Photoaffinity Labeling of the Adenine Binding Site of the Lectins from Lima Bean, *Phaseolus lunatus*, and the Kidney Bean, *Phaseolus vulgaris*¹

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8-Azidoadenine was employed as a photoaffinity probe of the adenine binding site of the seed lectin from lima beans and from *Phaseolus vulgaris* erythroagglutinin. This compound was shown to (a) bind competitively to the adenine binding site of these lectins and (b) exhibit enhanced binding in the presence of 1,8-anilinonaphthalenesulfonic acid in the same manner as adenine. The presence or absence of 1,8-anilinonaphthalenesulfonic acid during labeling caused no change in the peptide maps of either lectin when digested with trypsin. The peptide maps of each lectin showed one major peak of radioactivity. Sequencing of the corresponding tryptic peptide from lima bean lectin indicated the primary structure to be Val-Leu-Ile-Thr-Tyr-Asp-Ser-Ser-Thr-Lys. The sequence of the labeled peptide isolated from *P. vulgaris* erythroagglutinin was Thr-Thr-Thr-Trp-Asp-Phe-Val-Gly-Glu-Asn-Glu-Val-Leu-Ile-Thr-Tyr-Asp-Ser-Ser-Thr-Lys. The sequence of the labeled peptide from *P. vulgaris* has been described in detail using a number of techniques (Roberts and Goldstein, 1983; Roberts et al., 1986).

The adenine binding properties of the lectin from lima beans have been described in detail using a number of techniques (Roberts and Goldstein, 1983; Roberts et al., 1986).

The binding of adenine is quite sensitive to modifications of the purine ring (Roberts et al., 1986). Although most changes reduce binding, alterations at positions 2 and 8 are accommodated by the protein (Roberts et al., 1986).

The adenine binding site is unique with respect to the other binding sites of lectins in that there is only one such site per tetramer. Identification of sequences surrounding the site may lead to an understanding of the symmetry and location of this site within the lectin tetramer, its position relative to the four ANS sites, and the extent of conservation of these sequences among other legume lectins. To isolate these sequences, we have employed the photoactive compound 8-azidoadenine (N₃Ade) as an affinity probe of the primary structure of the adenine binding site. In this report we describe the specific labeling of two lectins with N₃Ade and the isolation and amino acid sequence of the labeled peptides.

EXPERIMENTAL PROCEDURES

Materials

Lima bean lectin was purified from green lima beans according to a published procedure (Roberts et al., 1982). PHA-E was provided by E-Y Laboratories (San Mateo, CA). 8-Azidoadenosine was purchased from Aldrich. [2-³H]-8-Azidoadenosine was obtained from Moravek Biochemicals (Brea, CA). [8-¹⁴C]Adenosine was from ICN. Adenosine-HCl was from Sigma. ANS was obtained from Eastman and recrystallized from hot water as the magnesium salt. TPCK-treated trypsin and chymotrypsin were purchased from Worthington. All HPLC solvents were from Fisher.

Methods

Preparation of 8-Azidoadenosine—N₃Ade was prepared by hydrolysis of 8-azidoadenosine as follows. 8-Azidoadenosine (17 mg) was dissolved in 0.5 M HCl (0.5 ml) in a Reactivial (Pierce). The reaction mixture was flushed with N₂, heated in the dark at 100 °C for 15 min, neutralized with NaOH, and immediately lyophilized. N₃Ade was purified by silica gel column chromatography (1 x 30 cm) in tert-butyl alcohol:methylethyleketon:Na₂SO₄:NH₄OH (50%) in the ratio 4:2:2:1. The purified material was detected by ultraviolet absorption on thin-layer chromatography silica plates. Integrity of the azido

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group was confirmed by UV absorption and infrared absorption at 2140 cm⁻¹. The purified material was stored as a 5 mM solution in methanol at -20 °C. In the case of [³H]N3Ade, the ribose was not removed from the mixture. Ribose was shown not to interfere with labeling (data not shown). The radiolabeled compound was diluted 1:10 with nonradioactive, purified N3Ade.

