

## Circular Dichroism and $^1\text{H}$ NMR Studies of $\text{Co}^{2+}$ - and $\text{Ni}^{2+}$ -substituted Concanavalin A and the Lentil and Pea Lectins\*

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Visible absorption, circular dichroism (CD) and magnetic circular dichroism spectra have been recorded for the  $\text{Ca}^{2+}$ - $\text{Co}^{2+}$  derivatives of the lentil (CCoLcH) and pea (CCoPSA) lectins ( $\text{Co}^{2+}$  at the S1 sites and  $\text{Ca}^{2+}$  at the S2 sites) and shown to be very similar for both proteins. The visible absorption and magnetic circular dichroism spectra indicate similar octahedral geometries for high spin  $\text{Co}^{2+}$  at S1 in both proteins, as found in the  $\text{Ca}^{2+}$ - $\text{Co}^{2+}$  complex of concanavalin A (CCoPL) (Richardson, C. E., and Behnke, W. D. (1976) *J. Mol. Biol.* 102, 441-451). The visible CD data, however, indicate differences in the environment around S1 of CCoLcH and CCoPSA compared to CCoPL.

$^1\text{H}$  NMR spectra at 90 MHz of the  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$  derivatives of the lectins show a number of isotropically shifted signals which arise from protons in the immediate vicinity of the S1 sites. Analysis of the spectra of the  $\text{Co}^{2+}$  derivatives in  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$  has permitted resonance assignments of the side chain ring protons of the coordinated histidine at S1 in the lectins. Differences are observed in the H-D exchange rate of the histidine NH proton at S1 in concanavalin A compared to the lentil and pea lectins. NMR data of the  $\text{Ni}^{2+}$ -substituted proteins, together with spectra of the  $\text{Co}^{2+}$  derivatives, also indicate that the side chains of a carboxylate ligand and of the histidine residue at S1 are positioned differently in concanavalin A than in the other two lectins. These results appear to account, in part, for the differences observed in the visible CD spectra of the  $\text{Co}^{2+}$ -substituted proteins. In addition, binding of monosaccharides does not significantly perturb the spectra of the lectins.

An unusual feature in the  $^1\text{H}$  NMR spectra of all three  $\text{Co}^{2+}$ -substituted lectins is the presence of two exchangeable downfield shifted resonances which appear to be associated with the two protons of a slowly exchanging water molecule coordinated to the  $\text{Ca}^{2+}$  ion at S2.  $T_1$  measurements of CCoLcH have provided an estimation of the distances from the  $\text{Co}^{2+}$  ion to these two protons of 3.7 and 4.0 Å.

Lectins are cell-agglutinating proteins of nonimmune origin (1) having diverse and unusual biological properties that relate to their saccharide binding specificities (2, 3). Among the most widely used lectins are concanavalin A (Con A)<sup>1</sup> and the lentil (LcH) and pea (PSA) lectins, which have all been classified as D-glucose/D-mannose-specific lectins (4). ConA exists as a dimer or tetramer depending upon the pH, ionic strength of the solution, and temperature (5), and possesses a single polypeptide subunit of  $M_r$  26,000 (6). LcH and PSA are dimers with subunits of  $M_r$  23,500 which consist of two noncovalently bound polypeptide chains of  $M_r$  5,500 and 18,000 (7, 8). All three lectins are metalloproteins containing 1  $\text{Mn}^{2+}$  and 1-2  $\text{Ca}^{2+}$ /monomer, and require the metal ions for their biological activities (9-11).

The x-ray data for ConA show that the  $\text{Mn}^{2+}$  ion at the S1 site (transition metal site) and the  $\text{Ca}^{2+}$  ion at the S2 site (calcium site) are 4.25 Å apart (12).  $\text{Mn}^{2+}$  at S1 is coordinated to the side chain oxygens from Glu-8, Asp-10, and Asp-19, to the  $N^{\epsilon}$ -2 nitrogen of His-24, and to two water molecules. Asp-10 and Asp-19 are bridging ligands to  $\text{Ca}^{2+}$  at S2, with Asp-14, Tyr-12, and two water molecules completing its coordination sphere. The side chain of Tyr-12 is believed to contribute to the saccharide binding site in ConA (12). Primary (13, 14) and secondary (15) structure data along with preliminary x-ray crystallographic data for LcH (7) and 3 Å data for PSA (16) indicate close structural homologies with ConA. The only reported difference in the metal binding sites of all three lectins is that Tyr-12 in ConA is substituted by phenylalanine residues in both LcH (13) and PSA (14).

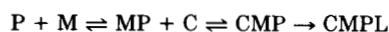
The mechanism of metal ion binding and activation of ConA by  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  has been extensively investigated. Using solvent proton nuclear magnetic relaxation dispersion techniques, Brown and co-workers (10, 17) showed that ConA exists as a mixture of two conformations: an "unlocked" conformation which weakly binds metal ions and saccharides and a "locked" conformation which strongly binds metal ions

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<sup>1</sup> The abbreviations used are: ConA, concanavalin A with unspecified metal ion content; CCoPL, ConA with  $\text{Co}^{2+}$  (Co) and  $\text{Ca}^{2+}$  (C) at the S1 and S2 sites, respectively, in the locked conformation (10); CCoP, ConA with  $\text{Co}^{2+}$  (Co) and  $\text{Ca}^{2+}$  (C) at the S1 and S2 sites, respectively, in the unlocked conformation (10); CoP, ConA with  $\text{Co}^{2+}$  at the S1 site in the unlocked conformation (10); CNiPL, ConA with  $\text{Ni}^{2+}$  (Ni) and  $\text{Ca}^{2+}$  at the S1 and S2 sites, respectively, in the locked conformation; LcH, lentil lectin; CCoLcH, LcH with  $\text{Co}^{2+}$  and  $\text{Ca}^{2+}$  in the S1 and S2 sites, respectively; CNiLcH, LcH with  $\text{Ni}^{2+}$  and  $\text{Ca}^{2+}$  in the S1 and S2 sites, respectively; PSA, pea lectin; CCoPSA, PSA with  $\text{Co}^{2+}$  and  $\text{Ca}^{2+}$  at the S1 and S2 sites, respectively; CNiPSA, PSA with  $\text{Ni}^{2+}$  and  $\text{Ca}^{2+}$  at the S1 and S2 sites, respectively; CD, circular dichroism; HDO, hydrogen deuterium oxide; deg, degree.

and saccharides. Their findings are summarized in the following equilibria.



Binding of a transition metal ion (M) to the S1 site of the apoprotein (P), which exists predominantly in the unlocked state, results in a weak affinity complex, MP, and the formation of S2. Binding of  $Ca^{2+}$  (C) to MP at the S2 site produces a metastable ternary complex, CMP, which undergoes a rapid first order conformational transition to the stable locked complex, CMPL, that possesses full saccharide binding activity (17).

ConA has also been substituted by other metal ions such as  $Co^{2+}$  (Co),  $Ni^{2+}$  (Ni), and  $Cd^{2+}$  at S1, which form active complexes with  $Ca^{2+}$  at S2 (*cf.* Refs. 18 and 19). Behnke and co-workers (20) have reported the "locking" effects of  $Ca^{2+}$  binding on the near ultraviolet CD, visible CD (21, 22), and magnetic circular dichroism (19) spectra of apo-ConA containing  $Co^{2+}$  at S1 (*i.e.*  $CoP \rightleftharpoons CCoP \rightarrow CCoPL$ ). Time-dependent changes in the intrinsic (20) and extrinsic Cotton effects (21, 22) were observed during the locking process, and different visible CD spectra were recorded for the respective binary and ternary  $Co^{2+}$  complexes (above) (21, 22). The results also demonstrated that  $Ca^{2+}$  binding induced a conformational change in the side chain of Tyr-12 at S2 (20).

Similar studies of metal ion binding in LcH and PSA have not been possible since their apoproteins are unstable and cannot be remetalized (11). However, Bhattacharyya *et al.* (11) recently developed a method of substituting different transition metal ions including  $Co^{2+}$  and  $Ni^{2+}$  into the S1 sites of LcH and PSA which results in fully active metalloprotein derivatives. These derivatives of the two lectins now permit a range of spectroscopic techniques to be used to investigate the metal ion and saccharide binding properties of LcH and PSA (23, 24).

The present paper is a comparative study of the metal ion binding sites of CCoPL, CCoLcH, and CCoPSA. The visible absorption and magnetic circular dichroism data are consistent with octahedral geometries for high spin  $Co^{2+}$  at the S1 sites of all three proteins. However, although the visible CD spectra of CCoLcH and CCoPSA are essentially identical, they differ from that observed for CCoPL, indicating differences in the environment around the  $Co^{2+}$  ion in the latter. These differences have been investigated by  $^1H$  NMR studies of the  $Co^{2+}$  and  $Ni^{2+}$  derivatives of all three lectins. In contrast to a previous NMR investigation of CCoPL at 220 MHz (25), a number of isotropically shifted resonances at 90 MHz are observed that arise from protons belonging to metal-coordinated ligands at S1 and S2. Similar shifts are observed for CCoLcH and CCoPSA. Together with studies of the corresponding  $Ni^{2+}$ -substituted lectins, the NMR results have allowed assignment of certain ligand protons around the S1 and S2 sites and, thus, direct comparison of the structural and dynamic properties of the metal ion binding sites of the proteins. Differences are observed in the spectra of the  $Co^{2+}$  and  $Ni^{2+}$  derivatives of ConA compared to the other two lectins.

