Demonstration of a Conserved Histidine and Two Water Ligands at the Mn^{2+} Site in Diocleinae Lectins by Pulsed EPR Spectroscopy†

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ABSTRACT: Lectins from the Diocleinae subtribe, including *Canavalia brasiliensis*, *Canavalia bonariensis*, *Canavalia grandiflora*, *Cratylia floribunda*, *Dioclea grandiflora*, *Dioclea guianensis*, *Dioclea rostrata*, *Dioclea violacea*, and *Dioclea virgata*, have been recently isolated and characterized in terms of their carbohydrate binding specificities. Although all of the lectins are Man/Glc specific, they possess different biological activities. In the present study, electron paramagnetic resonance (EPR) spectroscopy demonstrates that all nine Diocleinae lectins contain Mn^{2+}. The spectra of *C. floribunda* and *D. rostrata* suggest Mn^{2+} site symmetry different from that of the other seven lectins. However, electron spin−echo envelope modulation (ESEEM) spectroscopy indicates that all nine lectins are coordinated to a histidyl imidazole, with similar electron−nuclear coupling to the Mn^{2+}-bound imidazole nitrogen. ESEEM also demonstrates ligation of two water molecules to Mn^{2+} in all nine Diocleinae lectins. Thus, the EPR and ESEEM data indicate the presence of a Mn^{2+} binding site in the above Diocleinae lectins with a conserved histidine residue and two water ligands.

Lectins are a diverse group of proteins that reversibly bind to specific carbohydrate epitopes. Lectin−carbohydrate interactions have been implicated in a variety of important biological processes including infection, neural development, cell differentiation, and proliferation (cf. refs *1*, and *2*). Some of the best characterized lectins are those isolated from plants. The well-defined carbohydrate binding specificities of plant lectins have made them valuable tools for isolating and characterizing cell surface carbohydrates and glycoconjugates (*3*), and for exploring the membrane properties of both normal and transformed cells (cf. ref *4*).

Leguminous plants provide a rich source of lectins with varied carbohydrate binding specificities (*5, 6*).[1] One of the best known is the jack bean lectin, concanavalin A (ConA), which is a member of the Diocleinae subtribe. ConA has been well studied in terms of its carbohydrate (cf. ref *5*) and metal ion binding properties (cf. ref *7*). In addition, the X-ray crystal structure of this lectin is known to 1.75 Å resolution (*8*). ConA has long been known to bind Man and Glc residues (*9*). However, more recent studies have shown that the lectin binds with much higher affinity to 3,6-di-*O*-(D-mannopyranosyl)-D-mannopyranoside, a trisaccharide found in the core region of all asparagine-linked (N-linked) carbohydrates (cf. ref *10*). This high-affinity interaction is largely responsible for the binding of ConA to cell surface glycoconjugate receptors, and hence the biological properties of the lectin.

Recently, nine new lectins from the Diocleinae subtribe, *Canavalia brasiliensis*, *Canavalia bonariensis*, *Canavalia grandiflora*, *Cratylia floribunda*, *Dioclea grandiflora*, *Dioclea guianensis*, *Dioclea rostrata*, *Dioclea violacea*, and *Dioclea virgata*, have been isolated and characterized in terms of their carbohydrate binding specificities. Hemagglutination inhibition and isothermal titration microcalorimetry studies have shown that all are Man/Glc-binding lectins, and all have conserved high affinity for the core trimannoside, similar to ConA (*11*).

However, despite their similar carbohydrate binding properties, the above Diocleinae lectins possess different biological activities such as histamine release from rat peritoneal mast cells (*12*), lymphocyte proliferation and interferon production (*13*), peritoneal macrophage stimulation and inflammatory reaction (*14*), and induction of paw edema and peritoneal cell immigration in rats (*15*). Indeed, the

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[1] Abbreviations: ConA, lectin from jack bean (*Canavalia ensiformis*); *C. brasiliensis*, *Canavalia brasiliensis*; *C. bonariensis*, *Canavalia bonariensis*; *C. floribunda*, *Cratylia floribunda*; *D. rostrata*, *Dioclea rostrata*; *D. guianensis*, *Dioclea guianensis*; *D. virgata*, *Dioclea virgata*; *D. violacea*, *Dioclea violacea*; *D. grandiflora*, *Dioclea grandiflora*; *C. grandiflora*, *Canavalia grandiflora*; EPR, electron paramagnetic resonance; ESEEM, electron spin−echo envelope modulation.

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relative histamine release activities induced by the lectins have recently been found to correlate with their differential affinities for a biantennary complex carbohydrate (*11*). Thus, it is important to understand the molecular basis of the different biological activities of these lectins.

Metal ions appear to play an important role in the carbohydrate binding properties of the Diocleinae lectins, and hence their biological activities. The carbohydrate binding activity of ConA has been shown to be regulated by the binding of transition metal ions, including Mn^{2+} and Ca^{2+}, to the protein (cf. refs *7*, and *16*). X-ray crystallography shows that Mn^{2+} in ConA is coordinated to five oxygen atoms, three from carboxyl side chains (Asp 10, Asp 19, Glu 8) and two from water molecules, and one nitrogen atom from an imidazole side chain (His 24) (*8*). The ligands of Ca^{2+} consist of seven oxygen atoms (Asp 10, Asp 19, Tyr 12, Asn 14, and two water molecules). Mn^{2+} and Ca^{2+} share two bridging carboxylates (Asp 10, Asp 19) and are 4.25 Å apart. The Ca^{2+} site is adjacent to the carbohydrate binding site, and binding of Ca^{2+} determines the geometry of the protein at the carbohydrate binding site (*8, 16, 17*). Indeed, binding of Mn^{2+} and Ca^{2+} induces a nearby cis−trans isomerization in the polypeptide structure, leading to full saccharide binding activity of the lectin (*7, 16*).

