Kusumi, A., & Hyde, J. S. (1982) *Biochemistry 21*, 5978-5983.
Kusumi, A., Ohnishi, S., Ito, T., & Yoshizawa, T. (1978) *Biochim. Biophys. Acta 507*, 539-543.
Kusumi, A., Sakaki, T., Yoshizawa, T., & Ohnishi, S. (1980) *J. Biochem. (Tokyo) 88*, 1103-1111.
Mao, B., Ebrey, T. G., & Crouch, R. (1980) *Biophys. J. 29*, 247-256.
Marsh, D. (1980) *Biochemistry 19*, 1632-1637.
Matthews, R. G., Hubbard, R., Brown, P. K., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215-240.
Papermaster, D. S., & Dreyer, W. J. (1974) *Biochemistry 13*, 2438-2444.
Popp, C. A., & Hyde, J. S. (1981) *J. Magn. Reson. 43*, 249-258.
Ratner, V. L., Bagirov, I. G., & Fesenko, E. E. (1981) *Vision Res. 21*, 251-523.
Renk, G., Grover, T., Crouch, R., Mao, B., & Ebrey, T. G. (1981) *Photochem. Photobiol. 33*, 489-494.
Renk, G. E., Or, Y. S., & Crouch, R. K. (1987) *J. Am. Chem. Soc. 109*, 6163-6168.
Renk, G., Crouch, R. K., & Feix, J. B. (1988) *Biophys. J. 53*, 361-365.
Rousselet, A., Faure, G., Boulain, J., & Menez, A. (1984); *Eur. J. Biochem. 140*, 31-37.
Seigneuret, M., Zachowski, A., Hermann, A., & Devaux, P. F. (1984) *Biochemistry 23*, 4271-4275.
Thomas, D. D., Dalton, L. R., & Hyde, J. S. (1976) *J. Chem. Phys. 65*, 3006-3024.
Wald, G. (1967) *Les Prix Nobel en 1967*, pp 260-280, Nobel Foundation, Stockholm.
Yamamoto, K., & Shichi, H. (1983) *Biophys. Struct. Mech. 9*, 259-267.

ESR and Fluorescence Studies on the Adenine Binding Site of Lectins Using a Spin-Labeled Analogue†

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ABSTRACT: The techniques of electron spin resonance (ESR) and fluorescence spectroscopy have been used to study the interaction of a spin-labeled analogue of adenine, N^6-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)adenine (I), with several plant lectins. While most adenine derivatives enhanced lectin-induced fluorescence of 1,8-anilinonaphthalenesulfonic acid by binding to a separate, adenine-specific site [Roberts, D. D., & Goldstein, I. J. (1982) *J. Biol. Chem. 257*, 11274-11277], the spin label I caused a *decrease* in this fluorescence with certain lectins. ESR showed the ligand to interact strongly with lectins from lima bean (*Phaseolus lunatus*), *Dolichos biflorus*, and *Phaseolus vulgaris* (PHA); however, no binding was observed with *Griffonia simplicifolia* isolectins A_4 and B_4, soybean agglutinin, or *Amphicarpaea bracteata* lectins. The spin label was highly immobilized by each of these proteins ($2T_{\parallel}$ = 68 G). Apparent affinities of the spin label for the lectins decreased in the order lima bean lectin > PHA erythroagglutinin > PHA leukoagglutinin > *D. biflorus*. Spin-labeled adenine appeared to bind specifically to the adenine binding site of *D. biflorus* and PHA leukoagglutinin, as demonstrated by total abolition of the ESR spectrum of bound spin label by adenine. PHA erythroagglutinin and lima bean lectin bound the analogue with apparent dissociation constants of 5×10^{-5} and 3.2×10^{-5} M, respectively. Several lines of evidence indicate that this derivative binds to at least two sites on lima bean lectin: (1) [^{14}C]adenine was not completely displaced from the lectin by the spin-labeled analogue on equilibrium dialysis; (2) addition of adenine caused a shift in the bound ESR spectrum of the spin-labeled adenine; and (3) the bound ESR spectrum was abolished only by a combination of benzyladenine and 2-toluidinenaphthalene-6-sulfonic acid. Both of these ligands bind at the adenine binding site; however, at higher concentrations, the ligands also bind to the ANS sites [Robets, D. D., & Goldstein, I. J. (1983) *J. Biol. Chem. 258*, 13820-13824]. The distance between the carbohydrate and adenine binding sites on lima bean lectin from ESR studies of spin-labeled adenine I and a *N*-acetyl-D-galactosamine derivative must be greater than 12 Å since no indication of nitroxide-nitroxide interactions was observed.

The carbohydrate binding specificity of plant lectins has been extensively studied and is well described [for reviews, see Goldstein and Hayes (1978), Lis and Sharon (1981), and Goldstein and Poretz (1986)]. Additionally, it has been observed that several lectins also contain binding sites for hydrophobic ligands. For example, Con A[1] is known to bind TNS (Yang & Edelman, 1974) and the phytohormone indoleacetic acid (Edelman & Wang, 1978). Binding of such ligands has since been found to be a common feature of several legume and nonlegume lectins (Roberts & Goldstein, 1983a).

