differing membrane potentials would be inherently color-coded at the properly selected concentration of JC-1: green at low and red-orange at high membrane potentials. Within the category of slow membrane potential dyes, fluorescent J-aggregate-forming indicators could find a wide range of applications, including videomicroscopy with the emerging development of the color CCD camera.

ACKNOWLEDGMENTS

We thank Joseph Arruda for technical assistance and Michelle Somers for typing the manuscript.

Registry No. JC-1, 21527-78-6; K, 7440-09-7.

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Electron Spin Echo Envelope Modulation Studies of Lectins: Evidence for A Conserved Mn^{2+}-Binding Site†

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Received November 14, 1990; Revised Manuscript Received January 24, 1991

ABSTRACT: Electron spin echo envelope modulation (ESEEM) experiments have been used to investigate the Mn^{2+}-binding site in a series of lectins including concanavalin A, pea lectin (*Pisum sativum*), isolectin A from lentil (*Lens culinaris*), soybean agglutinin (*Glycine max*), *Erythrina indica* lectin, and *Lotus tetragonolobus* isolectin A. Together with model studies, the results provide direct evidence for a single nitrogen atom of a conserved residue bonded directly to Mn^{2+} in all of them. ESEEM measurements of the lectins exchanged with deuterium oxide, together with model studies, provide evidence for the presence of two water molecules coordinated to the Mn^{2+} in all of the proteins. In contrast to concanavalin A, the absence of solvent exchange at the Mn^{2+} site in the pea and lentil lectins demonstrated by nuclear magnetic relaxation dispersion measurements [Bhattacharyya, L., Brewer, C. F., Brown, R. D., III, & Koenig, S. H. (1985) *Biochemistry 24*, 4985-4990] must therefore be due to slow exchange of the water ligands of the bound Mn^{2+}. Binding of saccharides was observed to have little effect on the structural features of the Mn^{2+} site in the lectins as determined by ESEEM.

Lectins are cell-agglutinating proteins of nonimmune origin that bind to specific carbohydrate determinants without chemically modifying them. They are found in animal tissues and invertebrates, although the majority of the best studied lectins are from plants. It is this latter class that has been widely used to explore the membrane properties of both normal and transformed cells (Brown & Hunt, 1978; Lis & Sharon, 1981).

Legume lectins have been extensively studied for their carbohydrate-binding properties. They include those from Jack bean (concanavalin A) (Con A),[1] pea (PSA), lentil (LcH),

† This work was supported by National Institutes of Health Grant CA-16054 (C.F.B.), Core Grant P30 CA-13330 (C.F.B. and J.P.), Grant GM-40168 (J.P.) and RR-02583 (J.P.).

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0006-2960/91/0430-4486$02.50/0

soybean (SBA), *Erythrina indica* (EIL), and *Lotus tetragonolobus* seeds (LTL) (Goldstein & Poretz, 1986). Con A, PSA, and LcH are classified as Man/Glu[2] specific, SBA and EIL as Gal/GalNAc specific, and LTL as a Fuc specific lectin. The lectins exhibit a high degree of sequence homology (Strosberg et al., 1986). In view of their structural similarities, it is of interest to investigate the physicochemical properties of these molecules in order to understand the molecular basis of their biological activities (Lis & Sharon, 1986).

All of the above lectins are metalloproteins containing Mn^{2+} and Ca^{2+}, which are both required for maximal saccharide-binding activities (Goldstein & Poretz, 1986). However, only in the case of Con A has the role of the metal ions been elucidated in detail. Two metal ion binding sites exist on each monomer of Con A: the so-called transition metal ion site, S1, and a calcium-ion site, S2. The X-ray crystallographic structure of Con A at 1.75-Å resolution (Hardman et al., 1982) shows the Mn^{2+} and Ca^{2+} to be 4.25 Å apart, with the latter adjacent to the carbohydrate-binding site. Both ions possess pseudooctahedral symmetry, with Mn^{2+} having hexacoordinate geometry and Ca^{2+} heptacoordinate geometry. The ligands for Mn^{2+} consist of five oxygen atoms, three from carboxyl side chains (Asp 10 and 19 and Glu 8) and two from water molecules, and a single nitrogen atom from an imidazole side chain (His 24). The coordination sphere of Ca^{2+} consists of seven oxygen atoms, three from two carboxyl side chains (Asp 10 and 19), one from a peptide carbonyl (Tyr 12), one from an amide side chain (Asn 14), and two from water molecules. Mn^{2+} and Ca^{2+} share two bridging carboxylate ions (Asp 10 and 19).