Much less is known about the metal ion binding properties of the other Diocleinae lectins. The X-ray crystal structures of *C. brasiliensis* (*18*) and *D. grandiflora* (*19*) lectins have recently been reported, and show the presence of two metal ion binding sites in both proteins similar to those in ConA. In addition, complete primary sequence data have been reported for *C. floribunda*, *D. guianensis*, *D. grandiflora*, and *C. brasiliensis* as well as ConA (*20*), and the residues that form the two metal ion binding sites in ConA are conserved in these other lectins. The overall high degree of sequence homology of these lectins suggests that other members of the Diocleinae subtribe may possess relatively conserved sequences including residues involved in metal ion binding.

In the present study, electron paramagnetic resonance (EPR) spectroscopy was used to demonstrate the presence of Mn^{2+} in the following nine Diocleinae lectins: *C. brasiliensis*, *C. bonariensis*, *C. grandiflora*, *C. floribunda*, *D. grandiflora*, *D. guianensis*, *D. rostrata*, *D. violacea*, and *D. virgata*. The spectra of the lectins, in turn, were compared with that of Mn^{2+}−ConA. To obtain further structural information on the Mn^{2+} sites of the lectins, electron spin−echo envelope modulation (ESEEM) spectroscopy (*21*), a pulsed EPR technique, was employed. In ESEEM experiments, interactions between the paramagnet and nearby nuclei give rise to modulations in the electron spin−echo signal decay envelope generated by sequences of microwave pulses (*22, 23*). The frequencies of these modulations are determined by the nuclear Larmor frequency of coupled nuclei and the magnitude of the coupling, and therefore can be used to identify nearby nuclei such as those on a metal ligand and to measure the strength of the interaction. The amplitudes of the modulations, on the other hand, contain information on the number of coupled nuclei and their distance from the paramagnet (*21, 24*).

A previous ESEEM study of Mn^{2+}−ConA demonstrated the presence of one histidine and two water ligands of Mn^{2+} in the protein (*25*), a result that agreed with the X-ray crystal structure of the lectin (*8*). ESEEM data from the present study also indicate the presence of one histidine and two water ligands of Mn^{2+} in the nine new Diocleinae lectins. Thus, present and previous (*25*) ESEEM studies as well as X-ray crystal structures of ConA (*8*), *C. brasiliensis* (*18*), and *D. grandiflora* (*19*) lectins suggest a conserved Mn^{2+} binding site for all known Diocleinae lectins.

MATERIALS AND METHODS

Samples. ConA was purchased from Sigma Chemical Co. Mn^{2+}−ConA was prepared as previously described (*25*). Seeds of all the species used in this study were obtained from the States of Ceara and Rio Grande do Sul, Brazil. Lectins were purified by affinity chromatography using Sephadex G-50, as previously described (cf. ref *26*). Concentrations of the lectins were determined spectrophotometrically at 280 nm and expressed in terms of the monomer. The $A^{1\%}_{1\ cm}$ values at pH 7.2 and subunit molecular weights of the lectins used are as follows: 10.5 and 26 000 (*C. brasiliensis*) (*27*), 11.2 and 30 000 (*C. bonariensis*) (*28*), 11.4 and 29 500 (*C. floribunda*) (*29*), 11.2 and 30 000 (*D. rostrata)* (*30*), 10.5 and 30 000 (*D. guianensis*) (*31*), 10.2 and 30 000 (*D. virgata*) (*32*), 9.8 and 29 500 (*D. violacea*) (*33*), 12.0 and 25 000 (*D. grandiflora*) (*34*), and 10.2 and 26 000 (*C. grandiflora*) (present study). The $A^{1\%}_{1\ cm}$ values at pH 7.2 for *C. brasiliensis*, *C. bonariensis*, *D. guianensis*, and *D. virgata* were from Dam et al. (*11*).

Samples for EPR analysis contained 0.3−0.7 mM protein. Metal analysis showed close to 100% Mn^{2+} bound to ConA, and 20−40% to the other nine Diocleinae lectins. The buffers used were 50 mM Hepes, 150 mM NaCl, 5 mM $CaCl_2$ in H_2O or D_2O, pH 7.2.

Deuterium oxide (99.9 atom percent) was obtained from Aldrich Chemical Co. Buffers and salts were purchased from Sigma Chemical Co.

Spectroscopy. EPR spectra were collected at X-band and at liquid nitrogen temperature on a Varian E-112 spectrometer equipped with a Systron-Donner frequency counter and a PC-based data acquisition program. ESEEM data were collected at liquid (4.2 K) and pumped (∼1.4 K) helium temperatures on a home-built pulsed ESR spectrometer (*35, 36*) using a folded strip-line cavity (*37*). Two-pulse (*22*) data were collected at 2τ (τ is the interval between the first and the second microwave pulses) and with τ incrementing from 150 ns in steps of 3 ns. Three-pulse (*23*) data were collected at the time $2\tau + T$ (T is the interval between the second and third microwave pulses) and with T incrementing from 50 to 60 ns in steps of 5−10 ns. Each data set contained 1024−2048 points. Three-pulse time domain data were Fourier transformed after dead time reconstruction according to the methods of Mims (*38*).