The present results add to a series of recently reported  $^1H$  NMR studies of  $Co^{2+}$ -substituted proteins which have provided structural information on mononuclear and binuclear metal environments (*cf.* Refs. 26–31).

#### MATERIALS AND METHODS

ConA was purchased from Miles-Yeda. Apo-ConA (P), CoP, CCoPL, and CCoPL were prepared and characterized following the methods previously described (17). Seeds of lentil (*Lens culinaris* Med. sub. *Macrosperma*) and pea (*Pisum sativum* L. var. Columbian)

were purchased from a local food store. The two lectins were purified by affinity chromatography on Sephadex G-100 (32, 33). CCoLcH and CCoPSA, and CCoPSA and CCoPSA were prepared as previously reported (11). The concentrations of proteins in solutions were determined by using their respective extinction coefficients at 280 nm: ConA,  $A_{1\text{cm}}^{1\%} = 12.4$  (4); LcH,  $A_{1\text{cm}}^{1\%} = 12.6$  (4); and PSA,  $A_{1\text{cm}}^{1\%} = 15.0$  (11). Salts of different metals were the highest purity products available from either Mallinckrodt Chemical Works or Fisher. Monosaccharides were obtained from Sigma and Pfanstiel Laboratories.

Samples were prepared by dissolving the lyophilized  $Ca^{2+}$ - $Co^{2+}$  or  $Ca^{2+}$ - $Ni^{2+}$  proteins in  $H_2O$  or  $D_2O$  buffers and spinning down residual undissolved material. For ConA the buffer was 0.1 M potassium acetate, 0.9 M KCl, pH 6.4, and for LcH and PSA, 0.1 M potassium acetate, 0.1 M KCl, pH 6.4. The protein solutions are stable for several weeks at 4 °C. CoP was prepared by adding 0.8 eq of cobalt chloride to apo-ConA in 0.1 M potassium acetate buffer containing 0.9 M KCl, pH 6.4 (17).

**Visible Absorption, CD, and Magnetic Circular Dichroism Measurements**—Absorption spectra were measured with a Cary 17D spectrophotometer. Visible CD spectra were obtained with a Jasco 500 spectropolarimeter at 22 °C. Standardization of the instrument was accomplished by using an aqueous solution of 0.24 M nickel sulfate plus 0.36 M sodium potassium tartrate, as specified by Jasco. Quartz cells with a path length of 1.0 cm were used for all spectral measurements. Molar ellipticities,  $\theta$ , were calculated as specified by Varian and have units of  $\text{deg cm}^2 \text{dmol}^{-1}$ . Magnetic circular dichroism spectra were measured with a Cary 61 spectropolarimeter equipped with a Varian superconducting magnet. The field strength ran from 40 to 50 kG (4–5 tesla), the latter at a calibrated current of 29.3 A, and cobalt standardization was accomplished with commercial standard solutions.

**NMR Measurements**—Unless otherwise specified,  $^1H$  NMR spectra were recorded at 90 MHz using a Bruker CXP90 instrument at 27 °C. Spectra were acquired in the quadrature detection mode using a modified DEFT sequence (28, 34); a spectral width of 50,000 Hz ( $\pm 278$  ppm from the  $H_2O$  or residual HDO signal) and repetition times of 90 ms were used. (Preliminary runs were made over a 125,000-Hz spectral width to check for additional resonances.) Typically, 5–20 blocks (16,000 scans each) were averaged after Fourier transformation. A 20-Hz line broadening function was applied to improve signal-to-noise.  $T_1$  values were obtained using the modified DEFT sequence, as previously reported (34).  $^1H$  NMR spectra at 200 MHz were recorded using a Varian XL-200 spectrometer.

#### RESULTS

**Visible Absorption, CD, and Magnetic Circular Dichroism Spectra**—Fig. 1A shows the visible absorption spectra of CCoLcH and CCoPSA in the range 400–700 nm, in addition to those of CoP and CCoPL (18). The positions of the absorption maxima (520 nm) and low values of the extinction coefficients at the maxima are characteristic of octahedral or slightly distorted octahedral cobalt complexes in solution (35) and suggest such environments for cobalt in these proteins (18). Addition of 3-*O*-methyl D-glucose to CCoLcH and CCoPSA did not alter their spectra.

Fig. 1B shows the visible CD spectra of CCoLcH and CCoPSA. The spectra of CoP and CCoPL are shown for comparison (18, 21). CCoLcH and CCoPSA both exhibit strong positive bands with maxima near 490 and 525 nm, as previously reported for the former (23), and the two show similar molar ellipticities.

Visible magnetic circular dichroism spectra of CCoLcH and CCoPSA in the range of 400–600 nm are shown in Fig. 2 and are also similar. The visible magnetic circular dichroism spectrum of CCoPL is also shown for comparison (19). The spectra of all three proteins are typical of octahedral cobalt complexes in solution (36) and are characterized by a strong positive ( $B + C/kT$ ) term centered around 510 nm with a shoulder at 450 nm. This shoulder is less pronounced in the spectrum of CCoLcH, which resembles more closely those of  $Co(H_2O)_6^{2+}$  and  $Co(NH_3)_6^{2+}$  in solution (36). Binding of 3-*O*-methyl D-

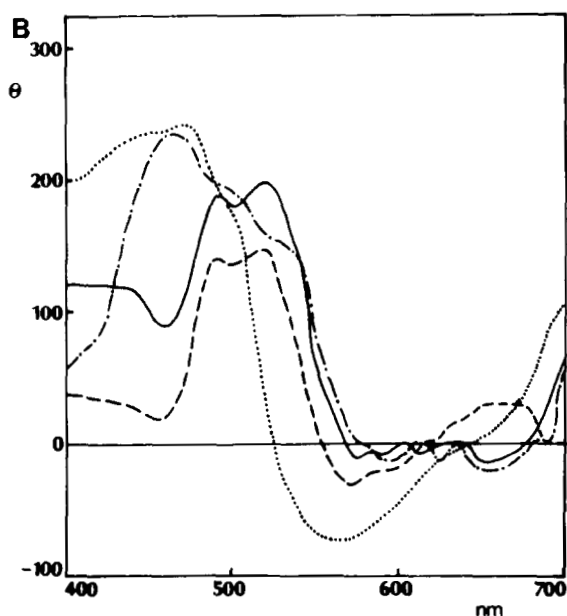
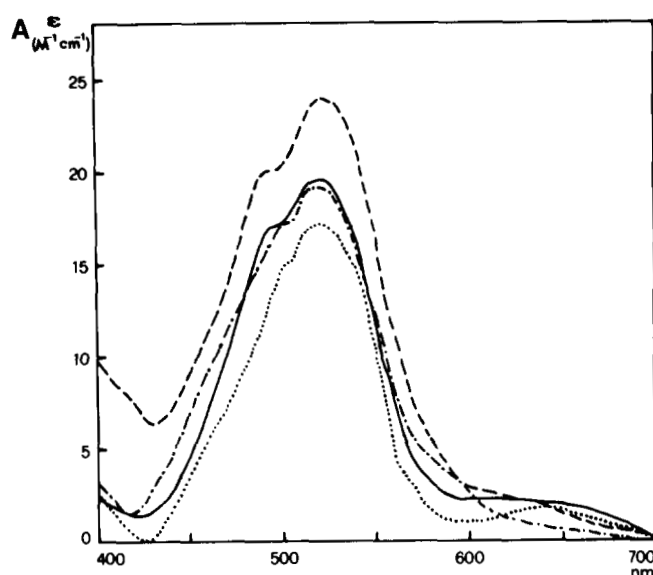


FIG. 1. A, the visible absorption spectra of CCoPSA (---) and CCoLcH (—) in 0.1 M potassium acetate buffer containing 0.1 M KCl, pH 6.4; CoP (---) in 0.1 M potassium acetate buffer containing 0.9 M potassium chloride, pH 6.4, and CCoPL (····) in 0.1 M potassium acetate buffer containing 0.9 M KCl, pH 6.4. The temperature was 22 °C. The protein concentrations were approximately 1 mM. B, visible CD spectra of CCoPSA (---), CCoLcH (—), CoP (---), and CCoPL (····) in the same buffers as in A. Protein concentrations were 0.3–0.5 mM. Molar ellipticities ( $\theta$ ) have units of  $\text{deg cm}^2 \text{ dmol}^{-1}$ .

glucose to CCoLcH and CCoPSA did not affect their respective spectra.

