPERIMENTAL PROCEDURES**

Reagents—[8-14C]Adenine (50 mCi/mmol, >98% radiochemical purity) and [2-3H]8-azidoadenosine (14 Ci/mmol, >98% radiochemical purity) were purchased from Moravek Biochemicals (Brea, CA). 8-Azidoadenosine and TLCK-treated chymotrypsin were obtained from Sigma. Ultrafree-FC filters with low binding cellulose membranes having a nominal molecular weight cutoff of 10,000 used for the competitive displacement assays were from Millipore. FPLC solvents were from Fisher. The *D. biflorus* lectins were isolated as previously described (Gegg et al., 1992).

Preparation of [2-3H]8-Azidoadenosine—172 nmol of 8-azidoadenosine and 1.7 nmol of [2-3H]8-azidoadenosine were hydrolyzed in 50 pl of 0.5 N HCl for 30 min at 100 °C then neutralized with 0.5 N NaOH as described by Malark and Goldstein (1988). [2-3H]8-Azidoadenosine was separated from [2-3H]8-azidoadenosine and ribose by preparative TLC on Silica Gel F using a mobile phase of CHCl3:MeOH:glacial acetic acid in a ratio of 17:2:1 (v/v). [2-3H]8-Azidoadenosine (RP = 0.87) and [2-3H]8-azidoadenosine (RP = 0.60) were visualized by ultraviolet absorbance and fluorography. [2-3H]8-Azidoadenosine (24 mCi/mmol) was eluted from the silica gel in MeOH and quantified by ultraviolet absorbance at 283 nm (ε = 20,000 M⁻¹ cm⁻¹ in 0.1 N NaOH). Functionality of the azido group was estimated spectroscopically by observing a reduction in absorbance at 283 and 235 nm after irradiation at 254 nm or alternatively a shift in λmax from 283 nm to 273 nm on reduction with dithiothreitol. Nonspecific 8-azidoadenosine was prepared from 8-azidoadenosine and characterized by the same procedure.

Competitive Displacement—Binding of unlabeled N3Ade was measured using a centrifugal ultrafiltration assay to determine concentrations of displaced [14C]adenine were used to calculate average relative affinity constants for N3Ade from the known concentrations of displaced [14C]adenine were used to calculate average relative affinity constants for N3Ade from the known competitive inhibition constant (Kc) were determined by averaging photoincorporation of label into the protein and the silica in MeOH and quantified by ultraviolet absorbance at 283 nm in 0.1 N NaOH. Functionality of the azido group was estimated spectroscopically by observing a reduction in absorbance at 283 and 235 nm after irradiation at 254 nm or alternatively a shift in λmax from 283 nm to 273 nm on reduction with dithiothreitol. Nonspecific 8-azidoadenosine was prepared from 8-azidoadenosine and characterized by the same procedure.

Photoaffinity Labeling—25 μg of seed lectin (227 pmol) or DB58 (451 pmol) were equilibrated with 25 μM N3[3H]Ade in 50 mM Tris-HCl, pH 7.2 for 30 min at room temperature then chilled 5 min in an ice bath. Samples (25-200 μl) in polypropylene microcentrifuge tubes were irradiated at 283 nm in an ice bath from a height of 5 cm (1000 microwatts/cm²) using a Spectroline hand lamp (Westbury, NY) with the filter removed. Immediately after photolysis, the reactions were quenched with dithiothreitol added to a final concentration of 10 mM. Label was recovered from the reaction mixture by addition mixture of 8 M urea, 0.2% SDS, 12.5% sucrose, and 0.0025% bromphenol blue and were immediately analyzed by SDS-urea-PAGE and fluorography (Carver and Etzler, 1975). Identification of Labeled Peptides—200 μg of seed lectin or 100 μg of DB58 were labeled at 1.0 and 0.75 mg/ml, respectively, with 25 μM N3[3H]Ade as described. The EtOH-precipitated lectins were resuspended in 8 M guanidine HCl for 20 min at room temperature then diluted 1:4 with 0.1 M Tris-HCl, pH 8.0. The diluted lectins were digested by overnight incubation at room temperature with TLCK-treated chymotrypsin at a ratio of 1:20 chymotrypsin:lectin (w/w).

The chymotryptic digests were initially separated by reverse phase HPLC on a 4.6 x 250 mm Aquapore RP-300 octyl column (Brownlee Lab) using a triethylamine-phosphatate (TEAP) buffer system at pH 7.0 and a 15-45% acetonitrile gradient. Peptide-containing peaks were detected by absorbance at 230 nm and plotted as a function of elution time. Radiolabeling reaction products were detected by scintillation counting 400 μl of each fraction. Peptide peaks containing radioactivity significantly above background were then dried and repurified on the same column using a trifluoroacetic acid buffer system at pH 2.2 with a 0-50% acetonitrile gradient. Detection of peptides and radioactivity was the same as before.