RESULTS AND DISCUSSION

Binding of Mn^{2+} to the Lectins. EPR spectroscopy indicates the presence of bound Mn^{2+} in all nine Diocleinae lectins. The X-band (9 GHz) EPR spectra, centered around $g = 2.0$, are characteristic of high-spin Mn^{2+} ($S = 5/2$) in a near octahedral environment and arise from the $M_s = -1/2$ to $M_s = 1/2$ transition of Mn^{2+}. The spectra contain a major six-line hyperfine pattern due to the ^{55}Mn ($I = 5/2$) nucleus. The EPR spectra of seven of the nine lectins, *C. brasiliensis*,

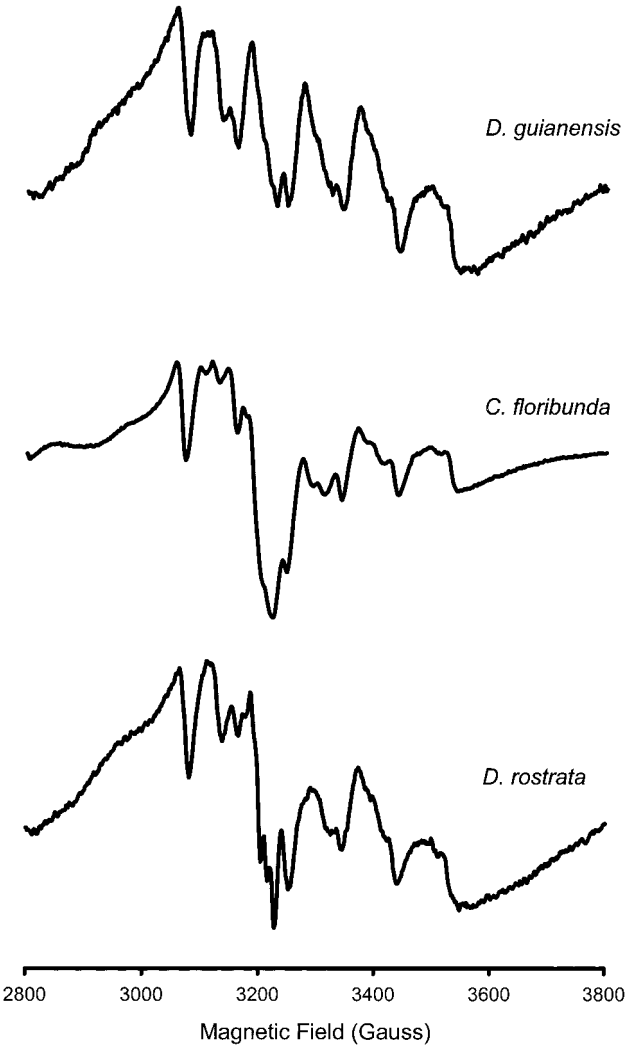

FIGURE 1: Continuous wave EPR spectra of *D. guianensis* (top), *C. floribunda* (middle), and *D. rostrata* (bottom) collected at magnetic field 3300 ± 500 G, microwave frequency 9.23 GHz, microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 8 G, and temperature 77 K.

C. bonariensis, *C. grandiflora*, *D. grandiflora*, *D. guianensis*, *D. violacea*, and *D. virgata*, are nearly identical to that of ConA (*39*, *40*), as exemplified by the spectrum of *D. guianensis* lectin (Figure 1). The relative intensities of the six major Mn hyperfine lines are similar. The lines are separated by 90−92 G; this separation is 92 G in ConA. Further splittings of about 30 G are observed in a few of the six major Mn hyperfine lines. Similar splittings have also been observed in the EPR spectrum of ConA (*39*, *40*). These minor splittings have been attributed to second-order zero-field effects on the $M_s = \pm 1/2$ states (*39*, *40*). Therefore, EPR indicates that seven of the Diocleinae lectins have similar Mn hyperfine coupling and axial (*D*) and rhombic (*E*/*D*) zero-field splitting parameters to those of the Mn^{2+} site in ConA. For ConA, a Q-band (35 GHz) single-crystal EPR study (*40*) has estimated $D = 232$ G and $E/D = 0.185$. Hyperfine coupling and *D* and *E*/*D* values are determined by the symmetry of the Mn^{2+} site and the strength of the Mn^{2+}−ligand interactions. The present results therefore suggest that the Mn^{2+} ligands and the interaction of the Mn^{2+} ligands with their protein environment in these seven lectins are nearly identical to those of ConA, and are consistent with the X-ray crystal structures of *C. brasiliensis* (*18*) and *D. grandiflora* (*19*) lectins.

The EPR spectra of *C. floribunda* and *D. rostrata* lectins (Figure 1) differ from those of the other seven lectins. Altered line intensities and increased splittings were observed between $g = 2.15$ and $g = 1.97$ (3070−3040 G, 9.23 GHz). A spectral simulation (*41*, *42*) has demonstrated that a change in relative intensities of the Mn hyperfine lines in the $M_s = -1/2$ to $M_s = 1/2$ transition spectra, as in the case of the current study, can result from a change in the values of *D* and *E*/*D*. The effect of change in *E*/*D* on the relative intensities of the Mn hyperfine lines increased as *D* was increased from 86 to 198 G (*41*). If the bound Mn^{2+} ions in *C. floribunda* and *D. rostrata* lectins have *D* values comparable to that for ConA (232 G), larger than those used in the spectral simulation (*41*), then the change in the relative intensities of the Mn hyperfine lines found for these two lectins would primarily be the result of a change in the *E*/*D* value, not both *D* and *E*/*D*. Thus, EPR suggests different *E*/*D* and/or *D* values, that is, different symmetry for the Mn^{2+} site in *C. floribunda* and *D. rostrata* lectins as compared to the other seven and ConA. This difference in symmetry can result from a change in the type of ligands and/or their environment. To further compare the Mn^{2+} sites and metal−ligand interactions of *C. floribunda* and *D. rostrata* lectins with those of the other seven Diocleinae lectins and ConA, ESEEM spectroscopy (*21*) was carried out.