**NMR Spectra of the  $\text{Co}^{2+}$  Derivatives**—The spectra of CCoPL, CCoLcH, and CCoPSA in  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$  are shown in Fig. 3. With  $\text{H}_2\text{O}$  as solvent, a number of well resolved isotropically (paramagnetically) shifted signals are apparent in the spectra in the 100 to  $-40$  ppm range, well outside the diamagnetic region of the spectrum. These resonances are clearly observable at relatively low magnetic fields (90 MHz), whereas experiments performed at 200 MHz show much broader resonances over this region. Such behavior arises from Curie broadening (40, 41), which, for the present  $\text{Co}^{2+}$ -

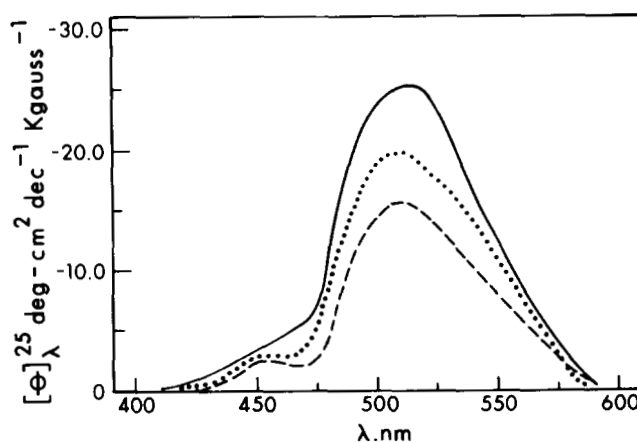


FIG. 2. Magnetic circular dichroism at 22 °C of cobalt-substituted lectins in the visible region; ---, CCoPSA; —, CCoLcH; and ····, CCoPL. Conditions are identical to those in Fig. 1. All spectra are corrected for CD contributions in the absence of magnetic field.

containing dimeric proteins of molecular weight of about 50,000, is expected to be sizable (42). The spectra of CCoPSA and CCoLcH are similar to each other, and show similarities and differences compared to that of CCoPL.

With  $\text{D}_2\text{O}$  as the solvent, the  $^1\text{H}$  NMR spectrum of CCoPL reveals additional differences compared to the other two lectins (Fig. 3). For CCoPSA and CCoLcH, the downfield signals at 75.9 and 74.1, 50.0 and 50.8, and 37.2 and 39.1 ppm, respectively, disappear in  $\text{D}_2\text{O}$ . For CCoPL, the signal at 72.2 ppm is still observed after several days in  $\text{D}_2\text{O}$  but vanishes after 2–3 weeks. CCoPL in  $\text{D}_2\text{O}$  also shows a signal at 39.7 ppm in the region where the protein in  $\text{H}_2\text{O}$  shows three signals at 43.0, 37.6, and 32.2 ppm (Fig. 3). (The broad resonance giving rise to the shoulder at 35 ppm in CCoPL in  $\text{D}_2\text{O}$  is discussed below.) It appears, therefore, that two of these three signals arise from exchangeable protons, whereas the third undergoes a shift from  $\text{H}_2\text{O}$  to  $\text{D}_2\text{O}$ . In order to establish a correspondence between the signal at 39.7 ppm observed for CCoPL in  $\text{D}_2\text{O}$  and the 43.0, 37.6, or 32.2 ppm signals observed in  $\text{H}_2\text{O}$ , the temperature dependence of the chemical shifts of the signals in both solvents was investigated between 4 and 50 °C (Fig. 4). The data suggest that the nonexchangeable proton at 39.7 ppm in  $\text{D}_2\text{O}$  is the signal at 37.6 ppm in the  $\text{H}_2\text{O}$  spectrum, based on their relative lack of temperature sensitivity.

The spectra of the three  $\text{Co}^{2+}$ -substituted lectins in  $\text{D}_2\text{O}$  also show evidence of two very broad signals in the downfield region (Fig. 3). These signals are approximately centered at 87 and 51 ppm in CCoLcH, 81 and 42 ppm in CCoPSA, and 77 and 35 ppm in CCoPL. These two resonances in CCoPL appear to be narrower than the corresponding pair in the other two lectins. These resonances are also present in the  $\text{H}_2\text{O}$  spectra but are less evident probably due to dynamic range problems.

A detailed comparison of many of the downfield shifted signals can now be made between CCoPL and the other two lectins (Fig. 5). Since the 72.2 ppm signal in CCoPL is an exchangeable proton (albeit slowly), it is easily related to the signal at 75.9 ppm of CCoPSA and 74.1 ppm of CCoLcH. Likewise, the exchangeable signal at 43.0 ppm in CCoPL can be related to that at 50.0 ppm in CCoPSA and 50.8 ppm in CCoLcH, while the 32.2 ppm signal in CCoPL would correspond to the 37.6 ppm signal in CCoPSA and 39.1 ppm in CCoLcH. The two broad signals in the spectra of all three proteins appear shifted differently (approximately 87 and 51



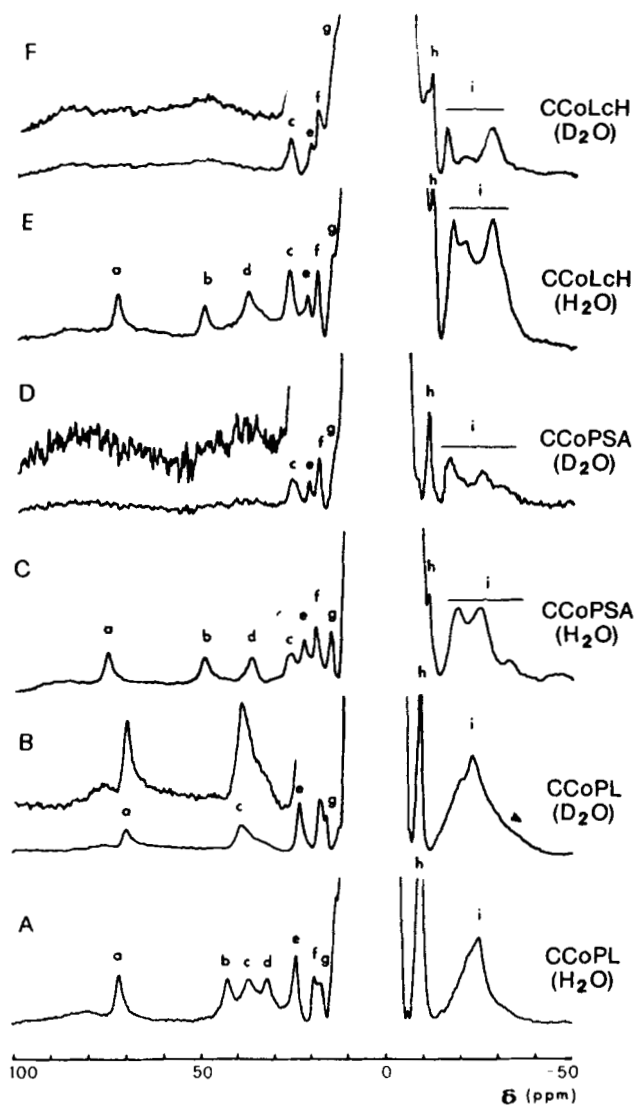


FIG. 3. 90 MHz  $^1\text{H}$  NMR spectra at 27  $^\circ\text{C}$  of CCoPL in  $\text{H}_2\text{O}$  solution (A), in  $\text{D}_2\text{O}$  solution (B); CCoPSA in  $\text{H}_2\text{O}$  solution (C), in  $\text{D}_2\text{O}$  solution (D); CCoLcH in  $\text{H}_2\text{O}$  solution (E), and in  $\text{D}_2\text{O}$  solution (F). Additional broad signals are evident in the vertical expansion regions. Sample buffers and pH were the same as those in Fig. 1. Protein concentrations were approximately 1 mM. The large resonance band in the spectra of the  $\text{D}_2\text{O}$  solutions is due to residual HDO and nonexchangeable protein resonances.

ppm in CCoLcH, 81 and 42 ppm in CCoPSA, 77 and 35 ppm in CCoPL), but their correspondence is straightforward.

A difference between CCoPL and the other two lectins is the presence of the nonexchangeable proton at 37.6 ppm in the former. A correspondence for this signal can be made with the nonexchangeable signals at 26.1 ppm in CCoPSA and 27.8 ppm in CCoLcH, mainly because these latter signals are also less sensitive to temperature than the others (data not shown). The other three signals at 22.8, 19.7, and 15.5 ppm in CCoPSA and 22.7, 20.1, and 15.8 ppm in CCoLcH then correspond to those at 24.5, 19.6, and 18.2 ppm, respectively, in CCoPL (Fig. 5).

A correspondence for the upfield shifted signals of the lectins is more difficult to establish. The featureless envelope of resonances, *i*, in CCoPL around  $-25$  ppm in  $\text{H}_2\text{O}$  probably contains the signals which are at  $-16.8$ ,  $-19.9$ , and  $-27.2$  ppm in CCoLcH and at  $-18.7$ ,  $-24.6$ , and  $-32.4$  ppm in CCoPSA. The three proteins also show a relatively sharp and