Photoaffinity Labeling of Lectin—Lectin (32 μM tetramer) in PBS was incubated in the dark with N3Ade for at least 30 min at 4 °C. Prior to and during photolysis, samples were frozen in a Dry Ice/isopropyl alcohol bath. Samples were placed in uncovered glass vials such that the sample depth was not greater than 2.5 mm. The samples were irradiated for 20 min at 300 nm in a Rayonet photochemical reactor. In experiments involving enhancement, or protection from labeling, the lectin was incubated with ANS or adenine, respectively, for 60 min at 4 °C prior to addition of N3Ade. Excess label was removed following photolysis either by extensive dialysis against 0.1 M NH4HCO3, pH 8.0, or passage through a centrifuged column of Sephadex G-15 equilibrated with 0.1 M NH4HCO3. Labeled lectin was then lyophilized and stored at -20 °C for further use.

Equilibrium Dialysis—Specific binding of N3Ade to LBL was quantified by a competitive assay using [³H]adenine as developed by Roberts and Goldstein (1983). Briefly, 0.15-ml samples of lectin (0.7 mg/ml) and buffer containing 5 mM [³H]adenine and varying concentrations of N3Ade were equilibrated in microdialysis cells (Technilab Instruments, Pequannok, NJ) for 24 h at 25 °C. Adenine concentrations were determined by scintillation counting.

Fluorescence Measurements—Fluorescence titrations were performed on an SLM 8000 photon counting spectrofluorimeter in ratio mode. All titrations were done at 25 °C with excitation at 420 nm and band width of 2 nm and emission at 480 nm and band width 4 nm. A 1.0-ml sample of lectin containing 1 × 10⁻⁵ M ANS was titrated with small volumes of N3Ade. A parallel blank titration was performed using buffer with ANS in the absence of lectin. The relative ANS fluorescence was calculated by correcting for the "blank" fluorescence and normalizing the fluorescence of lectin plus ANS in absence of extrinsic ligand (Roberts and Goldstein, 1983).

Proteolytic Digestion of Labeled Lectin—Typically, the lectin was dissolved in 0.1 M NH4HCO3, boiled for 5 min, and dissolved by the addition of 8 M urea in buffer. Trypsin or chymotrypsin was added in the ratio of 50 to 1, lectin/protease, by weight, and the digestion was carried out for 24 h at room temperature. The reaction was quenched by the addition of acetic acid.

Isolation of Labeled Peptides—Labeled peptides were purified by HPLC on a 10-μm octyl reverse-phase column (Alltech Associates, Deerfield, IL). The column was equilibrated with 0.1% H3PO4, pH 3.1, and a gradient run to 60% organic phase consisting of 3:1 acetonitrile/isopropyl alcohol over 60 min, at a flow rate of 0.5 ml/min.

Sequencing and Amino Acid Analysis—Amino acid analysis (Koop et al., 1989), manual sequence (Tarr, 1986), and gas-phase sequence analyses (Hewick et al., 1981) were performed by the University of Michigan Protein Sequencing Facility.

RESULTS

Characterization of the Incorporation of 8-Azidoadenine into Lectins—Our initial experiments were designed to investigate the specificity of N3Ade as covalent affinity probe of the lectin adenine binding sites from two related legumes. The sensitivity of adenine binding to modifications of the purine ring observed with LBL necessitated a comparison between N3Ade and adenine binding. The binding of N3Ade to the lectins was characterized by several approaches: 1) specific displacement of adenine in a competitive equilibrium dialysis assay; 2) enhancement by N3Ade of lectin-induced fluorescence of ANS; 3) protection from covalent modification of lectin by the probe in the presence of adenine.

Scatchard analysis of equilibrium dialysis results indicated N3Ade to be an effective competitor for the adenine binding site of LBL and PHA-E (Fig. 1). This approach also allowed the calculation of a dissociation constant for the binding of N3Ade to the lectins (Roberts and Goldstein, 1983). A Kd of 21 μM was determined for the binding of N3Ade to LBL and of 57 μM for the binding of N3Ade to PHA-E. The stoichiometry of binding was found to be 1.0 and 0.8 sites/tetramer for LBL and PHA-E, respectively. Although the dissociation constants were somewhat greater than that for the binding of adenine to LBL (12 μM) and PHA-E (8.6 μM) (Roberts and Goldstein, 1983), they were quite acceptable for use of N3Ade as a covalent probe.