FIG. 1. Competitive displacement of [14C]adenine by N3Ade. Displacement of [14C]adenine by N3Ade was determined in response to titration with 0-250 μM N3Ade in 50 mM Tris-HCl, pH 7.2, with 2 μM seed lectin (○) or 3.79 μM DB58 (●) in the presence of 2 μM [14C]adenine. Kc values for N3Ade were calculated as averages of all data points.

The isolated peptides were sequenced by automated Edman degradations on a model HPLC10000A sequenator (Hewlett-Packard) by the Protein Structure Laboratory at the University of California, Davis.

RESULTS

Lectin Affinity for 8-Azidoadenosine—N3Ade is an effective competitor for [14C]adenine binding to the seed lectin and DB58 (Fig. 1). Displacement of [14C]adenine by N3Ade is concentration-dependent, and, at 250 μM N3Ade, <20% of the [14C]adenine remained bound by either lectin. Affinity constants calculated from the displacement of [14C]adenine by 0.5-250 μM N3Ade are based on adenine affinities of 7.9 x 10⁶ liters/mol of seed lectin with two binding sites/tetramer and 1.3 x 10⁹ liters/mol of DB58 with 1 binding site/dimer (Gegg et al., 1992). The affinity constants determined for N3Ade binding are 1.27 x 10⁻⁶ liters/mol ± 0.37 for the seed lectin tetramer and 2.26 x 10⁻⁶ liters/mol ± 0.42 for the DB58 dimer.

Photoaffinity labeling of [2-3H]8-Azidoadenosine—Protection by adenine of the seed lectin and DB58 from N3[3H]Ade photoaffinity labeling is dependent on adenine concentration (Fig. 2). Photoaffinity labeling in the seed lectin is more responsive to low adenine concentrations than DB58; however, both lectins respond similarly to 500-2000 μM adenine. Background levels for nonspecific labeling by N3[3H]Ade were estimated by averaging photoincorporation of label into the proteins: trypsin, alkaline phosphatase, and γ-globulin. Although nonspecific labeling of the non-lectin proteins averaged 0.35 nmol/mg ± 0.17, this represents only 13.2% of the seed lectin labeling and 19.6% of DB58.

To test if protection by high adenine concentrations was due to chemical quenching of the photoactivated label, labeling reactions were performed with seed lectin as described in the identification of labeled peptides were analyzed by TLC as described for the preparation of [2-3H]8-azidoadenosine. The addition of 1000 μM concentration of the scavenger p-aminobenzoate (RP = 0.75) prevents photoaffinity labeling and causes the appearance of a new radiolabeled species (RF = 0.12) which is presumably the scavenger adduct. In contrast, the addition of 1000 μM adenine (RP = 0.66) does not yield a new radiolabeled species but instead causes an increase in the level of unincorporated radiolabel (RP = 0.29).

The dependence of photoaffinity labeling on irradiation time is demonstrated in Fig. 3. Maximum photoaffinity incorporation of N3[3H]Ade in both lectins is achieved after a 1-min irradiation with 15-18% of the seed lectin and DB58 binding sites becoming labeled. In the presence of adenine, maximum photoaffinity incorporation of N3[3H]Ade is achieved after 2 min for both lectins and at a lower level of labeling. A comparison of these curves...
Light dependence of photoincorporation. Photoaffinity labeling of 4.55 μM seed lectin (○) or 12.93 μM DB58 (●) with 25 μM N₃[³H]Ade was determined as a function of irradiation time. Each lectin was irradiated in the absence (○) or presence (●) of 1000 μM adenine. B/B₀ is defined as the amount of photoincorporated label divided by the amount of possible binding sites.

indicating the kinetics of photoincorporation in the presence of adenine is slower than in its absence. There was no radiolabel incorporation in the absence of ultraviolet irradiation or when the label was prephotolyzed prior to adding protein.

Photoincorporation into each of the lectin subunits was observed by SDS-urea-PAGE and fluorography (Fig. 4). Subunits I and II of the seed lectin tetramer and subunits α and β of DB58 were each labeled with N₃[³H]Ade; however, adenine effectively protects these subunits from photoincorporation of label. There was no evidence for ultraviolet light-induced cross-linking of subunits or photodegradation of either the seed lectin or DB58.