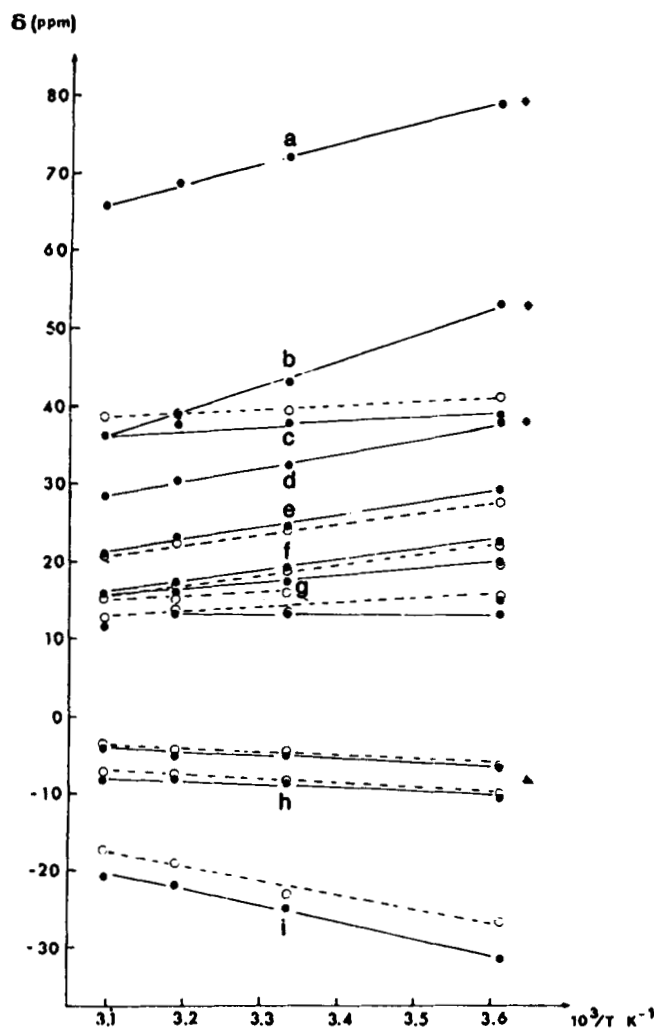


FIG. 4. Temperature dependence of the chemical shifts for CCoPL.  $\bullet$ , values obtained in  $\text{H}_2\text{O}$  solution;  $\circ$ , values obtained in  $\text{D}_2\text{O}$  solution. Sample conditions were as described in the legend to Fig. 3. Asterisks denote exchangeable protons. (The two split "h" peaks of CCoPL are not seen in the spectra shown in Fig. 3 but are observed in other spectra of CCoPL. These two peaks are sensitive to phasing and the size of the HDO and residual protons of the protein.)

intense signal *h* in the  $-9$ - to  $-11$ -ppm region. These correlations are shown in Fig. 5.

In order to gain information on the nature of the two exchangeable protons,  $^1\text{H}$  NMR spectra have been recorded of apo-ConA (P) with substoichiometric amounts of  $\text{Co}^{2+}$  (0.8 eq) in the absence of calcium. Under these conditions,  $\text{Co}^{2+}$  binds at the S1 site of P to give CoP, the binary complex in the unlocked conformation (17-22). The  $\text{H}_2\text{O}$  spectrum is shown in Fig. 6. Exchanging  $\text{D}_2\text{O}$  for  $\text{H}_2\text{O}$  results in the disappearance of the signal at 77.4 ppm. Addition of 1 eq of  $\text{Ca}^{2+}$  at ambient temperature results in the formation of the "locked" ternary complex (17) and appearance of the spectrum of CCoPL after a short time. The spectrum of CoP is different from that of CCoPL, but a correlation among certain signals can be established. The exchangeable proton at 77.4 ppm of CoP corresponds to signal *a* at 72.2 ppm in CCoPL, while the nonexchangeable signal at 37.6 ppm in CoP appears to correspond to nonexchangeable signal *c* at 37.6 ppm in CCoPL (Fig. 3). The nonexchangeable signals at 29.3, 26.2, and 25.6 ppm of CoP appear to relate to the nonexchangeable resonances at *e*, *f*, and *g* at 24.5, 19.6, and 18.1 ppm, respectively,

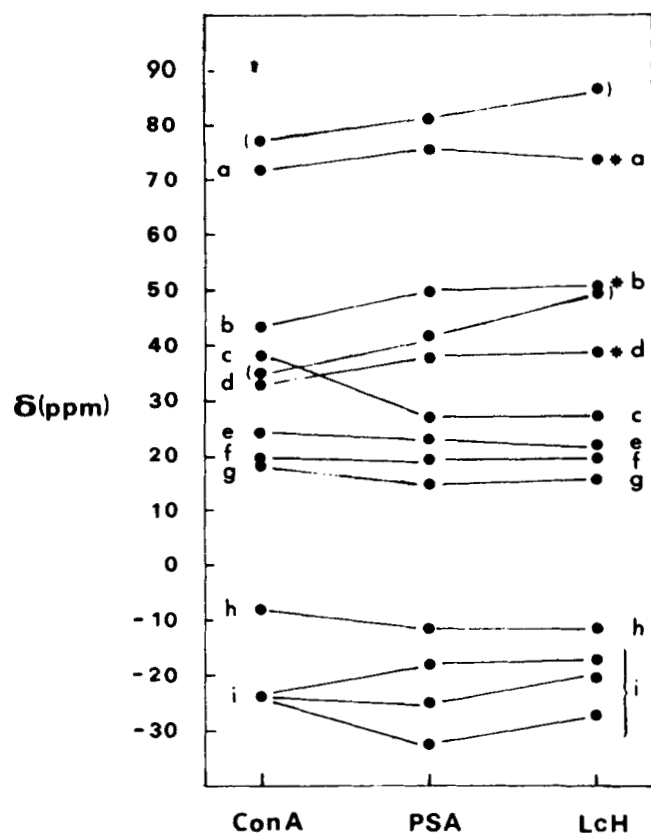


FIG. 5. Correspondence among the isotropically shifted proton signals in CCoPL, CCoPSA, and CCoLcH. Asterisks denote exchangeable protons. Parentheses indicate the two broad signals evidenced by vertical expansion in Fig. 3.

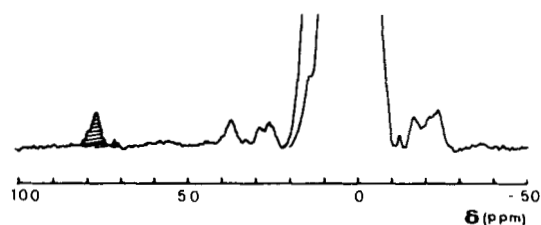


FIG. 6. 90 MHz  $^1\text{H}$  NMR spectra at 27 °C of CoP (1.1 mM apo-ConA plus 0.8 eq of  $\text{CoCl}_2$ ) in 0.1 M potassium acetate buffer containing 0.1 M potassium chloride at pH 6.4. The dashed signal disappears in  $\text{D}_2\text{O}$  solution.

in CCoPL. The two exchangeable proton signals *b* and *d* at 43.0 and 32.2 ppm, respectively, of CCoPL in  $\text{H}_2\text{O}$  (Fig. 3) are absent in CoP. These two signals are, therefore, related to calcium ion binding to the locked ternary complex of the protein (17).

A pH titration of CCoPSA was performed from 6.2 to 10.7. No pH dependence of the isotropically shifted signals was detected up to pH 10.4. Above this pH, oxidation of  $\text{Co}^{2+}$  was observed, with concomitant denaturation of the sample. These results indicate that no protein ionization occurs between pH 6.2 and 10.4 that induces changes in the metal ion binding region.

**Saccharide Binding**—Addition of methyl  $\alpha$ -D-mannopyranoside to CCoPL and of 3-O-methyl D-glucose to CCoPSA and CCoLcH solutions yields the  $^1\text{H}$  NMR spectra shown in Fig. 7. The spectra are very similar to those of Fig. 1, which indicate that the saccharides do not bind close ( $>6$  Å) to the S1 sites in all three lectins due to the lack of shifted saccharide resonances. This agrees with NMR (43) and x-ray crystallo-

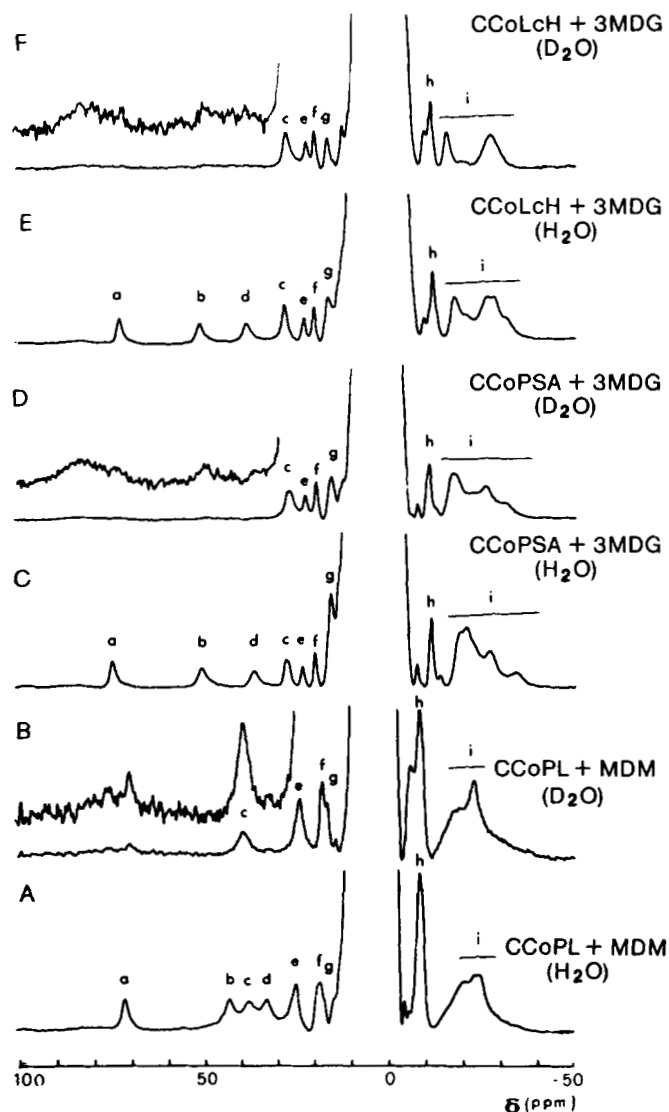


FIG. 7. 90 MHz  $^1\text{H}$  NMR spectra at 27 °C of CCoPL in  $\text{H}_2\text{O}$  (A) and  $\text{D}_2\text{O}$  solutions (B) in the presence of 0.1 M methyl  $\alpha$ -D-mannopyranoside (MDM); CCoPSA in  $\text{H}_2\text{O}$  (C) and  $\text{D}_2\text{O}$  solutions (D) in the presence of 0.1 M 3-O-methyl D-glucose (3MDG); and CCoLcH in  $\text{H}_2\text{O}$  (E) and  $\text{D}_2\text{O}$  solutions (F) in the presence of 0.1 M 3-O-methyl D-glucose. Except for added saccharides, sample conditions are as described in the legend to Fig. 3. The large resonance band in the spectra of the  $\text{D}_2\text{O}$  solutions is due to residual HDO and nonexchangeable protein resonances.