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[1] Abbreviations: Con A, concanavalin A; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonic acid; ANS, 1,8-anilinonaphthalenesulfonic acid; LBL, lima bean lectin; PBS, phosphate buffered saline; ESR, electron spin resonance; PHA-E and PHA-L, *Phaseolus vulgaris* isolectins erythroagglutinin and leukoagglutinin, respectively; GS I-A_4 and GS I-B_4, *Griffonia simplicifolia* isolectins A_4 and B_4, respectively; SBA, soybean agglutinin.

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In particular, the lectin from lima beans (*Phaseolus lunatus*) that binds both TNS and ANS was shown to have at least two classes of hydrophobic binding sites with apparent stoichiometries of one and four per tetramer, for the high- and low-affinity sites, respectively (Roberts & Goldstein, 1982).

Binding of nonphysiological hydrophobic ligands is a common feature of many proteins. Of interest to us is the finding that lima bean lectin specifically binds adenine as well as cytokinins, phytohormones that are N^6-derivatives of adenine (Roberts & Goldstein, 1983b). A single site per tetramer correlates with a high-affinity site for adenine/TNS. The high-affinity adenine binding site and the four low-affinity ANS binding sites have been shown to be thermodynamically linked. High-affinity sites for adenine are also found on several other lectins (Roberts & Goldstein, 1983b). Although many hypotheses have been presented, the physiological role of plant lectins is still unknown. Most speculations have centered on the sugar binding properties of these proteins. The possibility that lectins bind hormones may help elucidate the function of these proteins in vivo. Electron spin resonance (ESR) of spin-labeled reporter groups provides a sensitive method of measuring protein–ligand interactions at the molecular level. For example, detailed information on protein–carbohydrate interactions was obtained by using spin-labeled glycosides and several legume lectins (Goldstein et al., 1985; Berliner et al., 1986). Furthermore, the ESR technique allows a direct means of determining protein–ligand dissociation constants and potentially allows the measurement of intersite distances (Berliner, 1978, 1980). It was therefore of interest to explore the binding properties of a spin-labeled N^6 adenine derivative as a probe of hydrophobic ligand binding sites on a series of adenine binding lectins.

Materials and Methods

Lima bean lectin (Roberts et al., 1982) and *Griffonia simplicifolia* I isolectins A_4 and B_4 (Delmotte & Goldstein, 1980) were prepared as described. *Amphicarpaea bracteata* lectin was isolated by affinity chromatography on Synsorb A (Chembiomed, Alberta, Canada) and eluted with 0.1 M *N*-acetyl-D-galactosamine in PBS. *Dolichos biflorus* lectin was a gift of Dr. M. E. Etzler (University of California, Davis). Soybean agglutinin (SBA) and *Phaseolus vulgaris* isolectins E_4 and L_4 were obtained from Dr. E. Chu of E Y Labs (San Mateo, CA). Lectin concentrations expressed as intact tetramer were determined spectrophotometrically at 280 nm by using $E^{1\%}_{280}$ as follows: lima bean lectin, 12.3 (Gould & Scheinberg, 1970); *G. simplicifolia* I-A_4 and B_4, 14.1 (Hayes & Goldstein, 1974); *D. biflorus*, 11.9 (Etzler et al., 1981); SBA, 12.8 (Lotan et al., 1973); *A. bracteata*, 13.6; *P. vulgaris* isolectins, 11.8 (Leavitt et al., 1972).

Synthesis of N^6-(2,2,6,6-Tetramethyl-1-oxypiperidin-4-yl)adenine (I). 4-Amino-2,2,6,6-tetramethylpiperidino-1-oxy was obtained from Eastman Kodak (Rochester, NY). 6-Chloropurine was purchased from Aldrich Chemical Co. (Milwaukee, WI). A solution of 0.15 g (0.97 mmol) of 6-chloropurine, 1.5 mL of Methyl Cellosolve, and 0.5 g (2.9 mmol) of 4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy was heated under reflux in an oil bath for 2.5 h. The reaction mixture was evaporated to dryness under high vacuum and the residue extracted with ether. The ether-insoluble residue was purified by silica gel column chromatography with 9:1 (v/v) chloroform–methanol as the developing solvent. Fractions having an R_f of 0.5 were combined and evaporated to a red syrup, which crystallized from ethanol to yield analytically pure, red crystals in 68% yield; mp 222–223 °C. Anal. Calcd for $C_{14}H_{19}N_6O$: C, 58.31; H, 6.99; N, 29.15; O, 5.55. Found: C, 58.23; H, 7.26; N, 28.8; O, 5.61.