X-ray crystallographic analysis has also been carried out for PSA, but at lower resolution, 3 Å (Einspahr et al., 1986), than for Con A. These studies show that the Mn^{2+}- and Ca^{2+}-binding sites are similar to those in Con A. However, the presence of two water ligands on Mn^{2+} could not be established at this lower resolution. Primary sequence data for PSA, LcH, SBA, (Strosberg et al., 1986), and LTL-A (Konami et al., 1990) show conservation of the amino acids that form the S1 and S2 sites in Con A, except that Tyr 12 at the S2 site of Con A is replaced by a Phe residue in the other lectins. EPR spectra of PSA (Bhattacharyya et al., 1985b), LcH (Tichy et al., 1971), and SBA (Meirovitch et al., 1978) show site symmetries for Mn^{2+} similar to that observed for Con A (Reed & Cohen, 1970).

Nuclear magnetic relaxation dispersion (NMRD) studies show that solvent exchange at both the Mn^{2+} and Ca^{2+} sites in Con A contributes to solvent proton relaxation (Koenig et al., 1985). Similar studies with PSA and LcH demonstrate exchange from the Ca^{2+} site but not from the Mn^{2+} site (Bhattacharyya et al., 1985a). The question arises then as to whether there is water bound to Mn^{2+} in PSA and LcH or whether these waters, if present, exchange too slowly to be detected in the NMRD experiments.

In order to address this question, electron spin echo envelope modulation (ESEEM) studies have been carried out to elucidate the structure in the immediate environment of Mn^{2+} in Con A, PSA, and LcH as well as in several other related legume lectins. The structural information contained in these pulsed EPR experiments is obtained by measuring the modulation of the electron spin echo decay envelope that is generated by measuring the amplitude of the spin-echo signal as a function of microwave pulse spacing in both two-pulse (Mims & Peisach, 1976) and three-pulse stimulated echo experiments (Peisach et al., 1979). These modulations arise from weak superhyperfine interactions between the paramagnetic center and the surrounding nuclei. Fourier transform analysis of the ESEEM patterns yields a spectrum containing superhyperfine coupling frequencies similar to that obtained in an ENDOR experiment (Shimizu et al., 1979; Mims & Peisach, 1989). Detailed analysis of ESEEM data yields not only information concerning the hyperfine coupling and the type of nuclei involved (i.e., ^1H, ^2H, ^{14}N, etc.) but also information concerning the number of such nuclei (McCracken et al., 1981; Serpersu et al., 1988) and their distance from the paramagnetic center as well (Mims & Peisach, 1989; Cornelius et al., 1990; Mims et al., 1990).

In the present study, we report ESEEM spectra of Con A, PSA as well as isolectin B of PSA (PSA-B), isolectin A of LcH (LcH-A), SBA, EIL, and isolectin A of LTL (LTL-A). The data show evidence for a single nitrogen atom of a conserved (histidyl) residue bonded directly to the Mn^{2+} in all of the cases studied. From examination of protein samples exchanged against deuterium oxide, the ESEEM data provide evidence for the presence of two water molecules bonded to Mn^{2+} in Con A and EIL, as well as in PSA and LcH-A where solvent exchange could not be demonstrated with NMRD (Bhattacharyya et al., 1985a). The present results suggest that solvent exchange at the Mn^{2+} site in LcH and PSA occurs under NMR slow-exchange limits.