Ligation of Histidine to Mn^{2+}. *C. floribunda* and *D. rostrata* lectins show three-pulse ESEEM spectra similar to those of the other seven Diocleinae lectins, exemplified by that of *D. guianesis* (Figure 2). Spectral components at 1.5−1.6, 2.7−2.9, 3.3−3.4, and 4.9−5.1 MHz are resolved for all nine lectins. These spectral features resemble those of ConA, and are attributed to electron−nuclear coupling to the histidyl imidazole nitrogen (N_ϵ) directly coordinated to Mn^{2+} (*25*) on the basis of the following model compound studies. Nearly identical spectra were found for Mn^{2+}−histidine (*43*), Mn^{2+}−imidazole (*25*), and Mn^{2+}−1-methylimidazole complexes (*25*). Replacement of the proton associated with the noncoordinating imidazole nitrogen with a methyl group did not alter the ESEEM spectrum. In contrast, for Cu^{2+}−imidazole complexes where ESEEM spectra arise from electron−nuclear coupling to the noncoordinating (or remote) imidazole nitrogen, such replacement drastically altered the ESEEM spectrum (*44*). Therefore, it can be concluded that the Mn^{2+} site of all nine Diocleinae lectins contains a histidyl imidazole ligand. The presence of a conserved histidine has been suggested by primary sequence comparisons of *D. grandiflora*, *C. brasiliensis*, *C. floribunda*, and *D. guianensis* (*20*). Coordination of a histidine N_ϵ to Mn^{2+} has been demonstrated by the X-ray crystal structures of *C. brasiliensis* (*18*) and *D. grandiflora* (*19*) lectins.

Relatively few ESEEM studies of the interaction of a mononuclear Mn^{2+} center with imidazole nitrogen ligands have been reported (*25*, *43*, *45*). Furthermore, an ESEEM spectral simulation method for obtaining ligand electron−nuclear coupling for $S > 1/2$ systems with small zero-field splitting, such as Mn^{2+}, is not yet available. However, the four-component three-pulse ESEEM spectra of the present nine Diocleinae lectins and of ConA (*25*) resemble those of an $S = 1/2$ center coupled to a single ^{14}N at near exact

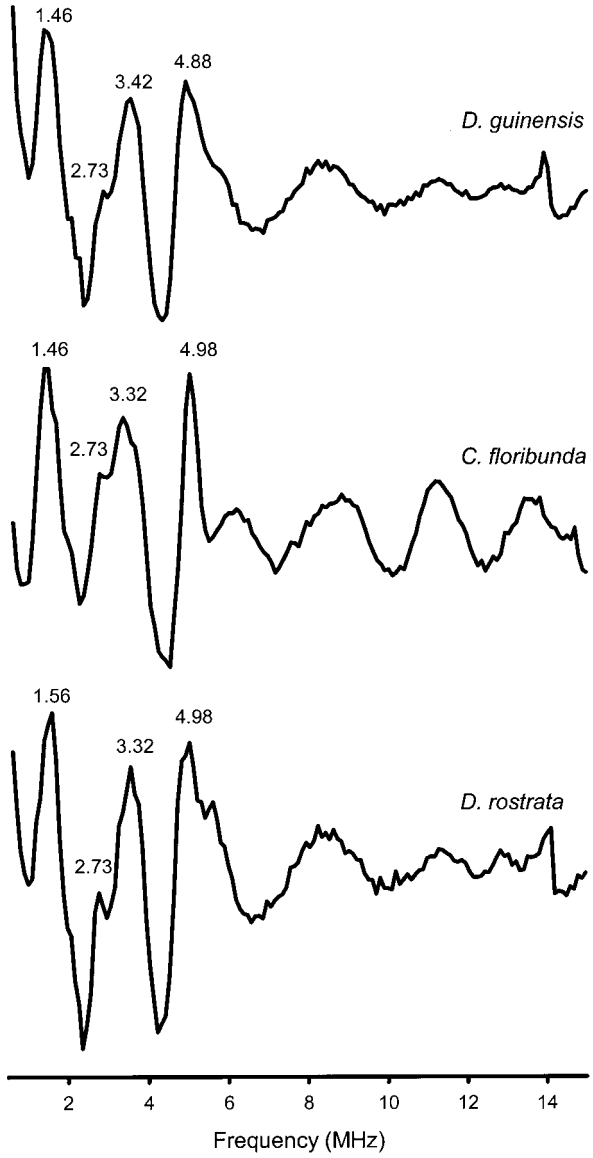

FIGURE 2: Three-pulse ESEEM spectra of *D. guianensis* (top), *C. floribunda* (middle), and *D. rostrata* (bottom). Experimental conditions are microwave frequency 9.16 GHz, magnetic field 3258 G, τ = 207 ns, *T* increment 5 ns, and temperature 4.1 K (*D. guianensis*); microwave frequency 9.64 GHz, magnetic field 3441 G, τ = 202 ns, *T* increment 5 ns, temperature 1.3 K (*C. floribunda*); and microwave frequency 9.28 GHz, magnetic field 3303 G, τ = 205 ns, *T* increment 5 ns, and temperature 4.1 K (*D. rostrata*).

