Cadmium-113 Nuclear Magnetic Resonance Investigation of Metal Binding Sites in Concanavalin A†

Allen R. Palmer,‡ David B. Bailey,§ W. D. Benhke,¶ Alan D. Cardin,¶ Ping P. Yang,‡ and Paul D. Ellis*,‡

ABSTRACT: Cadmium-113 nuclear magnetic resonance studies are presented on the ^{113}Cd-substituted protein concanavalin A (Con A). This protein has two different conformations, locked and unlocked, as described previously by Brown et al. [Brown, R. D., III, Brewer, C. F., & Koenig, S. H. (1977) *Biochemistry 16*, 3883]. The unlocked form of Con A gives one ^{113}Cd resonance, indicating rapid exchange of the metal ions between the solution and the binding sites. Solutions of the locked form show three resonances: a free cadmium resonance (68 ppm), a resonance assigned to ^{113}Cd occupying the manganese site (46 ppm), and a resonance assigned to ^{113}Cd occupying the calcium site (−125 ppm). In addition, Pb(II) is shown to bind to the calcium site and Zn(II) is shown to have high affinity for both sites. Data have been presented in previous literature that support a model in which Con A binds monosaccharides in a different manner than it binds oligosaccharides. However, if this difference exists, it does not affect the metal binding sites. Also, some heterogeneity in Con A has been reported in the literature; however, we have shown that the presence of these heterogeneities does not affect the ^{113}Cd NMR parameters.

Concanavalin A (Con A), a lectin isolated from jack beans, has been extensively studied due to its unusual biological properties [cf. Nicholson (1974) and Bittiger (1977)]. These properties include preferential agglutination of suspended malignant cells as compared to normal parent cells (Inbar & Sachs, 1969), stimulation of blastogenesis in lymphocytes (Douglas et al., 1969), stimulation of cell-mediated immunological responses (Stavey et al., 1971), and pronounced effects on delayed hypersensitivity (Leon & Schwartz, 1969). These properties all seem to arise from the ability of Con A to bind cell surfaces, which in turn may involve specific interactions between saccharides on the cell surface and the Con A moiety (Goldstein et al., 1965).

It has been known for some time that Con A contains certain metals which are necessary for its saccharide binding activity (Agrawal & Goldstein, 1968). The nature and location of these metals have been studied by X-ray crystallography (Edelman et al., 1972; Hardman & Ainsworth, 1972, 1976; Becker et al., 1975), ESR (Nicolan et al., 1969), NMR (Alter & Magnuson, 1974; Alter et al., 1977; Grimaldi & Sykes, 1975; Brewer et al., 1973ab; Villafranca & Viola, 1974; Barber & Carver, 1973; Sherry & Cottam, 1973; Brown et al., 1977; Koenig et al., 1978), and circular dichroism (Barber & Carver, 1974; McCubbin et al., 1971; Kalb & Levitski, 1968). These studies and others have shown that Con A is composed of identical subunits with a molecular weight of approximately 25 500 (Kalb & Lustig, 1968). Solutions of Con A consist of dimers and tetramers, with the percentage of each polymer being dependent on pH, temperature, and the ionic strength of the buffer (McKenzie et al., 1972). Each subunit requires two metals in order to bind saccharides. One of these sites, denoted S1, is occupied by Mn(II) in the native protein but will accept other metals as well. The second site, denoted S2, contains Ca(II) in the native protein but will also bind other metals (Shoham et al., 1973; Koenig et al., 1978). These two sites are approximately 5 Å apart (Edelman et al., 1972; Hardman & Ainsworth, 1972) and are 10–12 Å from the saccharide binding site (Hardman & Ainsworth, 1976). Other metal binding sites have also been reported for lead (Becker et al., 1975), lanthanides (Barber et al., 1975), and mercury (Shoham et al., 1973).

Recent developments in multinuclear Fourier transform NMR techniques (Peters et al., 1973; Traficante et al., 1974; Marshall et al., 1974) have provided NMR spectroscopists with the capability of using a variety of nuclei with sufficient sensitivity to investigate biological systems. One application is the use of metal nuclides as probes of metal–protein interactions in metalloproteins and enzymes. The native metals found in these proteins have, in general, poor NMR characteristics but in many cases may be replaced by metals with more favorable properties. One substitute nuclide with excellent NMR properties is ^{113}Cd. Several studies have been published concerning the ^{113}Cd NMR of inorganic and organometallic model systems (Maciel & Borzo, 1973; Kostelnik & Bothner-By, 1974; Cardin et al., 1975; Haberkorn et al., 1976). More recently, Armitage et al. (1976, 1978) and Chlebowski et al. (1977) have investigated the ^{113}Cd NMR of Cd(II)-substituted alkaline phosphatase, human and bovine carbonic anhydrase B, carboxypeptidase, and superoxide dismutase. Other reports have been published on the ^{113}Cd NMR of Cd(II) human carbonic anhydrase (Sudmeier & Bell, 1967), superoxide dismutase (Bailey et al., 1980), and carp parvalbumin (Drakenberg et al., 1978).