Materials and Methods

Con A from the jack bean *Canavalia ensiformis* was obtained from Miles-Yeda, and its metal ion complexes were prepared and characterized as described previously (Brown et al., 1977). Seeds of lentil (*Lens culinaris* Macrosperma) and pea (*Pisum sativum* Columbian) were purchased from a local food store. LcH was purified by affinity chromatography on Sephadex G-100 (Ticha et al., 1970). The separation of the two isolectins, LcH-A and LcH-B, was carried out on a CM-cellulose column (Howard et al., 1971). PSA and its two isolectins, A and B, were obtained according to the described procedure (Trowbridge, 1974). SBA (*Glycine max*) was isolated from Soyfluff (Central Soya, Gibson City, IL) as described (Bhattacharyya et al., 1988). Seeds of *Erythrina indica* were purchased from United Chemicals, India, and the lectin was purified as previously described (Bhattacharyya et al., 1981). Seeds of *Lotus tetragonolobus* (syn. *Tetragonolobus purpureas*) were purchased from Schumacher and Co., MA. Isolation of the isolectin A of LTL (LTL-A) was performed as described (Kalb, 1968). Metal salts were the highest purity available from Mallinckrodt and Fisher Scientific Co. Deuterium oxide (99.8% deuterons) was obtained from Stohler/Kor Stable Isotopes. Carbohydrates were obtained from Pfanstiehl Laboratories.

Protein Concentrations. The monomer concentration of Con A was determined spectrophotometrically at pH 5.6 by using $A^{1\%,1cm} = 12.4$ at 280 nm (Yariv et al., 1968). Those of LcH and PSA were determined at pH 6.4 by using $A^{1\%,1cm} = 12.6$ (Howard et al., 1971) and 15.0 (Bhattacharyya et al., 1985a), respectively, at 280 nm. The extinction coefficients of the isolectins are assumed to be the same as the native protein mixtures. The concentrations of SBA, EIL, and LTL-A were determined at 280 nm by using $A^{1\%,1cm} = 12.8$ (Lotan et al., 1974), 13.4 (Bhattacharyya et al., 1981), and 17.4 (Kalb, 1968), respectively.

[1] Abbreviations: EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; NMR, nuclear magnetic resonance; NMRD, nuclear magnetic relaxation dispersion; Con A, concanavalin A; PSA, pea lectin; LcH, lentil lectin; SBA, soybean lectin; EIL, *Erythrina indica* lectin; LTL, *Lotus tetragonolobus* lectin.

[2] Fucose is in the L configuration; all other sugars are in the D configuration.

Metal Ion Analysis. Protein solutions were acidified to pH 1.2 with concentrated HCl and allowed to stand overnight; precipitates were removed by centrifugation. The supernatants were subjected to metal ion analysis by atomic absorption meaurements with a Perkin-Elmer Model 603 spectrometer. Mn^{2+} concentrations in acidified solutions of Con A, PSA, LcH, and SBA were also determined by proton NMRD techniques (Brown et al., 1977). All the lectins were found to contain a single equivalent of Mn^{2+} per monomer. One equivalent of Ca^{2+} was found per monomer of Con A, with approximately 1.8–2.0 equivalents of Ca^{2+} per monomer for the other lectins studied.

Sample Preparations. Lectin solutions for ESEEM studies were prepared in 0.1 M potassium acetate buffer, pH 6.4, containing 0.9 M KCl. Samples in D_2O employed the same buffer. Samples were extensively dialyzed in order to remove adventitious metal ions. The absence of free or nonspecifically bound Mn^{2+} was judged from the room temperature EPR properties. Lectin samples were also prepared in the presence of 10 mM of the following glycosides: for Con A, methyl α-mannopyranoside; LcH and PSA, 3-O-methyl Glu; SBA, GalNAc; EIL, Gal; and LTL-A, Fuc.

Protein samples used for pulsed EPR studies were diluted 1:1 with ethylene glycol. (For samples in D_2O, the ethylene glycol was twice diluted 20-fold with D_2O and purified by vacuum distillation.) Final protein and hence Mn^{2+} concentrations ranged from 0.3 to 0.8 mM.