graphic data (12) for ConA-saccharide complexes. Slight differences in shifts are observed for the *a* signals of the proteins and somewhat larger differences for the two broad downfield signals, which are less evident than in the spectra obtained from the samples without saccharide. Since these resonances are assigned to the ring protons of the coordinated histidine at S1 (see "Discussion"), the results suggest a slight movement of this residue with respect to the metal ion in each protein. However, because of the high sensitivity of isotropically shifted signals to even small perturbations, it may be concluded that the geometry of the S1 site in each lectin remains essentially unaltered upon saccharide binding. Similar conclusions have been reached from the visible CD and magnetic circular dichroism data, as well as EPR studies of the native lectins (44, 45).

$T_1$  measurements were performed on the CCoLcH-sugar sample because of the quality of the spectra, and the values

obtained for most of the signals are shown in Table I.

**NMR Spectra of the Ni<sup>2+</sup> Derivatives**—The <sup>1</sup>H NMR spectra of the nickel derivatives of the three lectins in H<sub>2</sub>O and D<sub>2</sub>O are shown in Fig. 8. For CNIPL and CNIPLcH, only one isotropically shifted signal is observed (66.2 and 66.4 ppm, respectively) which disappears in D<sub>2</sub>O. CNIPL shows two downfield signals at 66.2 and 42.5 ppm; the former slowly decreases in intensity with time in D<sub>2</sub>O. A third signal is observed at -14.2 ppm. The most obvious choice is to assign

TABLE I  
Longitudinal relaxation times,  $T_1$  (ms), of <sup>1</sup>H NMR signals of CCoLcH in the presence of 3-O-methyl D-glucose

	Signals								
	a	b	d	c	e	f	h	i	i
ppm	73.4	51.7	38.8	28.5	23.1	20.4	-11.5	-17.2	-27.7
$T_1$ (ms)	12	12	8	20	20	26	21	18	9

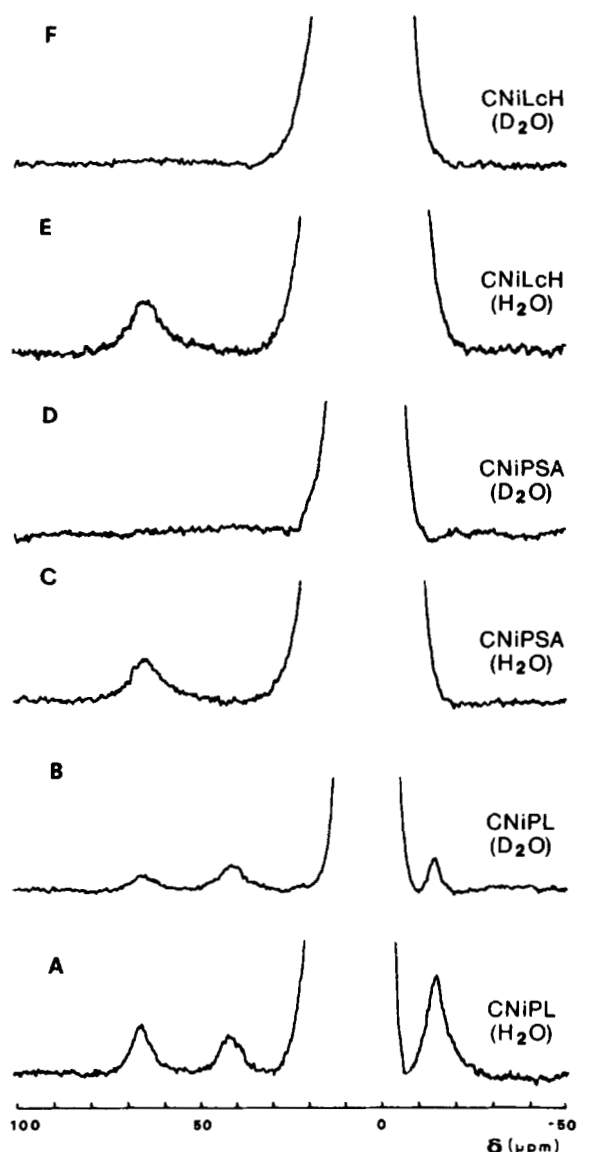


FIG. 8. 90 MHz <sup>1</sup>H NMR spectra at 27 °C of CNIPL in H<sub>2</sub>O solution (A), in D<sub>2</sub>O solution (B), CNIPLcH in H<sub>2</sub>O solution (C), in D<sub>2</sub>O solution (D), CNIPLcH in H<sub>2</sub>O solution (E), in D<sub>2</sub>O solution (F). Sample conditions are as described in the legend to Fig. 3. The concentrations of proteins were 1.0–1.5 mM.

the exchangeable signal in CNIPL and CNIPLcH, as well as the slowly exchanging peak of CNIPL, to the same proton that displays similar behavior in the cobalt derivatives (the a signals at 72.2, 75.9, and 74.1 ppm for CCoPL, CCoPSA, and CCoLcH, respectively). A correspondence can be analogously established between the signal of CNIPL at 42.5 ppm with signal c at 37.6 in CCoPL. The absence of such a signal in CNIPLcH is consistent with a smaller contact shift experienced by the latter signal in CCoPSA and CCoLcH with respect to CCoPL. The signal at -14.2 ppm in CNIPL appears to be absent or under the water envelope in the other two lectins.

## THEORY

Isotropic shifts in paramagnetic complexes arise from two different types of electron-nuclear spin interactions, namely contact and dipolar (pseudocontact) contributions. Contact shifts arise from the presence of fractions of unpaired electron spins delocalized onto s-type orbitals of the nuclei under investigation and, therefore, require that the nuclei be connected to the paramagnetic metal ion through covalent bonds (46). Dipolar shifts arise from through-space coupling of the electron and nuclear magnetic moments, and are independent of the presence of covalent bonds (47). Both contributions can be either up- or downfield. The sign of contact shifts depends on the kinds of molecular orbitals involved in the transmission of unpaired spin density to a particular nucleus (48). Its evaluation from first principles may be difficult, but for the cases of interest in the present study it can be predicted to be positive and sizable for histidine ring protons, positive but smaller for CH<sub>2</sub> protons adjacent to coordinated carboxylate groups, and slightly negative for the β-CH<sub>2</sub> group of the coordinated histidine (42, 48). The magnitude of the effect is expected to depend on the relative orientations of the metal-donor atom and the proton-heteroatom vectors (42, 49).

Dipolar shifts are a function of the reciprocal third power of the metal nucleus distance and of the angles between the metal-nucleus vector and the principal direction of the molecular anisotropy axis. In axially symmetric systems, the sign of the dipolar shifts goes from positive along the z axis to negative (and half in magnitude) in the XY plane (47). The shifts are expected to be sizable in hexacoordinated high spin Co<sup>2+</sup> complexes and of the same order of magnitude as the contact shifts for coordinated residues (42, 48). Hexacoordinated Ni<sup>2+</sup> complexes usually have much smaller anisotropy, and, therefore, the isotropic shifts are largely contact in origin. The latter are of the same order of magnitude as the corresponding contact shifts in Co<sup>2+</sup> complexes.

$T_1$  measurements are potentially useful for obtaining structural information in paramagnetic metalloproteins (42). To a first approximation,  $T_1$  values are mainly dipolar in origin and depend on the reciprocal sixth power of the metal-nucleus distance, as well as on a correlation time  $\tau_c$  for the electron-nucleus interaction. The latter is unknown in the present system, although it can be predicted to be fairly short on the grounds of theoretical expectations (27, 42) and of the lack of any measurable effect on the water proton nuclear magnetic relaxation dispersion (11). In principle, one should be able to evaluate  $\tau_c$  from the  $T_1$  of the His-24 NH signal and its distance from the metal ion calculated from x-ray data. However, ligand-centered dipolar contributions may play a sizable role in determining the relaxation rates of coordinated imidazole protons, thereby grossly affecting the evaluation of  $\tau_c$  (30). Moreover, in  $S > 1/2$  systems with sizable zero-field splitting, an angular dependence of  $T_1$  values has been recently predicted (47), making the use of  $T_1$  values for evalu-

ating the absolute metal proton distances impracticable unless additional information on the system is available. Qualitatively, however, the observed values confirm that all the signals come from fairly close protons, and their order should reflect their relative distances from the metal center.