cancellation conditions (*46*) in which the ^{14}N hyperfine coupling is close to twice that of the Zeeman interaction. For these systems, the positions of the three low-frequency lines are related to the zero-field quadrupole transitions for the coupled ^{14}N, and the highest frequency line is related to the nuclear hyperfine coupling. A multifrequency ESEEM study (*25*) has shown that the three low-frequency lines in the ESEEM spectra of the lectins studied show minimal magnetic field dependence, whereas the highest frequency line shifts with about twice the increase in ^{14}N Larmor frequency. These magnetic field dependencies of spectral features are characteristics of a ^{14}N at near exact cancellation conditions coupled to an $S = 1/2$ center. Therefore, on the basis of the similar frequencies of the spectral components for the nine Diocleinae lectins in this study, as well as ConA (*25*), and analogy to $S = 1/2$ systems, it is speculated that electron−nuclear coupling to the Mn^{2+}-bound histidyl imidazole nitrogen is similar in all nine Diocleinae lectins in this study and ConA. For $S = 1/2$ systems, electron−nuclear coupling to a metal-bound imidazole nitrogen is modulated by the charge of the metal and metal−imidazole bond lengths (*47*). Again on the basis of analogy to $S = 1/2$ systems, it is speculated that the Mn^{2+}−histidine bond length is similar in all Diocleinae lectins. For *C. brasiliensis* (*18*), *D. grandiflora* (*19*), and ConA (*8*), the Mn^{2+}−histidine bond length is 2.29, 2.24, and 2.29 Å, respectively, on the basis of X-ray crystallographic measurements.

Ligation of Water to Mn^{2+}. ESEEM has often been used to identify coordination of water molecules and to quantify their number bound to metal centers (*24*, *25*, *35*, *48*−*50*). These experiments involve ratioing ESEEM data for samples in D_2O solvent with those for samples in H_2O solvent, to better resolve modulations due to exchangeable deuterons, followed by analysis of the time or frequency domain of the ratio data. The number of ligated water molecules can be obtained by comparison with a standard system for which the number of water ligands is known.

ESEEM has been used to examine the coordination number of water molecules to Mn^{2+} in a series of legume lectins including ConA (*25*). In that study, modulations from the single D_2O ligand in Mn^{2+}−EDTA (*51*) were first isolated from those arising from ambient D_2O molecules (*25*, *48*). The intensity of the deuterium line in the Fourier-transformed (D_2O/H_2O) ratio data of the lectins was found to be twice that for the single D_2O ligand in Mn^{2+}−EDTA, and it was concluded that the Mn^{2+} sites of all the lectins studied contain two water molecules. For ConA, the ESEEM results agree with the X-ray crystal structure of the lectin (*8*).

In the present study, ESEEM has been used to quantify the number of water ligands at the Mn^{2+} site of the nine Diocleinae lectins. Because the presence of two water molecules at the Mn^{2+} site of ConA has been established by both ESEEM (*25*) and X-ray crystallography (*8*), ConA, rather than a model compound such as Mn^{2+}−EDTA, was used as the standard.

To obtain the D_2O/H_2O ratio data, it is necessary to first normalize individually the echo amplitude for the data of samples in D_2O and the data of samples in H_2O. Generally this is carried out by fitting a decay function to the echo envelope and extrapolate the zero-time amplitude, which would then be taken as unity (*24*, *25*, *48*−*50*). In the present study we normalized the echo amplitude without fitting the data to any decay function; rather, the echo amplitude at an actual data point was used as the maximum echo amplitude. This method greatly reduces the manipulation of data and simplifies the analysis. This method is applicable in the current study because of the observation that the two-pulse echo envelopes of the nine Diocleinae lectins are nearly identical to each other as well as to those of ConA. Data for *D. guianensis*, *C. floribunda*, and *D. rostrata* are shown in Figure 3. Thus, it is assumed that all the H_2O echo envelopes are described by a single decay function, and all the D_2O echo envelopes by another decay function. The ratio of the echo amplitude at a particular time point to that at zero time would then be constant for all H_2O data and for all D_2O data. Therefore, the maximum echo amplitude in the actual