Model compounds of Mn^{2+} with imidazole and *N*-methylimidazole were prepared by using ligand-to-metal ratios of 400:1. Other Mn^{2+} models were prepared by using a slight excess of stoichiometrically equivalent concentrations of millimolar EDTA or DTPA. Equivalent samples were prepared in D_2O, but with ligand that had been previously dissolved in a large excess of D_2O and then concentrated by lyophilization. Sample solutions were prepared in 50 μM Tris-HCl buffer at pH 7.4. For all models, samples were diluted 1:1 (v/v) with the appropriate ethylene glycol, as for the protein samples. Final Mn^{2+} concentrations varied from 100–200 μM.

Spectroscopy. Continuous wave EPR spectra at X-band were collected at 25 °C on a Varian E-112 spectrometer. Magnetic fields were calibrated with a Varian NMR gaussmeter, and the microwave frequency was determined with a Systron-Donner meter.

ESEEM decay envelopes were obtained at liquid helium temperatures on a home-built pulsed EPR spectrometer, operating from 8–18 GHz (McCracken et al., 1987). Data were collected by two-pulse (Mims & Peisach, 1976) and three-pulse or stimulated-echo (Peisach et al., 1979) methods. ESEEM spectra were obtained by Fourier transformation of decay envelopes subsequent to the dead-time reconstruction procedure described by Mims (1984). Echo envelopes were normalized to the intensity of the Mn^{2+} signal.

Results and Discussion

The ESEEM spectrum for Con A shown in Figure 1A contains lines at 1.4, 2.9, 3.5, and 4.9 MHz (Lobrutto et al., 1986). The spectrum resembles that obtained for a Mn^{2+}–histidine complex (Tipton & Peisach, in press) and bears resemblance to the spectrum for Mn^{2+} in the presence of a large excess of imidazole (Figure 1C). In the presence of methyl α-mannopyranoside, small changes in the spectrum of Con A are observed (Figure 1B).

The spectral lines obtained for the protein are ascribed to the interaction of the electron spin with the directly coordinated ^{14}N of imidazole. There are a number of reasons for this

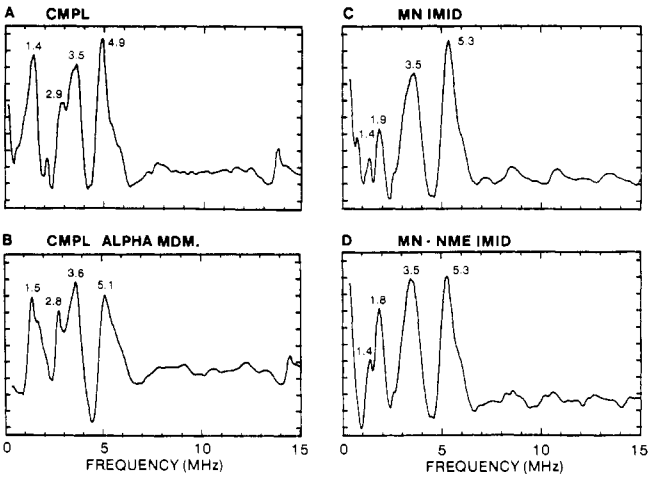

FIGURE 1: ESEEM spectra obtained by the three-pulse or stimulated echo procedure for the lectins and model compounds: (A) concanavalin A (CMPL); (B) the lectin in the presence of methyl α-mannopyranoside; (C) Mn^{2+}–imidazole; and (D) Mn^{2+}–*N*-methylimidazole. The respective experimental conditions were as follows: (A) microwave frequency = 9.271 GHz, magnetic field = 3200 G, and τ = 220 μs; (B) microwave frequency = 9.428 GHz, magnetic field = 3770 G, and τ = 209 μs; (C) microwave frequency = 9.955 GHz, magnetic field = 3540 G, and τ = 265 μs; and (D) microwave frequency = 9.465 GHz, magnetic field = 3370 G, and τ = 279 μs. The values of τ are given for the time spacing between the first two pulses of the sequence.