Isolation of Photoaffinity-labeled Peptides—Preliminary efforts to separate photoaffinity-labeled lectins from the unbound label by trichloroacetic acid precipitation indicated that the photoincorporated radiolabel was acid-labile. Isolation of radiolabeled peptides from tryptic or chymotryptic digests by reverse phase FPLC using a 0.1% trifluoroacetic acid:acetonitrile solvent system at pH 2.2 was also unsuccessful. These observations are consistent with those of other laboratories (Brinagar et al., 1988; Maliarik and Goldstein, 1988). Alternatively, a TEAP:acetonitrile solvent system at pH 7.0 was found to preserve ~15–20% of the photoincorporated radioactivity in discrete peptide-containing peaks.

Chymotryptic digests of the seed lectin and DB58, both photoaffinity-labeled with N₃[³H]Ade, were separated at pH 7.0 with the TEAP:acetonitrile solvent system and their respective chromatograms, and radioactivity profiles are shown in Fig. 5. The seed lectin digest produced two distinct peaks of radioactivity after the flow-through. The first radioactive peak included two small, poorly resolved peptide peaks with elution times of 32.63 min (peptide 1) and 33.53 min (peptide 2). The second radioactive peak contained a single small peptide peak at 37.16 min (peptide 3). Radioactivity in all three peaks was included two small, poorly resolved peptide peaks with elution times of 32.63 min (peptide 1) and 33.53 min (peptide 2). The second radioactive peak contained a single small peptide peak at 37.16 min (peptide 3). Radioactivity in all three peaks was

isolated using a 0.1% trifluoroacetic acid:acetonitrile solvent system. Solvent A was 0.3% triethylamine, 0.1% phosphoric acid, pH 7.0, and solvent B was 40% TEAP, 60% acetonitrile. Samples were loaded during 15 min at 85% solvent A:15% solvent B followed by a 60-min linear gradient to 55% solvent A:45% solvent B. The radioactivity profiles for each chromatogram are plotted for lectins labeled in the absence (---) or presence (-----) of 1000 μM adenine.
and 27.86 min (Fig. 6D). These contaminants as well as peptide 3 may have been derived from the large adjacent peak eluting at 40.18 min in Fig. 5A, as this peak yields three peaks at 25.36, 27.15, and 28.97 min in the 0.1% trifluoroacetic acid: acetonitrile solvent system (data not shown). Peak 4, from DB58, gave a large single peak at 26.84 min in the TEAP solvent carried over from the initial chromatographic separation. The small peaks at 21 and 38 min are also solvent-related. None of the peaks contained detectable levels of radioactivity after pH 2.2 chromatography.

Sequence Determination of Photoaffinity-labeled Peptides—The results of automated sequence analysis of the chromato-
graphed peaks 1–4 are given in Table I. The sequences determined for peak 1 correspond to residues 228–241 of the seed lectin (Schnell and Ettler, 1987) which is located near the carboxyl termini of both subunits I and II. Two sequences from the seed lectin were identified for peak 2; the more abundant sequence 2a corresponds to residues 90–104, while the 10-fold less abundant sequence 2b is a near match for residues 138–146. Peak 3 also gave two seed lectin sequences; however, both were in low yield. The first sequence 3a from peak 3 corresponds to residues 125–137 and is contiguous with the minor sequence 2b. Sequence 3a also contains 2 mismatched residues and a tryptophan. The second sequence 3b, obtained from peak 3, is a partial match to sequence 2a, and the residue corresponding to cycle 7 was indeterminable. Peak 4 from DB58 also gave two sequences in nearly equivalent amounts. The first sequence 4a aligns with residues 145–154 of DB58 (Schnell and Ettler, 1987) and overlaps the two mismatched residues at the carboxyl terminus of sequence 2b from the seed lectin. The other sequence 4b, obtained from peak 4, matches residues 248–251 which is at the extreme carboxyl terminus of the DB58 α subunit. The amino-terminal residues of all peptides with the exception of peptides 2b and 4a are consistent with chymotryptic cleavage sites. These discrepancies in peptide sequence and chymotryptic cleavage may represent sites of modification by the photoaffinity probe.

**DISCUSSION**

The 8-azidopurine nucleotides have been used successfully to label the nucleotide binding sites of a variety of different proteins (Potter and Haley, 1983; Haley, 1991; King, et al., 1991; their popularity stems from the relative nonspecific reactivity of the photoactivated nitrene which reacts by bond insertion (Bayley and Knowles, 1977). The yield of photoincorporation can be highly variable, 0.1–50%, and the new bond is frequently labile. In these experiments, it is essential to demonstrate the affinity and specificity of the target protein for binding the label and that photoincorporation is specific and dependent on irradiation.