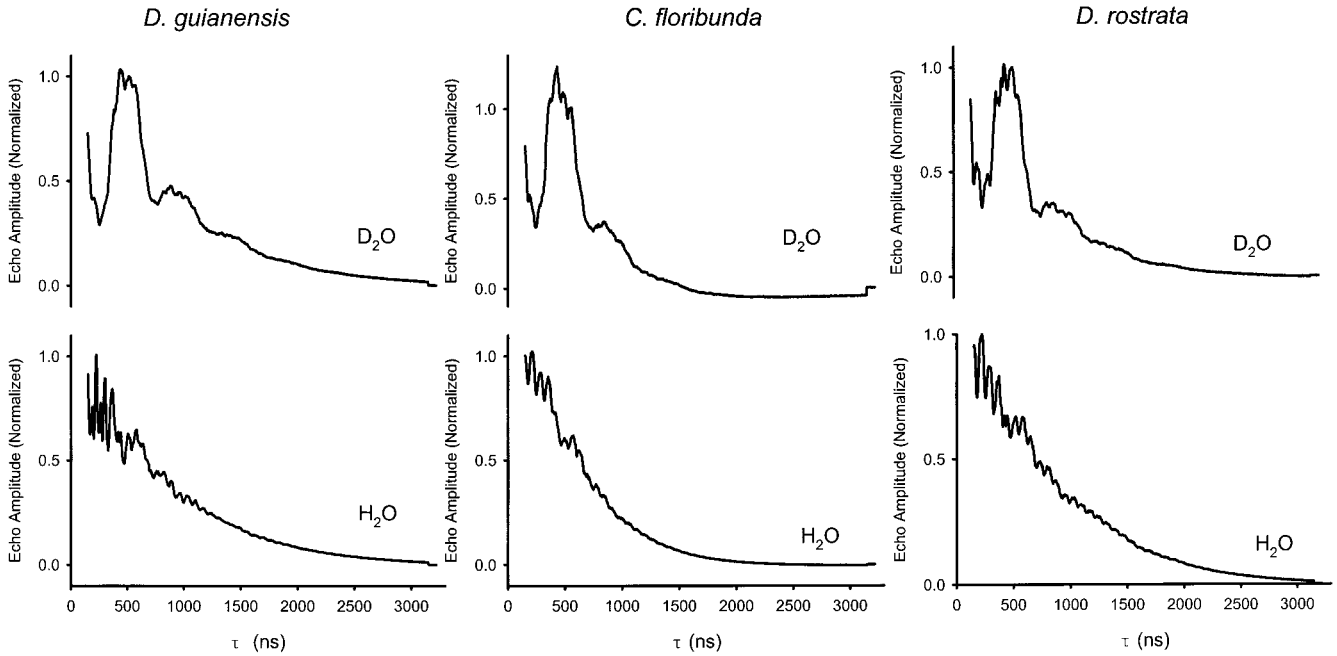

FIGURE 3: Two-pulse ESEEM data of *D. guianensis* (left), *C. floribunda* (middle), and *D. rostrata* (right). The experimental conditions are (*D. guianensis*) (D_2O) microwave frequency 9.25 GHz, magnetic field 3211 G, starting $\tau = 150$ ns, and temperature 1.5 K and (H_2O) microwave frequency 9.25 GHz, magnetic field 3211 G, starting $\tau = 150$ ns, and temperature 1.5 K; (*C. floribunda*) (D_2O) microwave frequency 9.40 GHz, magnetic field 3289 G, starting $\tau = 150$ ns, and temperature 1.5 K and (H_2O) microwave frequency 9.40 GHz, magnetic field 3289 G, starting $\tau = 150$ ns, and temperature 1.5 K; and (*D. rostrata*) (D_2O) microwave frequency 9.28 GHz, magnetic field 3211 G, starting $\tau = 150$ ns, and temperature 1.5 K and (H_2O) microwave frequency 9.34 GHz, magnetic field 3211 G, starting $\tau = 150$ ns, temperature 1.5 K.

data, rather than the zero-time echo amplitude, can be taken as unity.

For ConA (data not shown) in D_2O, the peak of the first modulation cycle occurred at $\tau = 516$ ns, whereas in H_2O, the peak was at 222 ns, and the echo amplitudes at these τ values were taken to be the maximum amplitudes. To normalize the echo amplitudes of ConA and the other nine Diocleinae lectins, the echo amplitudes at these τ values were taken to be 1.0 unit. Ratio data were then obtained by dividing the normalized D_2O data by the normalized H_2O data.

In previous studies (*25, 48*), the intensity of the deuterium line in the Fourier-transformed ratio data was used to quantify the number of water ligands. Because the current method of obtaining D_2O/H_2O ratio data avoided fitting the echo envelope to a decay function, the zero-time amplitude of the ratio data would not be available to carry out Fourier transformation. Thus, the depth of the deuterium modulation in the time domain of the ratio data was used to directly quantify the number of bound D_2O (*24, 49, 50*). The modulation depth was defined as the peak-to-trough distance (in normalized units) of the first deuterium modulation cycle in the ratio data.

For ConA, the two Mn^{2+}−water bonds are 2.20 and 2.32 Å (*8*). In D_2O buffer, each of the four deuterons on the two D_2O ligands in ConA would therefore be about ≥2.9 Å from Mn^{2+}, whereas the next closest exchangeable deuteron, that on the His24 imidazole (a Mn ligand), is about 5 Å from Mn^{2+}. The modulation depth of the His24 imidazole deuteron is thus $(2.9/5)^6$ or 0.038 (*49*) of that due to one deuteron on a bound D_2O. Similarly, the modulations due to exchangeable deuterons on any Mn^{2+} ligand amino acid side chain should be negligible compared to those from bound D_2O. That is, in the event that the Mn^{2+} site of a particular lectin does not contain any D_2O ligand, the total deuterium modulations from amino acid ligands of Mn^{2+} would not be significant so as to lead to an erroneous conclusion concerning the presence of a Mn-bound D_2O. Therefore, the deuterium modulation depth can be taken to be proportional to the number of bound D_2O ligands, with that of ConA as the calibration for two D_2O ligands.

Table 1: Peak-to-Trough Distance of Deuterium Modulation Envelopes of Diocleinae Lectins

lectins	peak-to-trough distance	no. of bound D_2O ligands	lectins	peak-to-trough distance	no. of bound D_2O ligands
ConA	1.02	2	*D. guianensis*	1.23	2.41
C. brasiliensis	0.95	1.86	*D. virgata*	0.85	1.67
C. bonariensis	0.99	1.94	*D. violacea*	0.94	1.84
C. floribunda	1.09	2.14	*D. grandiflora*	0.85	1.67
D. rostrata	0.99	1.94	*C. grandiflora*	0.82	1.61

To test the modified analysis that uses an actual data point as the maximum for normalization of the echo amplitude, it was first applied to *C. brasiliensis* and *D. grandiflora* lectins for which X-ray crystal structures are available (*18, 19*). With the deuterium modulation depth of ConA taken as to represent two D_2O ligands, *C. brasiliensis* and *D. grandiflora* lectins have, respectively, 1.94 and 1.67 D_2O ligands coordinated to Mn^{2+} (Table 1), in line with the two water ligands found by X-ray crystallography. Similarly, setting the deuterium modulation depth of either *C. brasiliensis* or *D. grandiflora* lectins as to represent two D_2O ligands, the deuterium modulation depth found for ConA would represent 2.14 and 2.40 D_2O ligands, respectively, bound to Mn^{2+}, in accordance with both X-ray crystallography (*8*) and previous

ESEEM analysis using the Fourier-transformed ratio data (*25*). Therefore, this simplified analysis is suitable for the current study of Diocleinae lectins.