assignment, the most outstanding being that Mn^{2+} is shown to be coordinated to the histidine side chain by X-ray crystal analysis (Becker et al., 1975). It is reasonable to assume that there is no major structural alteration at the metal-binding site for the protein in solution. Since the distance from the metal to the coordinated ^{14}N is reported as 2.3 Å (Hardman et al., 1982), one would expect contributions arising from nuclear quadrupole, contact, and dipolar interactions. As the zero field splitting is small (Dowsing & Gibson, 1969; Reed & Markham 1984), contributions from state mixing with all the transitions of the $S = 5/2$ spin manifold are expected. Proof that the lines arise from ^{14}N and not from ^{1}H comes from a multifrequency ESEEM measurement on the same sample. Unlike electron nuclear coupling to an $I = 1/2$ species such as ^{1}H, lines arising from coupling to an $I = 1$ ion, e.g., ^{14}N, do not scale directly with the magnetic field. This is easily demonstrated from the spectrum of PSA-B (which is the same as that of native PSA) studied at nearly identical g values but at disparate spectrometer frequencies, 9.4 and 11.4 GHz (Figure 2). The 4.9-MHz line observed at 3260 G at the lower microwave frequency exhibits the greatest spectral shift in the higher microwave frequency measurement, moving to 5.3 MHz in the spectrum obtained at 3920 G. Were this line to arise from ^{1}H and scale with the magnetic field, the expected shift would be 2.8 MHz and the line would be observed at 8.1 MHz rather than at 5.3 MHz. Thus, it as well as the other lines cannot arise from ^{1}H but rather from ^{14}N. As the spectrum for PSA-B, as well as for the other lectins, is nearly identical with that of Con A,[3] the conclusion can be made that a nitrogen ligand at the S1 site is conserved in all the proteins examined.

Although the spectral assignment is difficult, it can be argued on the basis of spectral similarity and primary sequence data that this nitrogenous species is imidazole in all instances.

[3] Although shifts in peak positions at constant g can be demonstrated at different spectrometer frequencies, the spectra remain rather invariant from one lectin to another (±0.1 MHz) when studied under the same experimental conditions.

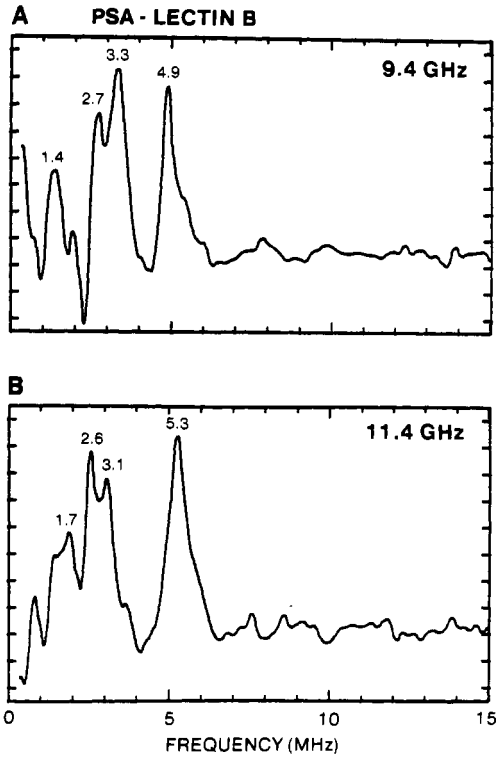

FIGURE 2: ESEEM spectra of PSA-B studied at two microwave frequencies: (A) microwave frequency = 9.435 GHz, magnetic field = 3260 G, and τ = 265 μs; (B) microwave frequency = 11.363 GHz, magnetic field = 3920 G, and τ = 240 μs.