The spin-labeled glycoside 1-[4-[[(methyl 2-deoxy-α-D-galactopyranosid-2-yl)amino]carbonyl]phenyl]-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea was available from a previous study (Berliner et al., 1986). ANS was purchased from Eastman Kodak and recrystallized from water as the magnesium salt. *N*-Acetyl-D-galactosamine and *N*-acetyl-D-glucosamine were purchased from Pfanstiehl (Waukegan, IL). All other reagents were from Sigma (St. Louis, MO).

EPR spectra were recorded on a Varian E-4 spectrometer using quartz microcapillary tubes (Berliner, 1978). Typical conditions were as follows: microwave frequency, 9.53 GHz; microwave power, 20 mW; modulation frequency, 100 MHz; applied field, 3395 G; scan range, 100 G; modulation amplitude, 0.5 G; time constant, 0.25–0.5 s; temperature, 25 ± 2 °C. Lectin spin-label dissociation constants were determined as reported previously (Goldstein et al., 1985). The K_d values are accurate to within 15% in most cases. "High-gain" spectra were measured at 5–10 times the receiver gain and 2–4 times the modulation amplitude in order to accurately resolve hyperfine extrema in immobilized spectra. The hyperfine extrema $2T_{\parallel}$ are estimated to within ±0.5 G and are denoted by arrows in the figures.

Fluorescence titrations were performed on an SLM 8000 photon-counting spectrofluorometer in ratio mode. All titrations were done at 25 °C with excitation at 420 nm and bandwidth of 2 nm and emission at 480 nm and bandwidth of 4 nm. A 1.0-mL sample of lectin containing 1×10^{-4} M ANS was titrated with small volumes of spin-labeled ligand. A parallel blank titration was performed by using buffer with ANS in the absence of lectin. The relative ANS fluorescence was calculated by correcting for the blank fluorescence and normalizing to lectin plus ANS fluorescence in the absence of extrinsic ligand (Roberts et al., 1986).

Equilibrium dialysis employing a competition assay using [^{14}C]adenine was performed in microdialysis cells (Technilab Instruments, Pequannock, NJ). Briefly, 0.3-mL samples of lectin (1.0 mg/mL) and buffer containing 5×10^{-6} M [^{14}C]adenine and varying concentrations of competing ligand were equilibrated for 24 h at 25 °C. Adenine concentrations were determined by scintillation counting of 100-μL aliquots in duplicate.

Results

The spin-labeled adenine derivative I was tested for its ability to bind to several adenine binding lectins using fluorescence and ESR spectroscopy. Of these, four lectins, lima bean, *D. biflorus*, and the *P. vulgaris* isolectins erythroagglutinin (E) and leukoagglutinin (L), bound the spin label. Previous work had shown that the binding of adenine to lima bean lectin was linked to the binding of the hydrophobic ligand ANS (Roberts & Goldstein, 1983b). Increased binding of ANS with a concomitant increase in the ANS fluorescence was used to assay for binding of various adenine derivatives to this lectin. However, such titrations with the spin-labeled adenine caused a *decrease* in ANS fluorescence with LBL and *D. biflorus* lectins, suggesting either negative heterotropic interactions or direct displacement of ANS by this ligand (Figure 1).

ESR provided a more direct method of measuring the binding of the spin-labeled analogue, independent of possible heterotropic interactions with ANS. Apparent dissociation constants were calculated from the decrease in the free label peak heights in the absence and presence of ligand (Table I). The degree of "immobilization" of the spin label, i.e., the decrease in tumbling rate of bound versus free ligand, is in-

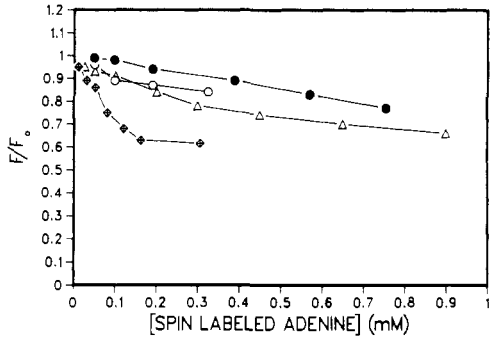

FIGURE 1: Decrease in ANS fluorescence by spin-labeled adenine in the presence of lectins. Lima bean lectin, 0.4 mg/mL (◈), *D. biflorus*, 0.7 mg/mL (△), PHA-E, 0.4 mg/mL (●), and PHA-L, 0.4 mg/mL (○), containing 100 μM ANS in 1.0 mL were titrated with spin-labeled ligand at 25 °C while ANS fluorescence emission was observed at 480 nm (λ_{ex} = 420 nm). The corrected fluorescence (F) was normalized by dividing by the fluorescence of lectin-bound ANS (F_0) in the absence of ligand.