The effect of N3Ade upon the binding of ANS to the two lectins was investigated by means of a fluorescence assay. The purpose of this was 2-fold: (a) exploitation of the linkage between the two sites to increase the specific binding of N3Ade to the lectin and (b) prevention of nonspecific binding to the hydrophobic sites in the presence of ANS. As shown in Fig. 2, N3Ade enhances the lectin-induced fluorescence of ANS, an effect which was attributed to cooperativity between the adenine and ANS binding sites (Roberts and Goldstein, 1983). Scatchard plots constructed using corrected fluorescence enhancement values (Glaudemans and Jolley, 1980) and low lectin concentrations relative to ligand allowed a second means of calculating dissociation constants for the binding of N3Ade to the lectins. The Kd values for binding to LBL (35 μM) and PHA-E (29 μM) were in reasonable agreement with those determined by equilibrium dialysis.

The preliminary investigations reported above were performed in the absence of photolysis. Quantitation of the incorporation of covalently bound probe was performed under various conditions which are summarized in Table I. To minimize nonspecific labeling, a 3-fold excess of lectin over probe was generally used. The addition of ANS to the reaction mixture significantly increased the amount of label incorporated, 7.5-fold in the case of LBL, 20-fold with PHA-E.
have no effect on the peptide maps, apart from an increased addition of ANS during the labeling procedure appeared to through a small column of Sephadex G-25 equilibrated with tized by radiolabeled N3Ade were desalted by centrifugation through a reverse-phase HPLC octyl column and aliquots removed for scintillation counting. Data are reported as the fraction of [3H]N3Ade bound in the presence (E) of adenine over [3H]N3Ade bound in the absence (B) of competing ligand.

Addition of 5 mM methyl N-acetyl-α-D-galactosaminide had no effect upon incorporation of label. It was also observed that freezing the samples in Dry Ice/isopropyl alcohol increased total incorporation of bound probe approximately 2-fold in the lima bean lectin. This is most likely due to an increased association of ligand and lectin as well as greater immobilization of the probe within the binding site during photolysis. As the inhibition curve in Fig. 3 demonstrates, incorporation of radioactivity could be inhibited by adenine in a dose-response manner over the range of 10-200 μM. The lectins retained hemagglutinating and adenine binding activities after photolysis, indicating that photolysis did not appear to denature the protein.

Isolation of Photoaffinity-labeled Peptides—Lectins derivatized by radiolabeled N3Ade were desalted by centrifugation through a small column of Sephadex G-25 equilibrated with digestion buffer and treated with trypsin as described under "Experimental Procedures." The peptides were separated on a reverse-phase HPLC octyl column and aliquots removed for scintillation counting (Fig. 4A and Fig. 5). Approximately 40-60% of the radioactivity eluted in the void volume. The peptide map of each lectin showed one major peak of radioactivity along with three or four minor radioactive peaks. The addition of ANS during the labeling procedure appeared to have no effect on the peptide maps, apart from an increased amount of label incorporated into the major peaks. Peptide maps of lectin photoaffixed in the presence of excess adenine showed decreased radioactivity in all peaks; however, the relative decrease was greatest in the peptides isolated.

The peaks corresponding to the major sites of label incorporation were repurified by shallow gradient elution and subjected to gas-phase sequencing (LBL peptide) or manual Edman degradation (PHA-E peptide). Results of each cycle are presented in Tables II and III, respectively. Comparison of sequence results with the amino acid composition (Table IV) indicated that sequencing of the PHA-E peptide ended prior to reaching the carboxyl terminus. The sequences obtained for the LBL and PHA-E peptides are, respectively,
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Val-Leu-Ile-Thr-Tyr-Asp-Ser-Ser-Thr-Lys and Thr-Thr-Thr- Thr-Trp-Asp-Phe-Val-Gly-Glu-Asn-Ala-Glu-Val-Leu-Ile- Thr-Tyr. The sequence from PHA-E corresponds to residues 173–190 in the cDNA-derived sequenced for PHA determined by Hoffman and Donaldson (1985). It is possible that other peptides labeled with acid-labile N3Ade were present in the digestion mixture. However, these could not be purified in great enough yield for sequencing. Therefore it should be noted that while the peptides isolated and sequenced in this report represent specifically labeled peptides, other peptides labeled in a specific fashion may also exist but have not been characterized.