Primary sequence data are consistent with the conservation of His 24 in Con A, which is a ligand for Mn^{2+}, being present in PSA, LcH, SBA (Strosberg et al., 1986), and LTL-A (Konami et al., 1990). In the simplest case, for an $S = 1/2$ ion interacting with ^{14}N and where the contact and Zeeman interactions (a and ω, respectively) are comparable in magnitude, the spectrum is largely dominated by three sharp lines arising from the nuclear quadrupole interaction, together with a broad higher frequency line arising from a $\Delta m = 2$ transition, whose frequency is approximately equal to $2\omega + a$ (Mims & Peisach, 1978). [This is the so-called condition of "exact cancellation" (Flanagen & Singel, 1987).] Where contact and Zeeman interactions are no longer comparable, the spectrum becomes more complicated and can contain as many as six lines. The quadrupole parameters can be determined by spectral simulation, and this method is often aided by multifrequency measurements at disparate magnetic fields. In this way, an imino, amino, or amido ^{14}N ligand can be differentiated. For Mn^{2+}, a method of spectral simulation in order to determine quadrupole coupling parameters has yet to be achieved.

For a Cu^{2+}–imidazole complex, coupling to directly coordinated nitrogen is about 40 MHz (van Camp et al., 1981) and to the remote nitrogen about 1.5 MHz (Mims et al., 1990). For Mn^{2+}–imidazole, one would predict that the coupling to the corresponding nitrogen would be smaller than for Cu^{2+}–imidazole considering that covalent interactions with Mn^{2+} would be less than with Cu^{2+}. Thus, the magnitude of electron nuclear coupling from the remote nitrogen might be too small to be easily observed by ESEEM, and the assignment of ^{14}N lines would then be to the directly coordinated nitrogen.

Evidence for this conclusion is based on model studies. In Cu^{2+}–imidazole models where the remote proton of the coordinated ligand is replaced by a methyl group, the resulting change in the electric field gradient leads to a large change in nuclear quadrupole parameters, thereby altering the ESEEM spectrum (Jiang et al., 1990). For Mn^{2+} models prepared with *N*-methylimidazole rather than with imidazole no change in the ESEEM spectrum is detected (Figure 1D). Therefore, coupling to the directly coordinated nitrogen is suggested.

Table I: Normalized Peak Intensity of the 2-MHz Line Attributable to Deuterium Interactions with Mn^{2+} in EDTA and in the Lectins[a]

sample	peak height
Mn^{2+}–EDTA	6.0
Con A	11.8
Con A + methyl α-mannopyranoside	11.8
PSA	10.5
LcH-A	10.9
EIL	12.3

[a] Spectral contributions from nonexchangeable ligands for protein samples and the EDTA complex were removed by the ratio method described in the text prior to Fourier transformation. For the Mn^{2+}–EDTA model, contributions from ambient water were removed by forming a ratio of the nuclear modulation data against a similar set for Mn^{2+}–DTPA (Serpersu et al., 1988). All studies were carried out at a spectrometer frequency of 9.27 GHz and a magnetic field of 3200 G.

X-ray crystallographic studies show that the Mn^{2+} in Con A is coordinated to two water molecules (Becker et al., 1975; Hardman et al., 1982), and NMRD studies show exchange of these water ligands with that solvent (Koenig et al., 1985). The absence of similar exchange in LcH and PSA (Bhattacharyya et al., 1985a) suggests either an absence or slow exchange of coordinated water at the Mn^{2+} site in these proteins. In order to resolve this question, the number of water molecules coordinated to Mn^{2+} in these lectins was determined by ESEEM techniques.

In order to quantify the number of water molecules bound to Mn^{2+} in Con A, the lectin was extensively exchanged against D_2O. The ESEEM spectrum for this sample was identical with that for the protein in H_2O, except that a line was observed near 2 MHz arising from electron nuclear coupling with ^2H. As the ESEEM decay envelope is a product function, containing modulations from all magnetic nuclei (Mims & Peisach, 1989), one is able to form a ratio of the modulation data for the D_2O sample with that for the H_2O sample and obtain a modulation pattern representative of the difference in isotopic composition. Modulations from ^{14}N are removed, and one obtains a modulation pattern dominated by the contribution from deuterium that has replaced exchangeable protons (McCracken et al., 1987; Mims et al., 1990). The cosine Fourier transform of the ratio data consists of a 2-MHz line arising from exchangeable deuterons and a line at higher frequency corresponding to lost protons whose overall depth of modulation is less than for the deuterons (Yudanov et al., 1969; Mims et al., 1977; Dikanov et al., 1987).