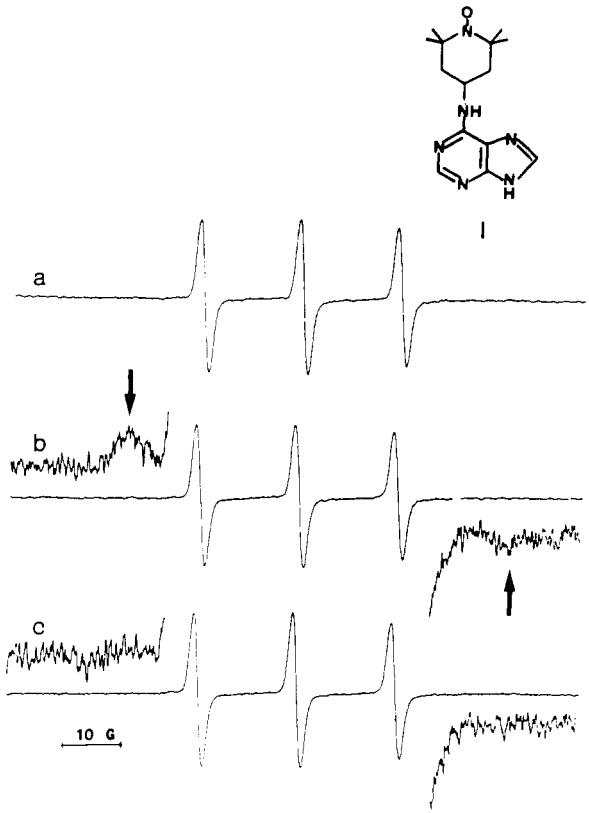

FIGURE 2: Titration of *D. biflorus* lectin with spin-labeled adenine: (a) Free spin label (87 μM); (b) spin label in (a) plus lectin (87 μM); (c) sample b plus 2.0 mM adenine. Hyperfine extrema in the bound spectra are denoted by a downward arrow. Complete displacement was confirmed by the absence of hyperfine extrema in the high-gain spectrum. Conditions were 0.1 M sodium phosphate, 0.15 M NaCl, 1.0 mM $CaCl_2$, and pH 7.1. High-gain spectra were measured at 10 times gain and 2–4 times modulation amplitude.

dicated by measurement of the hyperfine splitting extrema ($2T_{\parallel}$). In general, the greater the hyperfine extrema separation, the slower the piperidinoxy reporter group is tumbling. In every case, the label was strongly immobilized, with a $2T_{\parallel}$ close to 68 G (Table I). This splitting was most dramatically apparent in the high-gain spectra in the figure insets.

The affinity of the spin-labeled adenine derivative varied greatly among the lectins. The apparent dissociation constant calculated from ESR spectra for the *D. biflorus* lectin (Figure 2) was 2.2×10^{-4} M, 100-fold larger than that for adenine, 2×10^{-6} M (Roberts & Goldstein, 1983b). A similar K_d was found for PHA-L (1.9×10^{-4} M) (Figure 3). Addition of excess adenine almost completely displaced the spin label in the two lectins above (Figure 2c and 3c), indicating competitive binding at the adenine binding site. On the other hand, the addition of ANS or *N*-acetyl-D-galactosamine had no effect on the bound ESR spectra. PHA-E bound the spin label with an apparent dissociation constant of 5.0×10^{-5} M. This is approximately 6 times weaker than adenine ($K_d = 8.6 \times 10^{-6}$ M). In the presence of 3.0 mM adenine, the spin label was 70% displaced (Figure 3).

Interestingly, lima bean lectin bound the adenine spin label with an affinity in the same range as adenine, benzyladenine, various cytokinins (Roberts & Goldstein, 1983b), or the high-affinity TNS site (Roberts & Goldstein, 1982). Addition of adenine (2.4 mM) (Figure 4c), ANS (3.0 mM, Figure 4d), and benzyladenine (1.8 mM, Figure 4f) each affected the ESR spectrum in a manner indicating displacement of the spin label. Displacement was incomplete however, and the limited solubility of the compounds in the buffer made it difficult to

Table I: Binding Parameters of Spin-Labeled Adenine to Several Lectins

lectin	K_d (M)	$2T_{\parallel}$ (G)	K_d (M), adenine[a]
lima bean	3.2×10^{-5}	68.5	1.2×10^{-5}
D. biflorus	2.2×10^{-4}	68.0	2.0×10^{-6}
PHA-E_4	5.0×10^{-5}	68.0	8.6×10^{-6}
PHA-L_4	1.9×10^{-4}	68.2	ND[b]

[a] Taken from Roberts and Goldstein (1983b). [b] ND, not determined.