Attempts were made to determine the exact point(s) of attachment of the photoaffinity probe within the peptides. Scintillation counting of fractions collected during the amino acid analysis indicated that the radioactivity eluted prior to any amino acid and no modified amino acid residues could be identified. This suggested that the covalent bond was labile to acid hydrolysis. In addition, an aliquot of each sequencing cycle was counted in order to follow the release of probe. Radioactivity was detected through several cycles in both peptides, but no novel amino acid derivatives were found. This “leaching” effect may be due to hydrolysis during the acid cleavage step in sequencing.

The apparent lability of the photolabeled peptide bond posed a problem in both purity and quantitation of the peptide. In order to provide evidence that the peptide isolated was indeed not an artifact, several experiments were conducted.

A peptide was synthesized with the sequence determined for the peptide isolated from LBL. This unlabeled peptide was run under the same HPLC conditions as used in purification of the photoaffinity-labeled lectin peptides and was found to have a retention time different from that of the labeled peptide. The retention time of the synthetic peptide is marked by an arrow in Fig. 4, A–C.

A sample of photoaffiliated LBL was digested and run on HPLC as described earlier (Fig. 4A). The fractions containing the desired peak were collected and rechromatographed after storage in buffer for 24 h (Fig. 4B). Radioactivity was observed to appear in the void volume, and a small peak appeared with the retention time of the unlabeled synthetic peptide. The purified radioactive peptide was rechromatographed 2 weeks later and was now observed to have shifted completely to the unlabeled position; all counts were found in the void volume (Fig. 4C).

### Table II

**Automated sequence analysis of the photolabeled tryptic peptide from Lima bean lectin (5 nmol peptide sequenced)**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Residue</th>
<th>Yield* nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Val</td>
<td>2.42 (0.33 G)</td>
</tr>
<tr>
<td>2</td>
<td>Leu</td>
<td>2.41 (0.25 S)</td>
</tr>
<tr>
<td>3</td>
<td>Ile</td>
<td>1.74 (0.03 H)</td>
</tr>
<tr>
<td>4</td>
<td>Thr</td>
<td>1.12</td>
</tr>
<tr>
<td>5</td>
<td>Tyr</td>
<td>1.67 (0.14 G)</td>
</tr>
<tr>
<td>6</td>
<td>Asp</td>
<td>1.17 (0.12 I)</td>
</tr>
<tr>
<td>7</td>
<td>Ser</td>
<td>0.73</td>
</tr>
<tr>
<td>8</td>
<td>Ser</td>
<td>0.51</td>
</tr>
<tr>
<td>9</td>
<td>Thr</td>
<td>0.30</td>
</tr>
<tr>
<td>10</td>
<td>Lys</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Yields and identities for amino acids other than the identified amino acid residue are indicated in parentheses.