Although the ESEEM obtained is a measurement of electron nuclear coupling with all of the exchangeable deuterons in the sample, the greatest spectral contribution is from those deuterons in closest proximity, as the depth of modulation from a single nucleus that is dipole coupled to Mn^{2+} is dependent on the inverse sixth power of the distance (Mims et al., 1977, 1990). Thus, a deuteron on a coordinated water ligand would have a much larger spectral contribution, say, as one on a water in the secondary coordination sphere. Since the closest exchangeable protons for Con A are on coordinated water, the deuterium modulation obtained is for the most part from water deuterons. The Fourier transform of the data yields a 2-MHz line whose area depends on modulation depth and damping. For all the lectins studied, the half peak width is essentially the same while the amplitude of the 2-MHz line is nearly identical (Table I). Since the modulation obtained in the

study arises from $\Delta m_s = 1$ transitions in the $S = 5/2$ manifold for Mn^{2+} and the EPR spectra are similar (Reed & Cohen, 1970; Tichy et al., 1971; Meirovitch et al., 1978; Bhattacharyya et al., 1985b), the conclusion can be made that the deuterium contributions to the spectrum are comparable and that the modulations are derived from identical structures. Thus, two water molecules are shown to be bound to Mn^{2+} in all the lectins examined. [Interestingly, the number of coordinated water molecules bound to Mn^{2+} is not significantly altered by addition of specific saccharides to the lectins, although in the case of Con A the kinetics of solvent exchange at Mn^{2+} decreases (Brewer & Brown, 1979).]

In order to independently verify the above finding, a pair of Mn^{2+} models with EDTA and DTPA were studied, each in H_2O and in D_2O (Serpersu et al, 1988). There is a single water molecule bound to Mn^{2+} in the EDTA complex, but none in the DTPA complex (Stezowski & Hoard, 1984). Therefore, the contribution of deuterium from coordinated water in the EDTA complex should exceed the contribution from ambient or bulk solvent deuterons in both the EDTA and DTPA complexes. Thus, the ratio of data for Mn^{2+}–EDTA in D_2O to that in H_2O removes contributions from ^{14}N in the data set and provides modulation information about deuterons in coordinated water, C, and in ambient water, A, expressed as $C \times A$. A similar treatment with data for Mn^{2+}–DTPA provides deuteron modulations for ambient water, A.

To obtain modulation data for the single coordinated water, the modulation data $C \times A$ obtained for EDTA samples is divided by A obtained for DTPA samples. The result, subsequent to Fourier transformation, is a 2-MHz line whose amplitude is half of that for D_2O-exchanged Con A and other exchanged lectin samples and whose width is virtually the same as that of the 2-MHz line in the protein data. From this study, it is concluded that the Mn^{2+} sites in the various lectins examined contain the same number of coordinated water molecules. The lack of an exchange contribution to solvent proton relaxation at the Mn^{2+} site in PSA and LcH (Bhattacharyya et al., 1985a) as well as in SBA (Brewer, unpublished results) is thus due to slow solvent exchange rather than to the absence of coordinated water molecules. Although the Mn^{2+} ligands appear to be the same, differences in the amino acids near the Mn^{2+} site in the proteins (Strosberg et al., 1986) appear to influence the kinetics of solvent exchange at the metal ion. Indeed, Hardman et al. (1982) have observed that the water ligands of Mn^{2+} in crystalline Con A have access to the exterior of the protein and solvent through small gaps in the van der Waals packing between amino acids 17–18 and 32–34. The majority of the corresponding residues in PSA, LcH, and SBA differ from those in Con A (Strosberg et al., 1986).

Registry No. Mn, 7439-96-5; concanavalin A, 11028-71-0.