**Affinity, Specificity, and Photoincorporation of [2-3H]8-Azidoadenine—**Previous studies on the adenine binding site of the lima bean lectin (Roberts, et al., 1986) have shown that this binding site is most tolerant of modifications at C-2 and C-8 of adenine. Maliarik and Goldstein (1988) have determined that the single adenine binding sites on each of the tetrameric lectins from the lima bean and kidney bean have reduced affinities for 8-azidoadenine; however, N3Ade binding could be significantly enhanced in the presence of ANS. Similarly, the D. biflorus lectins bind N3Ade with affinity constants that are approximately 80% of those found for adenine binding. In contrast to the lima bean and kidney bean lectins, the D. biflorus lectins show a higher overall affinity for N3Ade with a binding stoichiometry of 2 sites/seed lectin tetramer and 1 site/DB58 dimer. Also, unlike the lima bean and kidney bean lectins, ANS has no effect on N3Ade binding to either D. biflorus lectin. From the affinity constants of N3Ade for the D. biflorus lectins, we calculate that 63% of the seed lectin binding sites and 77% of the DB58 binding sites are occupied by N3[3H]Ade at equilibrium under the conditions used for photolabeling.

The specificity of N3[3H]Ade for the adenine binding site is further evidenced by the protection from the non-lectin proteins. Although some nonspecific photoincorporation is indicated by the labeling observed with trypsin, alkaline phosphatase, and γ-globulin, the labeling of both the seed lectin and DB58 was at least 5-fold higher than the non-lectin proteins. Because protection from photoincorporation requires a large excess of adenine, despite the higher affinity of the lectins for adenine, it was considered possible that adenine was chemically quenching the photoactivated label. The TLC analysis of the labeling reactions indicates that, unlike the scavenger p-aminobenzozoate, adenine does not react directly with the photoactivated label to generate a chemical adduct with a unique mobility. Instead, adenine appears to protect the lectin so that the portion that would have become photoincorporated simply returns to the pool of unincorporated label. If 80% of the lectin labeling is specific and adenine is not chemically quenching the photoactivated label, then it is difficult to understand the high levels of adenine necessary for protection. An explanation for this may be derived from the comparatively long half-life of the activated affinity label, on the order of a millisecond, which could be very much larger than the rates of dissociation for both the label and adenine from the active site. In that context, several binding/dissociation events may occur during the life-
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Table 1
Sequence analysis of photolabeled chymotryptic peptides from the D. biflorus seed lectin (peaks 1–3) and DB58 (peak 4)

| Cycle | Peak 1 | Peak 2 | Peak 3 | Peak 4 |
|-------|--------|--------|--------|--------|
|       | a      | b      | a      | b      |
| 1     | S (95) | A (317)| D (30) | A (15) |
|       |        |        |        |        |
| 2     | F (305)| L (218)| T (12) | L (13) |
| 3     | A (366)| V (326)| L (19) | V (14) |
| 4     | S (65) | P (220)| S (5)  | P (11) |
| 5     | K (177)| V (215)| N (12) | V (10) |
| 6     | L (195)| G (137)| A (6)  | G (11) |
| 7     | P (248)| S (24) | G (14) | T (20) |
| 8     | D (260)| E (82) | W (2)  | E (5)  |
| 9     | D (216)| P (70) | D (7)  | P (6)  |
| 10    | S (33) | R (89) | P (9)  | R (7)  |
| 11    | T (63) | R (116)| R (8)  |        |
| 12    | A (114)| D (9)  | M (3)  |        |
| 13    | E (82) | G (10) | K (3)  |        |
| 14    | P (8)  | G (14) |        |        |
| 15    | Y (8)  |        |        |        |

* Picosome yields of PTH-derivatives. Italic boldface indicates residues inconsistent with the predicted sequences of the D. biflorus lectin. X is no residue determined.

The dependence of N3[3H]Ade photolabeling on irradiation time indicates that label incorporation occurs through a photoactivated intermediate, most probably a nitrene. The absence of labeling from prephotolyzed N3[3H]Ade shows that the intermediate nitrene is not so long-lived that the observed labeling continues after the irradiation is discontinued. Photoincorporation is complete for both lectins after 1 min of irradiation; however, when 1000 μM adenine is included, labeling saturation is not complete until 2 min of irradiation. The change in labeling kinetics may be due to quenching by the ultraviolet-absorbing adenine. The yield of photoincorporation in the absence of adenine is ~15–17% of the total binding sites present during labeling or ~22–24% of the binding sites estimated to be occupied by N3[3H]Ade.