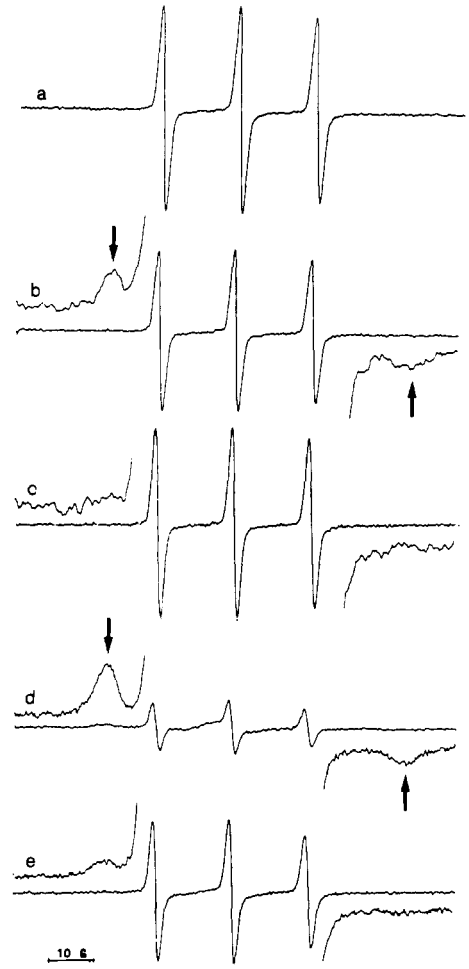

FIGURE 3: ESR titration of *P. vulgaris* isolectins with adenine spin label: (a) Free spin label (128 μM); (b) spin label in (a) plus 27 μM PHA-L; (c) sample b plus 3.0 mM adenine; (d) spin label in (a) plus 77 μM PHA-E; (e) sample d plus 3.3 mM adenine.

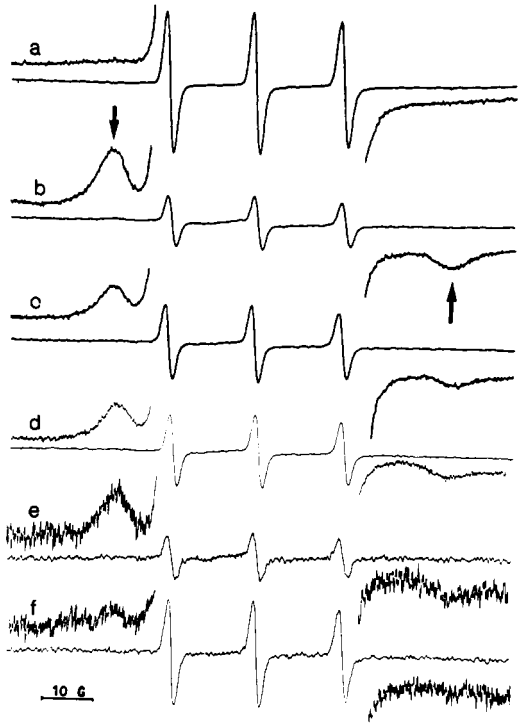

FIGURE 4: ESR titration of lima bean lectin with spin-labeled adenine: (a) Free spin label (168 μM); (b) spin label in (a) plus 156 μM lectin; (c) sample b plus 2.4 mM adenine; (d) sample b plus 3.0 mM ANS; (e) lectin (95 μM) plus spin label (43 μM); (f) sample e plus 1.8 mM benzyladenine.

achieve large excesses of these ligands. On the other hand, complete displacement was attained by the addition of saturating quantities of benzyladenine plus TNS.

In order to further examine the relationships between adenine and spin-labeled adenine I binding to LBL, several additional equilibrium titrations were performed (data not shown): (1) An ESR titration of LBL with spin-labeled adenine I yielded a $K_d = 32 \pm 3$ μM with a stoichiometry of 1.0–2.4 mM ligand concentration. (2) The same titration as above was repeated in the presence of 1.0 mM adenine. The resulting K_d was raised to 172 μM, which rules out a unique, common site model for both adenine and its spin-labeled counterpart. The data further suggest that additional, weaker binding loci *become available* for spin label I in the presence of adenine; furthermore, the $2T_{\parallel}$ values for the bound spin label decreased by 1.5 G upon adenine binding, which correlates with a change in the nitroxide binding environment. (3) Equilibrium dialysis experiments in which spin-labeled adenine (up to 500 μM) was titrated against [^{14}C]adenine (5 μM) plus (8.6 μM) LBL also showed that both ligands bound simultaneously, as only a partial displacement of bound radiolabel was observed.

Thus, these results confirm that both ligands bind simultaneously according to some linked noncompetitive inhibition model, such as the scheme

$$\begin{array}{ccc} \text{LBL} & \overset{K_2}{\rightleftharpoons} & \text{LBL—I} \\ {\scriptstyle K_1}\downarrow\uparrow & & \uparrow\downarrow{\scriptstyle K_3} \\ \text{Ad—LBL} & \underset{K_4}{\rightleftharpoons} & \text{Ad—LBL—I} \end{array}$$

where Ad is adenine and the dissociation constants, which have been determined from this work, are $K_1 = 12 \pm 2$ μM, $K_2 = 32 \pm 3$ μM, and $K_4 = 172 \pm 26$ μM. The remaining constant, $K_3 = K_1K_4/K_2$, may be calculated at 65 ± 26 μM. It is also interesting to note that the fluorescence results in Figure 1, which showed that spin label I binding reduced the affinity for LBL for ANS, were completely consistent with ESR results in parts b and d of Figure 4, which demonstrated reduced affinity of LBL for spin label I in the presence of ANS. It also appears that in the case of ANS (and also benzyladenine) these results support some type of a noncompetitive inhibition model with spin label I. The binding behavior of PHA-E appears to be similar to LBL (Figure 3d,e), while PHA-L and *D. biflorus* showed apparent competitive behavior between adenine and spin label I.