#### DISCUSSION

**Visible Absorption, CD, and Magnetic Circular Dichroism Measurements**—The visible absorption and magnetic circular dichroism spectra of CCoLcH and CCoPSA are similar to each other and resemble visible absorption (18) and magnetic circular dichroism data (19) for CoP and CCoPL. These results indicate overall octahedral geometries for high spin  $\text{Co}^{2+}$  in all three lectins, which provides evidence that  $\text{Co}^{2+}$  binds to S1 of CCoLcH and CCoPSA. The visible CD spectra of CCoLcH and CCoPSA are also similar to each other; however, they differ from that of CCoPL and other  $\text{Co}^{2+}$  complexes of ConA (18, 21). The visible CD spectrum of the binary complex of apo-ConA in the unlocked conformation, CoP, shows strong positive bands at 470, 505, and 535 nm (21). Addition of  $\text{Ca}^{2+}$  to CoP forms the unstable ternary complex, CCoP, in the unlocked conformation, which possesses maxima at 475 and 505 nm and a weak shoulder at 525 nm (21). CCoP spontaneously converts to the stable CCoPL complex with reported maxima at 483 and 505 nm (21). The visible CD spectra of CCoLcH and CCoPSA with strong bands at 490 and 525 nm thus show marked differences from the spectra of the binary and ternary  $\text{Co}^{2+}$  complexes of ConA. These results indicate differences in the environment of the  $\text{Co}^{2+}$  at S1 in LcH and PSA as compared to ConA, even though the ligands of S1 in all three proteins are conserved (13, 14).

**$^1\text{H}$  NMR Spectra of the  $\text{Co}^{2+}$ -substituted Lectins**—The  $^1\text{H}$  NMR spectra of CCoPL, CCoPSA, and CCoLcH show a variety of hyperfine shifted resonances. However, an unusual feature of the NMR data is the observation of three exchangeable protons, *a*, *b*, and *d*, in the downfield region (30–76 ppm) in all three lectins. Judging from investigations of other  $\text{Co}^{2+}$ -substituted proteins and from theoretical considerations, the only exchangeable protons expected to undergo sizable isotropic shifts belong to NH groups of coordinated histidines (26–31, 42). Such signals are usually observed in the 30–80-ppm region (42). The x-ray data for ConA shows one histidine residue (His-24) coordinated at S1 through the  $N^{\epsilon}$ -2 nitrogen (Fig. 9) (12, 50). Therefore, one of the three exchangeable resonances would belong to the  $N^{\epsilon}$ -1 proton of this histidine residue. Primary sequence (13, 14) and  $^{113}\text{Cd}$  NMR data (24) indicate that this residue is conserved as a ligand at S1 in PSA (His-136) (14) and LcH (His-138) (13). The observation of more exchangeable protons than histidine NHs is unprecedented in  $^1\text{H}$  NMR spectra of  $\text{Co}^{2+}$ -protein complexes, and, therefore, conclusions about the identity of the two other resonances must be made with caution.

Histidine ring CHs are known to slowly undergo deuterium exchange under appropriate conditions (51), which could be the case for the CCoPL signal *a* at 72.2 ppm. However, it seems unlikely that the corresponding *a* resonances at 75.9 and 74.1 ppm for CCoPSA and CCoLcH, respectively, could be fully exchanged for deuterons within a few hours after dissolution of the protein in  $\text{D}_2\text{O}$ . Other candidates for the two extra exchangeable protons could be those belonging to the hydrogen bonding network around the S1 site involving carboxylate or water donors, such as the OH proton of Ser-34 of ConA which hydrogen bonds to one of the water ligands of the metal ion at S1 (12). It might even be tempting to assign the two extra exchangeable protons to one of the two

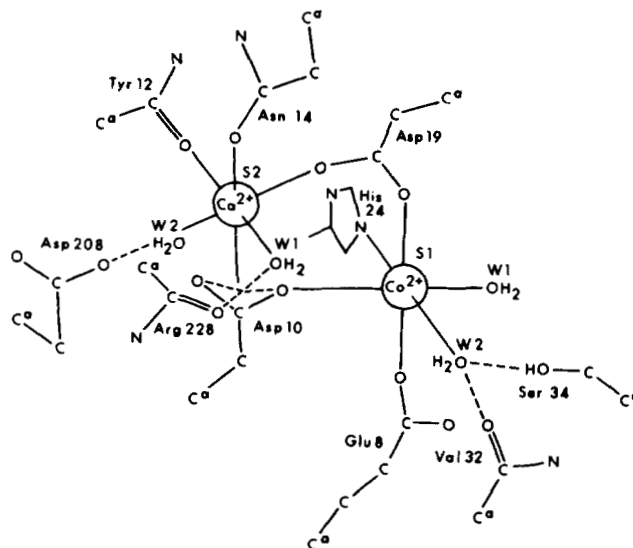


FIG. 9. Schematic illustration of the binding of  $\text{Co}^{2+}$  and  $\text{Ca}^{2+}$  to the S1 and S2 sites in CCoPL, respectively. The  $\text{Co}^{2+}$  and  $\text{Ca}^{2+}$  ions are both octahedrally coordinated by four protein ligands and two water molecules, W1 and W2. Drawn from the data in Refs. 12 and 50.

water ligands at S1. Such protons have been proposed to be in slow exchange on the NMR time scale from experiments with native  $\text{Ca}^{2+}$ - $\text{Mn}^{2+}$ -PSA and -LcH (43, 51). However, the proton resonances of these water molecules are expected to be sizably broader than the coordinated histidine NHs, since they are closer to the metal ion. No large difference in the line widths of signals *a*, *b*, and *d* in all three proteins is observed.

Experiments with CoP help provide insight into the assignments of these three downfield resonances in CCoPL. The spectrum of CoP shows an exchangeable signal at 77.4 ppm, which corresponds to signal *a* at 72.2 ppm in CCoPL, while the other two exchangeable signals, *b* and *d*, at 43.0 and 32.2 ppm in CCoPL are missing in CoP. Since  $\text{Co}^{2+}$  is known to bind to the S1 site in CoP (19) and thus to His-24, the resonance at 77.4 ppm in CoP and signal *a* at 72.2 ppm in CCoPL can be associated with the  $N^{\epsilon}$ -1 proton of this coordinated residue. Furthermore, since this histidine is conserved in the other two lectins (13, 14, 24), the corresponding *a* resonances at 75.9 and 74.4 ppm in CCoPSA and CCoLcH, respectively, can also be assigned to the  $N^{\epsilon}$ -1 proton of this residue. These assignments are consistent with the NMR results obtained for the  $\text{Ni}^{2+}$ -substituted lectins, as discussed below. The observation of relatively slow exchange of the *a* signal of CCoPL in  $\text{D}_2\text{O}$  compared to rapid exchange of the *a* signal in CCoLcH and CCoPSA suggests different structural and dynamic properties of the S1 site of ConA compared to the other two lectins.

Addition of  $\text{Ca}^{2+}$  to CoP to form CCoPL results in the appearance of resonances at 43.0 and 32.2 ppm, which correspond to signals *b* and *d* of the latter complex. Thus, these two resonances are associated with  $\text{Ca}^{2+}$  binding in CCoPL. EPR data indicate that the coordination environment of  $\text{Mn}^{2+}$  at S1 is largely unchanged upon  $\text{Ca}^{2+}$  binding at S2 (44). Changes in the visible CD spectrum of CoP upon  $\text{Ca}^{2+}$  binding have been observed (21, 22), although no changes in the MCD of CoP upon  $\text{Ca}^{2+}$  binding were reported (19). These results are consistent with the lack of major structural changes at S1 upon  $\text{Ca}^{2+}$  binding, as would be required to give rise to signals *b* and *d* in CCoPL. Evidence also suggests that there is a cis-trans isomerization in the amide linkage between Ala-207 and

Asp-208 upon  $\text{Ca}^{2+}$  binding to S2 (12, 17). Since the side chain of the latter residue is a ligand at S2 via a bridging water molecule (12), these findings indicate changes in the secondary structure of ConA in the S2 region upon  $\text{Ca}^{2+}$  binding (12). Changes in the conformation of Tyr-12 at S2 upon  $\text{Ca}^{2+}$  binding have also been reported (20). However, most of the above changes near S2 are too far from the  $\text{Co}^{2+}$  at S1 ( $>6 \text{ \AA}$ ) to give rise to *b* and *d*, which are dipolar shifted resonances that require closer proximity to the  $\text{Co}^{2+}$  ion for the magnitude of the observed shifts (see below). Since S2 is only  $4.25 \text{ \AA}$  from S1 (12), it is reasonable to suspect that a ligand of the  $\text{Ca}^{2+}$  ion at S2 may be responsible for these signals. Furthermore, since *b* and *d* are exchangeable signals, aliphatic side chain protons of carboxylate residues at S2 can be ruled out. This raises the intriguing possibility of a water ligand of the  $\text{Ca}^{2+}$  as the source of these two resonances.