Photoaffinity labeling of the adenine binding sites of seed lectin and DB58 with N3[3H]Ade yields specific photoincorporation into both subunits I and II of the seed lectin α and β of DB58. Taken in the context of adenine binding stoichiometry, which is two identical sites/seed lectin tetramer and one site/DB58 dimer, this observation indicates that the binding site must occur at a heterologous subunit interface. Similarly, photoaffinity labeling of the lima bean and kidney bean lectins with N3[3H]Ade has identified a tryptic peptide that aligns with the corresponding seed lectin sequence. One sequence each from the seed lectin and DB58 corresponds to carboxyl-terminal peptides. In two cases, peptides were identified which did not correspond to chymotryptic peptides as judged by their amino-terminal residues. In a third peptide, an aromatic residue was detected within the sequence. Three inconsistencies may represent sites of N3[3H]Ade modification.

Considerable sequence homology has been observed among the legume lectins (Sharon and Lis, 1990), despite in some cases dramatic differences in quaternary structure (Goldstein and Poretz, 1986). Based on these differences in structure, three subclasses have been proposed for the legume lectins (Becker, et al., 1983). The circularly permuted ConA is a member of the first subclass, the "two-chain" lectins refav and pea belong to the second subclass, and, among the third, are the G. simplicifolia isolectins and the GalNAc binding Erythrina coralloidendron and D. biflorus lectins. The three-dimensional crystal structures have been solved for ConA (Hardman and Ainsworth, 1972; Reeke et al., 1975), the broad bean lectin, favin (Reeke and Becker, 1986), pea lectin (Einspahr et al., 1986), and the lectins from E. coralloidendron (Shaanan et al., 1991) and G. simplicifolia (Delbaere et al., 1990). The protomers from each lectin subclass are nearly identical in secondary and tertiary structure and are quite similar to the predicted secondary structure of the D. biflorus seed lectin and DB58 (Schnell and Etzler, 1988).

The striking similarity in tertiary structure among the legume lectins supports using the three-dimensional structure of ConA as a model for an approximation of the topographical location of the adenine binding sites of the D. biflorus lectins. When mapped to the α-carbon backbone of ConA we find that peptides 2a and b, 3a and b, and 4a comprise three loops which approach to within 10 Å of each other in the metal binding domain of ConA. Peptide 3a aligns with the metal binding loop of ConA (residues 10–23) and the residues determined in sequencing cycles 6 and 11 which are inconsistent with the seed

* time of the activated label, providing multiple opportunities for photoincorporation (Ruoho et al., 1973). Therefore, adenine becomes less effective as a protectant because it would eventually be displaced by the activated label. Although the dissociation of activated label from the binding site may lead to higher nonspecific labeling, the highest levels of photoincorporation should occur at multiple sites in and around the binding site, decreasing as a function of distance away from the true binding site orientation.

Localization of Photolabeled Peptides—The acid lability of the photoprobe was partially overcome by performing the initial separations of photoaffinity-labeled peptides by reverse phase FPLC at pH 7.0. Even at pH 7.0, only ~5–20% of the photoincorporated radioactivity was recovered in discrete peptide peaks, the rest eluting in the flow-through or as an elevation in background throughout the gradient. The background radioactivity observed in the chromatograms of Fig. 5 may derive from nonspecific labeling of the lectins. Alternatively, the background radioactivity may represent small populations of specifically labeled peptides and peptide fragments from around the binding site which are labile in response to the acetonitrile gradient. Rechromatography at pH 2.2 allowed the further isolation of peaks 1–4, but resulted in a complete loss of label.

Sequence analysis of the photoaffinity-labeled peaks 1–4 gave six different peptide sequences, each of which corresponds to unique seed lectin or DB58 peptides. Two of the seed lectin sequences are contiguous, and one DB58 sequence overlaps the carboxyl terminus of these contiguous peptides on alignment with the corresponding seed lectin sequence. One sequence each from the seed lectin and DB58 correspond to carboxyl-terminal peptides. In two cases, peptides were identified which did not correspond to chymotryptic peptides as judged by their amino-terminal residues. In a third peptide, an aromatic residue was detected within the sequence. These three inconsistencies may represent sites of N3[3H]Ade modification.

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The consequences of localizing the adenine binding site in the case of benzyladenine, the oxidation product is benzyladenine (Gegg et al., 1992). The localization of a binding site for cytokinin near the metal ions and adjacent to the carbohydrate site may be consistent with a catalytic role for this domain in protecting the plant and its seeds from oxidative damage and senescence.

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