The data for the other seven lectins were similarly analyzed. The results are summarized in Table 1 and compared with those of ConA. If the peak-to-trough distance from ConA data is taken to represent two bound D_2O molecules, the nine Diocleinae lectins in this study contain 1.61−2.41 D_2O ligands bound to Mn^{2+}. Therefore, it can be concluded that all nine Diocleinae lectins contain two water ligands at the Mn^{2+} site. For *C. brasiliensis* (*18*) and *D. grandiflora* (*19*), ESEEM results are consistent with their X-ray crystal structures.

CONCLUSIONS

EPR and ESEEM studies of nine Diocleinae lectins demonstrate binding of Mn^{2+} and coordination of one histidine and two water molecules to Mn^{2+}. The environment of the histidine is believed to be similar in all nine lectins. For *C. brasiliensis* and *D. grandiflora* lectins, the frozen solution structure of the Mn^{2+} site revealed by ESEEM agrees with their respective X-ray crystal structures (*18*, *19*). These findings extend the observation that all legume lectins thus far investigated possess conserved Mn^{2+} binding sites which include one histidine and two water ligands (*52*).

EPR spectra of the *C. floribunda* and *D. rostrata* lectins, however, suggest differences in their Mn^{2+} site symmetry as compared to that of the other seven. These differences likely arise from different environments for the remaining Mn^{2+} ligands.