The fact that TNS binds to two classes of sites (Roberts & Goldstein, 1982) does not allow for distinction of the exact mode of binding by the spin-labeled adenine analogue. The effect of ANS on binding also leaves open the possibility of either negative heterotropic interactions or direct competition between the ligands for the same site. It is very possible that the molecule binds to two sites on the protein.

Nitroxide–nitroxide interactions can be used to determine distances between binding sites of ligands containing the nitroxide reporter group. Such an attempt was made in a "double-label" experiment with lima bean lectin. Assuming pairwise interaction between spin labels in an immobilized system, i.e., strongly immobilized by lectin, which is dominated by dipolar interactions, the separation between two unpaired spins can be determined (Berliner, 1980). A spin-labeled glycoside, 163 μM 1-[4-[[(methyl 2-deoxy-α-D-galactopyranosid-2-yl)amino]carbonyl]phenyl]-3-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)-2-thiourea and 148 μM adenine spin label I were added to 59 μM LBL. Under these conditions at least one saccharide binding site per tetramer was saturated while the spin-labeled adenine site was ca. 80% occupied. The resulting spectrum was compared on a Varian E-935 data system with that of each ligand bound separately. No significant broadening or splitting of hyperfine lines indicative of nitroxide–nitroxide interaction was observed, suggesting that the distance between the nitroxide moieties on the reporter groups at the hydrophobic and carbohydrate binding sites is greater than 12 Å (Berliner, 1978, 1980).

Discussion

Previously we reported on the hydrophobic binding properties of the lima bean lectin (Roberts & Goldstein, 1982, 1983a,b). This phenomenon was found to be of rather general occurrence in that other lectins also displayed this effect. We have attempted to further describe the adenine binding site in several lectins, using the spin-labeled derivative I. Considerable variation exists in binding of the spin label to lectins that bind adenine. *D. biflorus* and PHA-L appear to bind the probe specifically at the adenine site, although with reduced affinity relative to adenine. On the other hand, binding of the spin-labeled adenine to PHA-E and LBL suggests the presence of a secondary, weaker binding site for the probe, as well as the possibility of negative cooperativity between the adenine spin label I and ANS sites. However, binding of this ligand may also sterically interfere with the binding of ANS to its hydrophobic site.

It is interesting to compare the binding to LBL of the spin-labeled adenine derivative I with other derivatives as studied fluoromerically (Roberts et al., 1986). The amino group at the N^6-position on the purine ring is required for binding; however, several modifications are apparently acceptable at this position, including the introduction of ethyl groups, isoprenoid moieties (cytokinins), and benzylic groups. While affinity decreased in some cases, an *increase* in ANS fluorescence was always observed. The compounds that behaved most like the spin-labeled adenine (tight binding, re-

duction of ANS fluorescence enhancement) were those modified at the N^9-position by a benzyl or 4-nitrobenzyl group. Evidence that the spin label may bind at more than one type of site includes the following: (a) Equilibrium dialysis using competition of the spin label with [^{14}C]adenine resulted in only 40% inhibition of radiolabel binding at millimolar concentration of spin-labeled adenine. (b) Attempts to competitively displace the spin label with high adenine concentrations (3.0 mM) failed to completely eliminate the bound ESR signal. The signal was abolished only in the presence of benzyladenine and TNS, ligands known to bind to both types of hydrophobic sites at high concentrations. However, addition of adenine caused a change in $2T_{\parallel}$ of 1.5 G. This suggested that displacement by adenine caused the probe to shift to a secondary site. It is possible that both heterotropic effects and heterogeneous binding by the ligand occur.

The symmetry and location of the adenine binding site within the lima bean lectin tetramer are of great interest. The double-spin-label experiment provides only an estimate of nitroxide–nitroxide separation. This is, the two spin labels may be oriented "head-to-head" or "tail-to-tail" or aligned in parallel. In the first two cases, the intramolecular distance between the NO group and the ligand moiety (i.e., adenine or galactosaminyl ring) must be factored into any distance estimate between the corresponding ligand binding sites. Thus, the assertion that the minimum distance between the two sites exceeds 12 Å is valid in all cases except that of the tail-to-tail orientation. In this situation, the adenine and glycoside moieties may bind to adjacent (or abutting) sites while the NO moieties were $\geq$12 Å apart. Previous fluorescence energy transfer studies indicated that the ANS site is 28 Å from the sulfhydryl group essential for carbohydrate binding (Kella et al., 1984). It is possible the ANS and adenine binding sites are close to one another in the lectin.