From the x-ray structure of ConA (12, 50), one of the two waters coordinated to  $\text{Ca}^{2+}$  (W1) forms an oxygen-S2 metal-S1 metal angle smaller than  $90^\circ$  (Fig. 9) and, therefore, could be a reasonable candidate. This water molecule must be in slow exchange with bulk solvent because its two protons give rise to separate shifted signals in CCoPL, and they are not broadened compared to the other resonances in the spectrum. The slow exchange kinetics of this water ligand is consistent with the position of W1 toward the interior of the protein, away from the surface (12, 50). The presence of the same two resonances, *b* and *d*, in CCoPSA and CCoLcH is also consistent with the apparent conservation in structure of the S2 sites of the two lectins compared with ConA (13, 14). Nuclear magnetic relaxation dispersion (52, 53) and  $^{113}\text{Cd}$  NMR (24) data indicate that the water ligands at S2 in LcH and PSA are conserved with those in ConA. Thus, it is reasonable to assign the *b* and *d* signals in CCoPSA and CCoLcH with the same slowly exchanging water ligand (W1) at S2.  $T_1$  measurements (discussed below) have allowed calculation of the distance separating the  $\text{Co}^{2+}$  ion from the two protons associated with *b* and *d* in CCoLcH ( $4.0$  and  $3.7 \text{ \AA}$ , respectively). These calculated distances are consistent with the protons of the W1 water at S2 by analogy with the x-ray data for ConA (12, 50). The absence of signals *b* and *d* in the spectra of the corresponding  $\text{Ni}^{2+}$ -substituted lectins is also consistent with this assignment, as discussed below. To our knowledge, this is the first apparent observation of individual proton resonances of a slow exchanging water molecule coordinated to the metal ion in a protein.

The exchange time,  $\tau_m$ , of this  $\text{Ca}^{2+}$ -coordinated water molecule (W1) in the three lectins can be calculated and shown to be much greater than  $35 \mu\text{s}$  (considering one of the proton resonances is shifted downfield approximately  $50 \text{ ppm}$  in all three lectins without appreciable broadening, *i.e.*  $\tau_m \gg 1/(2\pi \times (90 \text{ Hz} \times 50))$ ). Solvent proton relaxation data of the  $\text{Ca}^{2+}$ - $\text{Mn}^{2+}$  complexes of ConA, LcH, and PSA indicate that water proton relaxation occurs primarily through exchange at S2 for the latter two lectins (52) and at both S2 and S1 for the former lectin (53). The  $\tau_m$  of the rapidly exchanging water at S2 in all three proteins was calculated to be approximately  $10 \text{ ns}$  (52). Thus, if the W1 water molecule is in slow exchange at S2, then W2, the second  $\text{Ca}^{2+}$ -coordinated water molecule, which is more exposed to the solvent in ConA (12), must be responsible for the observed solvent relaxation (nuclear magnetic relaxation dispersion) data at S2 for all three lectins.

By analogy with the data for other  $\text{Co}^{2+}$ -substituted proteins (27), the two nonexchangeable broad downfield signals centered at  $87$  and  $51 \text{ ppm}$  in CCoLcH,  $81$  and  $42 \text{ ppm}$  in CCoPSA, and  $77$  and  $35 \text{ ppm}$  in CCoPL can be assigned to the  $H^\epsilon$ -2 and  $H^\epsilon$ -1 protons of the histidine residue at S1, which

are in ortholike positions with respect to the coordinating nitrogen. These resonances have large line widths due to both scalar and dipolar broadening effects.

$T_1$  Measurements of CCoLcH— $T_1$  values of many of the isotropically shifted protein resonances of the 3-*O*-methyl D-glucose-CCoLcH complex are in a range typical of metalloproteins containing hexacoordinated  $\text{Co}^{2+}$  (27). In particular, the value of  $12 \text{ ms}$  for signal *a* at  $74.1 \text{ ppm}$  is consistent with its assignment as the NH proton of the S1 coordinated histidine (26, 27). The other two exchangeable protons *b* and *d* also have relatively short  $T_1$  values, thus confirming their close proximity to the metal ion. The  $T_1$  values of the two broad downfield signals could not be accurately measured, but their values are shorter than those of all the signals, in agreement with their positions on the coordinated histidine ring (42).

The distance from the  $\text{Co}^{2+}$  ion of the two downfield exchangeable protons in CCoLcH can be guessed to be shorter than that of the coordinated histidine NH proton ( $5.2 \text{ \AA}$ ), because the latter proton experiences ligand-centered contributions to relaxation which sizably shorten its  $T_1$  value (30). Such effects are not present on the protons of water coordinated to  $\text{Ca}^{2+}$ . By estimating 80% ligand-centered contribution to  $T_1$  of the NH proton of the coordinated histidine (30), the distances from the  $\text{Co}^{2+}$  to the other two exchangeable protons, *b* and *d* in Fig. 1, would be  $4.0$  and  $3.7 \text{ \AA}$ , respectively, which is consistent with their being the protons of a water ligand (W1) of the  $\text{Ca}^{2+}$  ion in CCoLcH, by analogy to the x-ray data for ConA (12, 50).

Comparison of the  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$  Complexes—Several conclusions can be made by comparing the spectra of the nickel and cobalt derivatives of the three lectins. Pseudooctahedral  $\text{Ni}^{2+}$  complexes are theoretically expected and experimentally found to display much smaller dipolar contributions to the isotropic shifts than the corresponding  $\text{Co}^{2+}$  complexes (42, 48). Therefore, the spectra of the nickel lectins show that very few protons experience contact shifts larger than a few ppm. In particular, only one exchangeable proton is observed among them at approximately  $66 \text{ ppm}$ . This signal corresponds to the *a* signals found in the  $72$ – $76$ -ppm range for the cobalt-substituted lectins which has been assigned to the  $N^\delta$ -1 proton of the coordinated histidine residue at S1. Thus, the  $66$ -ppm signals for the nickel derivatives of the lectins can be similarly assigned to the  $N^\delta$ -1 proton of the same S1-coordinated histidine residue for which a sizable contact shift is expected (42, 48). The two exchangeable protons *b* and *d* observed in the  $\text{Co}^{2+}$  derivatives must be primarily shifted by dipolar mechanisms, since they are absent in the spectra of the  $\text{Ni}^{2+}$  derivatives. This is in agreement with their assignment as protons of a  $\text{Ca}^{2+}$ -coordinated water. The other downfield proton signal observed at  $42.5 \text{ ppm}$  in CNiPL, which has been related to the nonexchangeable *c* resonance at  $37.6 \text{ ppm}$  in CCoPL, may belong to one of the  $\gamma$ - $\text{CH}_2$  protons of Glu-8 or  $\beta$ - $\text{CH}_2$  protons of Asp-10 or Asp-19, which are also expected to experience downfield contact shifts. Since this resonance is missing or hidden in the spectra of CNiPSA and CNiLcH, this suggests that the orientation of the side chain of one of the above carboxylate ligands at S1 is different in both CCoPL and CNiPL compared to the corresponding derivatives of the other two lectins.

The resonance at  $-14.2 \text{ ppm}$  in CNiPL, which appears missing for CNiPSA and CNiLcH, may be assigned to one of the  $\beta$ - $\text{CH}_2$  protons of the coordinated histidine residue (26). These results are similar to the differences observed for the corresponding  $\text{Co}^{2+}$  derivatives in this region of the spectra. This suggests that the orientation of the side chain of the



histidine residue at S1 in ConA is somewhat different from that of the same residue in LcH and PSA. These findings may relate to the much slower exchange rate of the  $N^{\delta}$ -1 proton of the histidine at S1 in ConA compared to the other two lectins.

Last, the ortholike protons of the histidine coordinated at S1 in the  $Ni^{2+}$  derivatives of the lectins are also expected to undergo sizable downfield contact shifts (41, 47) and are expected to be very broad. This appears to account for their lack of detection in the spectra of the  $Ni^{2+}$ -substituted proteins.

*Further Comments*—A comment is due to the sizable up- and downfield dipolar shifts experienced by all the signals in the cobalt-lectin derivatives. In principle, their factorization should permit a further step in the individual assignment as well as an evaluation of the direction of the principal axis of the magnetic susceptibility tensor of  $Co^{2+}$  (37, 38, 42, 48, 49, 54, 55). Attempts along this line have already been reported for ConA using the small perturbations induced by  $Co^{2+}$  on the shifts of either noncoordinated histidine protons far from the metal ion (25) or of  $^{13}C$  signals in a protein sample whose lysines had been derivatized with  $^{13}C$ -enriched formaldehyde (39). While these studies have the advantage of dealing with noncoordinated residues and, therefore, with purely dipolar shifts, the measured effects are small, which may induce errors in the conclusions. As a matter of fact, the  $z$  axis of the magnetic susceptibility tensor  $\chi$  was placed along the  $Co^{2+}$ -His-24 bond in the former report (25) and  $40$ – $60^\circ$  off it in the latter (39). If we assume that the contact shift experienced by the His-24  $H^{\delta}$ -1 proton in CNIPL and CCoPL is similar, then the dipolar shift experienced by that proton in both  $Ni^{2+}$  and  $Co^{2+}$  derivatives is rather small, in better agreement with the  $z$  axis being sizably off the  $Co^{2+}$ -His-24 bond.

#### SUMMARY

The visible absorption and magnetic circular dichroism spectra for CCoLcH and CCoPSA are very similar for both lectins and similar to those of  $Co^{2+}$  complexes of ConA (18, 19). However, the visible CD data of CCoLcH and CCoPSA, which are very similar to each other, differ from that of  $Co^{2+}$  complexes of ConA (21), indicating differences in the S1 environments of the former two lectins compared to the latter.