### Table III

**Manual sequence analysis of photolabeled tryptic peptide from PHA-E (2 nmol sequenced)**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Residue</th>
<th>Yield* nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thr</td>
<td>0.16</td>
</tr>
<tr>
<td>2</td>
<td>Thr</td>
<td>0.12 (0.12 T)*</td>
</tr>
<tr>
<td>3</td>
<td>Thr</td>
<td>0.12 (0.12 T)</td>
</tr>
<tr>
<td>4</td>
<td>Thr</td>
<td>0.10 (0.11 T)</td>
</tr>
<tr>
<td>5</td>
<td>Asp</td>
<td>0.05 (0.003 W)</td>
</tr>
<tr>
<td>6</td>
<td>Phe</td>
<td>0.07 (0.07 A)</td>
</tr>
<tr>
<td>7</td>
<td>Val</td>
<td>0.09 (0.07 F)</td>
</tr>
<tr>
<td>8</td>
<td>Lys</td>
<td>0.08 (0.10 V)</td>
</tr>
<tr>
<td>9</td>
<td>Gly</td>
<td>0.06 (0.10 K)</td>
</tr>
<tr>
<td>10</td>
<td>Glu</td>
<td>0.04 (0.04 G)</td>
</tr>
<tr>
<td>11</td>
<td>Asn</td>
<td>0.059 (0.03 E)</td>
</tr>
<tr>
<td>12</td>
<td>Ala</td>
<td>0.036 (0.04 N)</td>
</tr>
<tr>
<td>13</td>
<td>Gln</td>
<td>0.02 (0.02 A)</td>
</tr>
<tr>
<td>14</td>
<td>Val</td>
<td>0.03 (0.02 E)</td>
</tr>
<tr>
<td>15</td>
<td>Leu</td>
<td>0.01 (0.03 V)</td>
</tr>
<tr>
<td>16</td>
<td>Ile</td>
<td>0.01 (0.02 L)</td>
</tr>
<tr>
<td>17</td>
<td>Thr</td>
<td>0.01 (0.02 I)</td>
</tr>
<tr>
<td>18</td>
<td>Tyr</td>
<td>0.01 (0.01 T)</td>
</tr>
</tbody>
</table>

*See Footnote a, Table II.

* This secondary sequence is identical to the primary sequence but out of phase. This is due to incomplete cleavage during the first cycle, which may be due to blockage at that residue. Whether this is a threonine modified by N3Ade is unclear.
We have attempted to determine the primary structure of the adenine binding site region in two legume lectins. The binding specificity of LBL has been well described (Roberts et al., 1986); however, its complete primary structure is not yet known. PHA-E is a closely related lectin which also appears to have a single high affinity adenine binding site (Roberts and Goldstein, 1985). It was hoped that covalent modification of these two lectins could provide complementary information on the primary structure of the adenine binding site as well as placement of the site within the lectin subunits.

Irradiation of the two lectins in the presence of 8-azidoadenosine resulted in specific incorporation of the photoactive probe into the adenine binding site. A peptide was isolated from the tryptic digest of each lectin, which contained a majority of the radioactive peptide map. This supports the concept of a specific orientation for N\textsubscript{6}Ade within the adenine binding site. The amino acid sequences of the isolated peptides show identity between the 5 amino-terminal amino acids found based on extensive primary structure homologies found. The peptide also shows extensive homology with sequences from A, favin, and pea lectin (are less homologous in this region).

The concept of a specific orientation for N\textsubscript{6}Ade within the adenine binding site region in two legume lectins. The amino acid sequences of the isolated peptides resulted in specific incorporation of the photoactive probe into the adenine binding site. The majority of the radioactivity in the peptide map. This supports the view that lectins from the third subclass may also be variants on the same model (Olsen, 1983). An estimation of the placement of the adenine binding region peptide can be made, using Con A as a prototype lectin. The region around the sequence V-L-I-T-Y-D-S-S-T aligns with amino acid residues 50-60 in Con A (162-172 in the \(\beta\)-chain of the pea lectin). In Con A, this region comprises a portion of the antiparallel \(\beta\)-structure involved in contacts between two Con A dimers to form the tetrameric lectin. In pea lectin this region is also part of the \(\beta\)-structure but makes no intermolecular contacts (consistent with the fact that it remains a dimer) (Reeke and Becker, 1986). Location of this peptide in a region that makes intermolecular contact to form a tetramer is consistent with the observed single site stoichiometry for adenine binding. These residues do not align with amino acids which make up the hydrophobic binding cavities in Con A subunits. Crystallographic analysis of adenine binding lectins will be necessary to determine accurately the placement of this ligand binding site. The binding of 2,3-diphosphoglycerate to hemoglobin provides an example similar to that of the binding shown by adenine. The 2,3-diphosphoglycerate binding site is located between the \(\beta_1\) and \(\beta_2\) subunits (Arnone, 1972) and serves to control red blood cell structure. Whether adenine or cytokinins serve a similar purpose with respect to hydrophobic ligand binding in lectins is unknown.

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