Although not completely characterized, the hydrophobic binding properties of many plant lectins appear to be similar in that many bind and enhance the fluorescence of ANS and TNS (Roberts & Goldstein, 1983a). Several lectins specifically bind adenine and produce a concomitant enhancement of ANS fluorescence (Roberts & Goldstein, 1983b). The similar behavior displayed by these proteins with the spin-labeled analogue suggests conservation, not only of specific hydrophobic sites but of specific interactions between these sites. It was also apparent that modifications of the purine ring drastically affect binding of other hydrophobic ligands whether via allosteric effects or direct competition for sites. These lectins bind adenine in a specific manner with a stoichiometry of four per tetramer, additional nonspecific adenine binding (on, e.g., LBL or PHA-E) may simply be a coincidence. In all cases these sites appear to be independent of carbohydrate binding.

The difference in binding between PHA-E and PHA-L for ANS has been reported previously (Roberts & Goldstein, 1983a). Subunits E_4 and E_3L showed cooperative binding of ANS, whereas isolectins E_2L_2, EL_3, and L_4 did not. Further dissimilarities have been observed with spin-labeled adenine: PHA-E binds the ligand 25 times more tightly than PHA-L and appeared to exhibit a more complex binding mechanism for the spin label. It is possible that, in vivo, these differences may serve regulatory functions in the isolectins.

Conclusions

The significance of these results is profound. A survey of eight lectin samples (six species and their isozymes) with a newly synthesized spin-labeled analogue of adenine I showed that four bound the paramagnetic probe uniquely: LBL, PHA-E, PHA-L, and *D. biflorus*. On the other hand, GSI-A_4 and B_4 isolectins as well as SBA and *A. bracteata* lectins did not bind the probe.[2] More subtle structural differences are suggested by the adenine displacement studies where label I was essentially completely displaced from *D. biflorus* and PHA-L but not from PHA-E. It would be interesting and valuable in the future to correlate the binding interactions above with the physiological properties with analogous phytohormones on these lectins.

Hydrophobic binding appears to be more complex than carbohydrate binding in the lectins studied. It should be noted that in several cases adenine binds with a much greater affinity than the haptenic sugars used to define lectin specificity. Whether these adenine and other nonpolar sites act in conjunction with sugar binding and what these functions may be are still unknown.

Added in Proof

Using the photoaffinity probe 8-azidoadenine, we have succeeded in the specific labeling of the adenine binding sites of the lima bean and *P. vulgaris* E_4 lectins. Labeled peptides isolated from each protein were shown to exhibit homologous sequences, suggesting a possible conserved physiological role for this site in certain legume lectins (Maliarik & Goldstein, 1988).

Acknowledgments

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Registry No. I, 61468-65-3; adenine, 73-24-5; 4-amino-2,2,6,6-tetramethylpiperidinyl-1-oxy, 14691-88-4; 6-chloropurine, 87-42-3.

References

Berliner, L. J. (1978) *Methods Enzymol. 49G*, 418–480.
Berliner, L. J. (1980) in *Spectroscopy and Biochemistry* (Bell, J. E., Ed.) Vol. 2, CRC, West Palm Beach, FL.
Berliner, L. J. Musci, G., Maliarik, M. J., Plessas, N. R., & Goldstein, I. J. (1986) *Biochemistry 25*, 4457–4461.
Delmotte, F. M., & Goldstein, I. J. (1980) *Eur. J. Biochem. 112*, 219–223.
Edelman, G. M., & Wang, J. L. (1978) *J. Biol. Chem. 253*, 3016–3022.
Etzler, M. E., Gupta, S., & Borrebaeck, C. (1981) *J. Biol. Chem. 256*, 2367–2370.
Goldstein, I. J., & Hayes, C. E. (1978) in *Adv. Carbohydr. Chem. Biochem. 35*, 127–340.
Goldstein, I. J., & Poretz, R. (1986) in *The Chemistry and Biology of Plant Lectins* (Liener, I. E., Sharon, N., & Goldstein, I. J., Eds.) pp 33–247, Academic, New York.
Gould, N. R., & Scheinberg, S. L. (1970) *Arch. Biochem. Biophys. 137*, 111.
Hayes, C. E., & Goldstein, I. J. (1974) *J. Biol. Chem. 249*, 1904.
Kella, N. K. D., Roberts, D. D., Shafer, J. A., & Goldstein, I. J. (1984) *J. Biol. Chem. 259*, 4777–4281.
Leavitt, R. D., Felsted, R. L., & Backus, N. B. (1972) *J. Biol. Chem. 252*, 2961–2966.
Lis, H., & Sharon, N. (1981) in *The Biochemistry of Plants* (Marcus, A., Ed.) pp 372–447, Academic, New York.
Lotan, R., Seigelman, N. W., Lis, N., & Sharon, N. (1973) *J. Biol. Chem. 299*, 1219–1224.
Maliarik, M. J., & Goldstein, I. J. (1988) *J. Biol. Chem. 263*, 11274–11279.

[2] Yet SBA binds adenine and ANS (Roberts & Goldstein, 1983a,b).