REFERENCES

1. Sharon, N., and Lis, H. (1993) *Sci. Am. 268*, 82−89.
2. Zanetta, J.-P. (1997) in *Glycosciences* (Gabius, H.-J., and Gabius, S., Eds.) pp 439−458, Chapman & Hall, New York.
3. Cummings, R. D. (1997) in *Glycosciences* (Gabius, H.-J., and Gabius, S., Eds.) pp 191−200, Chapman & Hall, New York.
4. Lis, H., and Sharon, N. (1986) *Annu. Rev. Biochem. 55*, 35−67.
5. Goldstein, I. J., and Poretz, R. D. (1986) in *The Lectins* (Liener, I. E., Sharon, N., and Goldstein, I. J., Eds.) pp 35−244, Academic Press, Inc., New York.
6. Van Damme, E. J. M., Peumans, W. J., Pusztai, A., and Bardocz, S. (1997) *Handbook of Plant Lectins: Properties and Biomedical Applications*, Wiley, New York.
7. Brewer, C. F., Brown, R. D. I., and Koenig, S. H. (1983) *J. Biomol. Struct. Dyn. 1*, 961−997.
8. Hardman, K. D., Agarwal, R. C., and Freiser, M. J. (1982) *J. Mol. Biol. 157*, 69−86.
9. Goldstein, I. J., Hollerman, C. E., and Smith, E. E. (1965) *Biochemistry 4*, 876−883.
10. Gupta, D., Dam, T. K., Oscarson, S., and Brewer, C. F. (1997) *J. Biol. Chem. 272*, 6388−6392.
11. Dam, T. K., Cavada, B. S., Grangeiro, T. B., Santos, C. F., de Sousa, F. A. M., Oscarson, S., and Brewer, C. F. (1998) *J. Biol. Chem. 273*, 12082−12088.
12. Gomes, J. C., Rossi, R. R., Cavada, B. S., Moreira, R. A., and Oliveira, J. T. A. (1994) *Agents Actions 41*, 132−135.
13. Barral-Netto, M., Santos, S. B., Barral, A., Moreira, L. I. M., Santos, C. F., Moreira, R. A., Oliveira, J. T. A., and Cavada, B. S. (1992) *Immunol. Invest. 21*, 297−303.
14. Rodrigues, D., Cavada, B. S., Oliveira, J. T. A., Moreira, R. D. A., and Russo, M. (1992) *Bras. J. Med. Biol. Res. 25*, 823−826.
15. Bento, C. A. M., Cavada, B. S., Oliveira, J. T. A., Moreira, R. A., and Barja-Fidalgo, C. (1993) *Agents Actions 38*, 48−54.
16. Bouckaert, J., Poortmans, F., Wyns, L., and Loris, R. (1999) *J. Biol. Chem. 271*, 16144−16150.
17. Derewenda, Z., Yariv, J., Helliwell, J. R., Kalb, A. J., Dodson, E. J., Papiz, M. Z., Wan, T., and Campbell, J. (1989) *EMBO 8*, 2189−2193.
18. Sanz-Aparicio, J., Hermoso, J., Grangeiro, T. B., Calvete, J. J., and Cavata, B. S. (1997) *FEBS Lett. 405*, 114−118.
19. Rozwarski, D. A., Swami, B. M., Brewer, C. F., and Sacchettini, J. C. (1998) *J. Biol. Chem. 273*, 32818−32825.
20. Calvete, J. J., Thole, H. H., Raida, M., Urbanke, C., Romero, A., Grangeiro, T. B., Ramos, M. V., Almeida da Rocha, I. M., Guimaraes, F. N., and Cavada, B. S. (1999) *Biochim. Biophys. Acta 1430*, 367−375.
21. Mims, W. B., and Peisach, J. (1989) in *Advanced ESR: Applications in Biology and Biochemisty* (Hoff, A. J., Ed.) pp 1−57, Elsevier, Amsterdam.
22. Mims, W. B., and Peisach, J. (1976) *Biochemistry 15*, 3863−3869.
23. Peisach, J., Mims, W. B., and Davis, J. (1979) *J. Biol. Chem. 254*, 12379−12384.
24. Mims, W. B., Davis, J. L., and Peisach, J. (1990) *J. Magn. Reson. 86*, 273−292.
25. McCracken, J., Peisach, J., Bhattacharyya, L., and Brewer, F. (1991) *Biochemistry 30*, 4486−4491.
26. Agrawal, B. B. L., and Goldstein, I. J. (1967) *Biochim. Biophys. Acta 147*, 262−271.
27. Moreira, R. A., and Cavada, B. S. (1984) *Biol. Plant. (Prague) 26*, 113−120.
28. Cavada, B. S., Moreira-Silva, L. I. M., Grangeiro, T. B., Santos, C. F., Pinto, V. P. T., Barral-Netto, M., Roque-Barreira, M. C., Gomes, J. C., Martins, J. L., Oliveira, J. T. A., and Moreira, R. A. (1996) in *Lectins: Biology, Biochemistry, Clinical Biochemistry* (Van Driessche, E., Rouge, P., Beeckmans, S., and Bog-Hansen, T. C., Eds.) Vol. 11, pp 74−80, TEXTOP, Hellerup, Denmark.
29. Oliveira, J. T. A., Cavada, B., S., and Moreira, R. D. A. (1991) *Rev. Bras. Botan. 14*, 61−66.
30. Cavada, B. S., Grangeiro, T. B., Ramos, M. V., Cordeiro, E. F., Oliveria, J. T. A., and Moreira, R. A. (1996) *Rev. Bras. Fisiol. Veg. 8*, 31−36.
31. Vasconcelos, I. M., Cavada, B. S., Moreira, R. D. A., and Oliveira, J. T. A. (1991) *J. Food Biochem. 15*, 137−154.
32. Cavada, B. S., Ramos, M. V., Cordeiro, E. F., Grangeiro, T. B., Oliveira, J. T. A., Carvalho, A. F. F. U., and Moreira, R. A. (1996) *Rev. Bras. Fisiol. Veg. 8*, 3742.
33. Moreira, R. D. A., Cordeiro, E. D. F., Ramos, M. V., Grangeiro, T. B., Martins, J. L., Oliveria, J. T. A. D., and Cavada, B. S. (1996) *Rev. Bras. Fisiol. Veg. 8*, 23−29.
34. Moreira, R. A., Barros, A. C. H., Stewart, J. C., and Pusztai, A. (1983) *Planta 158*, 63−69.
35. McCracken, J., Peisach, J., and Dooley, D. M. (1987) *J. Am. Chem. Soc. 109*, 4064−4072.
36. Bender, C. J., Rosenzweig, A. C., Lippard, S. J., and Peisach, J. (1994) *J. Biol. Chem. 269*, 15993−15998.
37. Britt, R. D., and Klein, M. P. (1987) *J. Magn. Reson. 74*, 535−540.
38. Mims, W. B. (1984) *J. Magn. Reson. 59*, 291−306.
39. Antanaitis, B. C., Brown, R. D., III, Chasteen, N. D., Freedman, J. H., Koenig, S. H., Lilienthal, H. R., Peisach, J., and Brewer, C. F. (1987) *Biochemistry 26*, 7932−7937.
40. Meirovitch, E., Luz, Z., and Kalb, A. J. (1974) *J. Am. Chem. Soc. 96*, 7538−7542.
41. Coffino, A. R., and Peisach, J. (1996) *J. Magn. Reson. 111*, 127−134.
42. Reed, G., and Markham, G. D. (1984) in *Biological Magnetic Resonance* (Berliner L. J., and Reuben, J., Eds.) Vol. 6, pp 73−142, Plenum, New York.
43. Tipton, P. A., and Peisach, J. (1991) *Biochemistry 30*, 739−744.
44. Jiang, F., McCracken, J., and Peisach, J. (1990) *J. Am. Chem. Soc. 112*, 9035−9044.
45. Espe, M. P., Hosler, J. P., Ferguson-Miller, S., and Babcock, G. T. (1995) *Biochemistry 34*, 7593−7602.

46. Flanagan, K. L., and Singel, D. J. (1987) *J. Chem. Phys. 50*, 294−303.
47. Lee, H. C., Scheuring, E., Peisach, J., and Chance, M. R. (1997) *J. Am. Chem. Soc. 119*, 12201−12209.
48. Serpersu, E. H., McCracken, J., Peisach, J., and Mildvan, A. (1988) *Biochemistry 27*, 8034−8044.
49. Mims, W. B., Peisach, J., and Davis, J. (1977) *J. Chem. Phys. 66*, 5536−5550.
50. Mims, W. B., Davis, J. L., and Peisach, J. (1984) *Biophys. J. 45*, 755−766.
51. Stezowski, J. C., and Hoard, J. L. (1984) *Isr. J. Chem. 24*, 323−334.
52. Loris, R., Hamelryck, T., Bouckaert, J., and Wyns, L. (1998) *Biochim. Biophys. Acta 1383*, 9−36.

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