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Uniform ^{13}C Isotope Labeling of Proteins with Sodium Acetate for NMR Studies: Application to Human Carbonic Anhydrase II†

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ABSTRACT: Uniform double labeling of proteins for NMR studies can be prohibitively expensive, even with an efficient expression and purification scheme, due largely to the high cost of [$^{13}C_6$, 99%]glucose. We demonstrate here that uniformly (>95%) ^{13}C and ^{15}N double-labeled proteins can be prepared for NMR structure/function studies by growing cells in defined media containing sodium [1,2-$^{13}C_2$, 99%]acetate as the sole carbon source and [^{15}N, 99%]ammonium chloride as the sole nitrogen source. In addition, we demonstrate that this labeling scheme can be extended to include uniform carbon isotope labeling to any desired level (below 50%) by utilizing media containing equal amounts of sodium [1-^{13}C, 99%]acetate and sodium [2-^{13}C, 99%]acetate in conjunction with unlabeled sodium acetate. This technique is less labor intensive and more straightforward than labeling using isotope-enriched algal hydrolysates. These labeling schemes have been used to successfully prepare NMR quantities of isotopically enriched human carbonic anhydrase II. The activity and the ^1H NMR spectra of the protein labeled by this technique are the same as those obtained from the protein produced from media containing labeled glucose; however, the cost of the sodium [1,2-$^{13}C_2$, 99%]acetate growth media is considerably less than the cost of the [$^{13}C_6$, 99%]glucose growth media. We report here the first published ^{13}C and ^{15}N NMR spectra of human carbonic anhydrase II as an important step leading to the assignment of this 29-kDa zinc metalloenzyme.

The size of proteins whose backbone assignment and subsequent structure determination can be effectively elucidated by high-field NMR spectroscopy has increased rapidly as advances in pulse sequences, probe design, and instrumentation have been made. One major contributing factor to this advance has been the ability to utilize ^{13}C and ^{15}N isotopically labeled proteins in both backbone and side-chain assignment strategies (Marion et al., 1989; Wang et al., 1990; Westler et al., 1988; Bax et al., 1990; Fesik et al., 1990; Kay et al., 1990; Ikura et al., 1990).

The traditional first step in a protein NMR solution structure study is to assign the backbone protons as completely as possible. For modestly sized proteins, this is usually accomplished by using homonuclear ^1H 2-D methodologies including through-bond or COSY-type experiments in conjunction with through-space or NOESY experiments. The data sets generated are subsequently analyzed and assigned on the basis of published strategies (Wüthrich, 1986; Englander & Wand, 1987). These strategies break down, however, as the size of the protein exceeds 10 kDa due to spectral overlap, a decrease in sensitivity resulting from line broadening, decreases in T_2 relaxation times, and a marked increase in assignment ambiguities. Most of these problems can be overcome by recently developed heteronuclear experiments, including heteronuclear multiple quantum coherence experiments (HMQC, HMQC-COSY, NOESY-HMQC, and TOCSY-HMQC) (Marion et al., 1989; Wang et al., 1990; Westler et al., 1988), ^{13}C-^{13}C magnetization-transfer experiments (HC-CH) (Bax et al., 1990; Fesik et al., 1990; Kay et al., 1990), and the 3-D experiments [HNCO, HNCA, HCACO, HCA(CO)N] introduced by Ikura et al. (1990). All of these experiments derive sequential connectivities through one-bond J-couplings and are, therefore, independent of the local geometry of the molecule. In addition, assignments using these experimental methods are less ambiguous because they do not depend on NOE information and because they offer several independent pathways in determining sequential connectivities. Moreover, since only large couplings (H-C, ~125-160 Hz; C-C, ~33-45 Hz; C-N, ~7-15 Hz; and H-N, ~90 Hz) are

†Supported by grants to C.A.F. from the National Institute of Health (R29GM40602) and from the American Cancer Society (JFRA246) and to L.D.S. from the National Institute of Health (R01GM41829) and Glaxo Pharmaceuticals, Inc.
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0006-2960/91/0430-4491$02.50/0