Roberts, D. D., & Goldstein, I. J. (1982) J. Biol. Chem. 257, 11274–11277.
Roberts, D. D., & Goldstein, I. J. (1983a) Arch. Biochem. Biophys. 224, 479–484.
Roberts, D. D., & Goldstein, I. J. (1983b) J. Biol. Chem. 258, 13820–13824.
Roberts, D. D., Etzler, M. E., & Goldstein, I. J. (1982) J. Biol. Chem. 257, 9198–9204.
Roberts, D. D., Arjunan, P., Townsend, L. B., & Goldstein, I. J. (1986) Phytochemistry 25, 589–593.
Yang, D. C. H., Gall, W. E., & Edelman, G. M. (1974) J. Biol. Chem. 249, 7018–7023.

The Structural Basis for Substrate-Induced Changes in Redox Potential and Spin Equilibrium in Cytochrome P-450$_{CAM}$[†]

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ABSTRACT: The crystal structures of cytochrome P-450$_{CAM}$ complexed with the alternative substrates norcamphor and adamantanone have been refined at 2.0-Å resolution and compared with the native, camphor-bound form of the enzyme. Norcamphor lacks the 8-, 9-, and 10-methyl groups of camphor. Thus, specific interactions between these groups and phenylalanine 87 and valines 247 and 295 are missing in the norcamphor complex. As a result, norcamphor binds about 0.9 Å further from the oxygen-binding site than does camphor, which allows sufficient room for a water molecule or hydroxide ion to remain coordinated with the heme iron atom. The larger adamantanone occupies a position closer to that of camphor and, as in the camphor-bound enzyme, the heme iron remains pentacoordinate with no solvent molecule coordinated as a sixth ligand. A comparison of crystallographic temperature factors indicates that norcamphor is more "loosely" bound than are either camphor or adamantanone, as might be expected from the relative sizes of the different substrates. The looser fit of norcamphor in the active-site pocket results in a less specific pattern of hydroxylation. The presence of an aqua ligand is the likely structural basis for the norcamphor–P-450$_{CAM}$ complex having both a lower redox potential and higher percentage of low-spin heme than do either the camphor–P-450$_{CAM}$ or adamantanone–P-450$_{CAM}$ complexes.

Cytochromes P-450 are a group of *b*-type heme proteins that catalyze the hydroxylation of aromatic and aliphatic substrates in a variety of metabolic processes. The most extensively studied P-450 is the camphor hydroxylase from *Pseudomonas putida*, or P-450$_{CAM}$ (Wagner & Gunsalus, 1982; Gunsalus et al., 1974; Debrunner et al., 1978; Gunsalus & Sligar, 1978). P-450$_{CAM}$ is a 45 000-Da polypeptide containing a single ferric protoporphyrin IX. As with many *b*-type heme proteins, P-450$_{CAM}$ equilibrates between low-spin, $S = {}^1/_2$, and high-spin, $S = {}^5/_2$, states (Sharrock et al., 1976; Tsai et al., 1970). The binding of the substrate, camphor, shifts the spin equilibrium toward the high-spin form and also shifts the redox potential from about −300 to −170 mV (Philson, 1976; Sligar, 1976; Sligar & Gunsalus, 1976; Fisher & Sligar, 1985). A structural basis for these changes has been provided by a comparison between the substrate-free and -bound crystal structures (Poulos et al., 1985, 1986, 1987). In the substrate-free structure, the camphor pocket is filled with solvent molecules, and one of these, a water molecule or hydroxide ion, coordinates with the heme iron atom. However, in the camphor-bound structure, the heme iron is pentacoordinate and no ordered solvent molecules are observed at the active site. It is likely that the lower redox potential of the substrate-free structure is due to additional stabilization of the ferric (Fe^{3+}) state as a result of the higher dielectric environment provided by the active-site solvent molecules. Since the low-spin state is favored by the substrate-free enzyme, we believe that the strong-field hydroxide ligand, OH^-, is axially coordinated with the heme iron atom, rather than the weak-field ligand, H_2O.

Fisher and Sligar (1985) have further analyzed the relationship between redox potential and spin equilibrium by measuring both the redox potentials and the spin equilibria of various substrate–P-450$_{CAM}$ complexes and have found a linear free energy relationship between the two. The substrate norcamphor is metabolized with relatively low regiospecificity, giving at least three detectable hydroxylated products (Atkins & Sligar, 1988). Binding of this substrate results in an enzyme with a low redox potential (−206 mV) and with a significant population fraction (54%) of the heme iron in the low-spin state. In contrast, substrates such as adamantanone and camphor, from each of which a single hydroxylated product is formed, shift the heme iron spin equilibrium almost entirely (>95%) to high spin and exhibit significantly higher redox potentials, −175 and −170 mV, respectively. Interestingly, there is no readily discernible relationship between binding constant and spin state (Fisher & Sligar, 1985). We have postulated (Poulos et al., 1986) that these substrate-associated shifts in redox potential and spin equilibrium are controlled by the degree of hydration at the active site and by water

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