$^1H$  NMR spectra of the  $Co^{2+}$  and  $Ni^{2+}$  derivatives of the three lectins show a number of isotropically shifted signals which arise from protons in the immediate vicinity of the metal ion at S1. Assignments have been made of the protons of the side chain of the histidine at S1 in the  $Co^{2+}$  derivatives of the lectins. Differences in the H-D exchange rate of the NH proton of this histidine in ConA compared to the other two lectins is observed in both the  $Co^{2+}$  and  $Ni^{2+}$  derivatives. The  $^1H$  NMR spectra of the  $Ni^{2+}$ -substituted proteins, together with data for the  $Co^{2+}$  derivatives, also indicate that the side chains of a carboxylate ligand at S1 and the histidine residue at S1 are positioned differently in ConA than in the other two lectins. These results may account, in part, for the differences observed in the visible CD spectra of the lectins and the metal ion exchange properties of the proteins (11). In addition, all three  $Co^{2+}$ -substituted lectins possess two unusual downfield shifted resonances which appear to be associated with the two protons of a slowly exchanging water molecule coordinated to the  $Ca^{2+}$  ion at S2.  $T_1$  measurements of CCoLcH have provided an estimation of the distances from the  $Co^{2+}$  to these two protons of 3.7 and 4.0 Å.

#### REFERENCES

- Goldstein, I. J., Hughes, R. C., Monsigny, M., Osawa, T., and Sharon, N. (1980) *Nature* **285**, 66
- Liener, I. E., Sharon, N., and Goldstein, I. J. (eds) (1986) *The Lectins*, Academic Press, Orlando, FL
- Lis, H., and Sharon, N. (1986) *Annu. Rev. Biochem.* **55**, 35–67
- Goldstein, I. J., and Poretz, R. D. (1986) in *The Lectins* (Liener, I. E., Sharon, N., and Goldstein, I. J., eds) pp. 33–247, Academic Press, Orlando, FL
- Mckenzie, G. H., Sawyer, W. H., and Nichol, L. W. (1972) *Biochim. Biophys. Acta* **263**, 283–293
- Reeke, G. N., Jr., Becker, J. W., Cunningham, B. A., Wang, J. L., Yahara, I., and Edelman, G. M. (1975) *Adv. Exp. Med. Biol.* **55**, 13–33
- Leburn, E., Van Rapenbusch, R., Foirers, A., and Hoebeke, J. (1983) *J. Mol. Biol.* **166**, 99–100
- Meehan, E. J., Jr., McDuffie, J., Einspahr, H., Bugg, C. E., and Suddath, F. L. (1982) *J. Biol. Chem.* **257**, 13278–13282
- Kalb, A. J., and Levitzki, A. (1968) *Biochem. J.* **46**, 669–672
- Brewer, C. F., Brown, R. D., III, and Koenig, S. H. (1983) *J. Biomol. Struct. Dyn.* **1**, 961–997
- Bhattacharyya, L., Brewer, C. F., Brown, R. D., III, and Koenig, S. H. (1985) *Biochemistry* **24**, 4974–4980
- Hardman, K. D., Agarwal, R. C., and Freiser, M. J. (1982) *J. Mol. Biol.* **157**, 69–86
- Foirers, A., Leburn, E., Van Rapenbusch, R., de Neve, R., and Strosberg, A. D. (1981) *J. Biol. Chem.* **256**, 5550–5560
- Higgins, T. J. V., Chandler, P. M., Zurawski, G., Button, S. C., and Spencer, D. (1983) *J. Biol. Chem.* **258**, 9544–9549
- Herrmann, M. S., Richardson, C. E., Setzler, L. M., and Behnke, W. D. (1978) *Biopolymers* **17**, 2107–2120
- Einspahr, H., Parks, E. H., Sugana, K., and Subramanian, E. (1986) *J. Biol. Chem.* **261**, 16518–16527
- Brown, R. D., III, Brewer, C. F., and Koenig, S. H. (1977) *Biochemistry* **16**, 3883–3896
- Kalb, J., and Pecht, I. (1973) *Biochim. Biophys. Acta* **303**, 264–268
- Richardson, C. E., and Behnke, W. D. (1976) *J. Mol. Biol.* **102**, 441–451
- Cardin, A. D., Behnke, W. D., and Mandel, F. (1979) *J. Biol. Chem.* **254**, 8877–8884
- Cardin, A. D., Behnke, W. D., and Mandel, F. (1980) *Biochemistry* **19**, 5439–5445
- Cardin, A. D., Behnke, W. D., and Mandel, F. (1981) *J. Biol. Chem.* **256**, 37–40
- Stafford, E., Behnke, W. D., Bhattacharyya, L., and Brewer, C. F. (1986) *Biochem. Biophys. Res. Commun.* **136**, 438–444
- Bhattacharyya, L., Marchetti, P. S., Ellis, P. D., and Brewer, C. F. (1987) *J. Biol. Chem.* **262**, 5616–5621
- Carver, J. P., Barber, B. H., and Fuhr, B. J. (1977) *J. Biol. Chem.* **252**, 3141–3146
- Bertini, I., Canti, G., Luchinat, C., and Mani, F. (1981) *J. Am. Chem. Soc.* **103**, 7784–7788
- Bertini, I., and Luchinat, C. (1984) in *Advances in Inorganic Biochemistry* (Eichhorn, G. L., and Marzilli, L. G., eds) pp. 71–111, Elsevier, Scientific Publishing Co., Inc. New York
- Bertini, I., Gerber, M., Lanini, G., Luchinat, C., Maret, W., Rawer, S., and Zeppeauer, M. (1984) *J. Am. Chem. Soc.* **106**, 1826–1830
- Bertini, I., Luchinat, C., and Monnanni, R. (1985) *J. Am. Chem. Soc.* **107**, 2178–2179
- Banci, L., Bertini, I., Luchinat, C., and Scozzafava, A. (1987) *J. Am. Chem. Soc.* **109**, 2328–2334
- Banci, L., Bertini, I., Gallori, E., Luchinat, C., Paoletti, F., Polsinelli, M., and Viezzoli, M. S. (1987) *J. Inorg. Biochem.*
- Ticha, M., Entlicher, G., Kostir, J. V., and Kocourek, J. (1970) *Biochim. Biophys. Acta* **221**, 282–289
- Trowbridge, I. S. (1974) *J. Biol. Chem.* **249**, 6004–6012
- Hochmann, J., and Kellerhals, H. (1980) *J. Magn. Reson.* **38**, 23–29
- Carlin, R. L. (1965) in *Transition Metal Chemistry* (Carlin, R. L., ed) Vol. 1, pp. 1–28, Marcel Dekker, Inc., New York
- Kaden, T. A. (1974) in *Metal Ions in Biological Systems* (Sigel, H., ed) Vol. 4, pp. 1–27, Marcel Dekker, Inc., New York
- Horrocks, W. D., Jr., and Hall, D. D. (1971) *Inorg. Chem.* **10**, 2368–2370
- La Lancette, E. A., and Eaton, D. R. (1964) *J. Am. Chem. Soc.* **86**, 5145–5148
- Sherry, A. D., and Teherani, J. (1983) *J. Biol. Chem.* **258**, 8663–8669
- Gueron, H. J. (1975) *J. Magn. Reson.* **19**, 58–66

41. Vega, A. J., and Fiat, D. (1976) *Mol. Physiol.* **31**, 347-355
42. Bertini, I., and Luchinat, C. (1986) in *NMR of Paramagnetic Molecules in Biological Systems* (Gray, H. B., and Lever, A. B. P., eds) Benjamin/Cummings Publishing, Menlo Park, CA
43. Brewer, C. F., Sternlicht, H., Marcus, D. M., and Grollman, A. P. (1973) *Biochemistry* **12**, 4448-4457
44. Reed, G. H., and Cohn, M. (1970) *J. Biol. Chem.* **245**, 662-664
45. Bhattacharyya, L., Freedman, J. H., Brewer, C. F., Brown, R. D., III, and Koenig, S. H. (1985) *Arch. Biochem. Biophys.* **240**, 820-826
46. McConnell, H. M., and Chesnut, D. B. (1958) *J. Chem. Phys.* **28**, 107-117
47. Kurland, R. J., and McGarvery, B. R. (1970) *J. Magn. Reson.* **2**, 286-301
48. La Mar, G. N., Horrocks, W. D., Jr., and Holm, R. H. (eds) (1973) *NMR of Paramagnetic Molecules*, Academic Press, Orlando, FL
49. Fitzgerald, R. J., and Drago, R. S. (1968) *J. Am. Chem. Soc.* **90**, 2523-2527
50. Becker, J. W., Reeke, G. N., Jr., Wang, J. L., Cunningham, B. A., and Edelman, G. M. (1975) *J. Biol. Chem.* **250**, 1513-1524
51. Hill, H. A. O., Lee, W.-K., Bannister, J. V., and Bannister, V. H. (1980) *Biochem. J.* **185**, 245-252
52. Bhattacharyya, L., Brewer, C. F., Brown, R. D., III, and Koenig, S. H. (1985) *Biochemistry* **24**, 4985-4990
53. Koenig, S. H., Brown, R. D., III, and Brewer, C. F. (1985) *Biochemistry* **24**, 4980-4984
54. Bertini, I., Luchinat, C., Mancini, M., and Spina, G. J. (1984) *J. Magn. Reson.* **59**, 213-222
55. Horrocks, W. D., Jr., and Greenberg, E. S. (1971) *Inorg. Chem.* **10**, 2190-2194