We recently reported the preliminary results of a ^{113}Cd NMR investigation of the metal binding sites of Con A (Bailey et al., 1978). In that report we presented a ^{113}Cd NMR

† From the Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208, and the Department of Biological Chemistry, University of Cincinnati, Cincinnati, Ohio 45276. *Received October 25, 1979.* Financial support for this research came in part from the National Institutes of Health, GM26295, the Environmental Protection Agency, R804359, and the National Science Foundation, CHE78-18723 (University of South Carolina group), and the National Institutes of Health, R01-H123741-01 (University of Cincinnati group). This paper is based on a portion of the dissertation of A.R.P., to be submitted to the Graduate School of the University of South Carolina in partial fullfillment of the requirements for the degree of Doctor of Philosophy.

* Address correspondence to this author. Alfred P. Sloan Foundation Fellow, 1977–1979.

‡ Present address: Department of Chemistry, University of South Carolina, Columbia, SC 29208.

§ Present address: Department of Chemistry, State University of New York at Stony Brook, Stony Brook, NY 11790.

¶ Present address: Department of Biological Chemistry, University of Cincinnati, College of Medicine, Cincinnati, OH 45276.

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spectrum of Con A containing 2 equiv of $^{113}Cd(II)$ per subunit. This spectrum consisted of three peaks, at 68, 46, and −125 ppm. Based on metal ion competition experiments using diamagnetic metals, we assigned the resonance at 68 ppm to correspond to cadmium at the calcium site (S2), the resonance at 46 ppm for cadmium at the transition metal site (S1), and the resonance at −125 ppm for cadmium at a third site available to Zn(II), Cd(II), Ca(II), and other metals.

The resonance at −125 ppm was over 100 ppm more shielded than any ^{113}Cd resonance which had been observed at that time. Recently, however, a ^{113}Cd NMR study of carp muscle parvalbumin using ^{113}Cd to replace Ca(II) showed ^{113}Cd resonances at −90 and −100 ppm (Drakenberg et al., 1978). The parvalbumin metal binding site (Moews & Kretzinger, 1975) and the Con A S2 site (Edelman et al., 1972) are similar, each containing six oxygen ligands in an approximately octahedral environment. This suggested that the resonance at −125 ppm could correspond to cadmium at the S2 site. In light of this study, we designed several experiments to confirm our original assignments. We are reporting here the data from these experiments, including corrected ^{113}Cd assignments and data on the conformational states of Con A.

Materials and Methods

Native Con A was isolated from jack bean meal (Pfaltz and Bauer, Inc., or Sigma Chemical Co.) as described by Agrawal & Goldstein (1967). Preparation of intact subunits of Con A was carried out according to the procedure of Cunningham et al. (1972) and Williams et al. (1978a) and assayed for homogeneity by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis (Laemmli, 1970). Preparations made in this manner were 87% homogeneous as assayed by densitometric analysis of the gels. Enriched ^{113}CdO (96%) was purchased from Oak Ridge National Laboratory and converted to the chloride or nitrate form by using metal-free mineral acid solutions. The saccharides used were the generous gift of Dr. Fred Brewer. All buffers used in the dialysis of the apoprotein and for assays were rendered metal free by dithizone extraction. All other buffers and salts were reagent grade chemicals and were used without further purification.

Protein concentrations were measured spectrophotometrically at 280 nm by using $\epsilon^{0.1\%} = 1.24$ (Goldstein et al., 1965) and 25 500 as the molecular weight of the monomer (Wang et al., 1971). A polysaccharide light-scattering activity assay was used as described by Goldstein et al. (1965). The assay was maintained at 25 °C, and the optical density at 420 nm was monitored with a Gilford-300 microsample spectrophotometer. Each NMR sample was assayed before a spectrum was taken, and each sample in metal competition experiments was assayed after the spectrum to ensure that no erroneous data would be collected due to protein denaturation. Unlocked Con A is not active; therefore, these samples were warmed to 25 °C and held for 24 h before assaying. Unlocked Cd:Cd Con A[1] samples were also checked by locking and then examining their ^{113}Cd NMR spectra.

Apo Con A was prepared by using a modified version of the procedure given by Brown et al. (1977). Approximately 1.3 g of native Con A was dissolved in 8.0 mL of deionized water, and the pH was lowered to 1.3 by dropwise addition of 3 M HCl while stirring at 25 °C. After being stirred for 45 min at 25 °C, the solution was centrifuged to remove any solid remaining and clamped tightly in a dialysis bag (Spectropore 2 dialysis bags were used with plastic clamps, both produced by Spectrum Industries). Dialysis was then carried out at 4 °C against constantly flowing deionized water (about 30 L) for 16 h. The bag was then suspended in cold water, and a concentrated, metal-free sodium acetate buffer solution, pH 5.2, was slowly added over a period of 15 min until the final acetate concentration was 0.05 M. A final dialysis was performed against a 40% D_2O–acetate buffer of the same pH to provide an internal lock for NMR experiments. The solution was then centrifuged to remove any precipitate, and a solid salt (either sodium chloride or sodium nitrate) was added with stirring to reach the desired ionic strength. This procedure minimized the loss of protein as the solution is restored to an active pH and avoids entirely a concentration step. Typical protein concentration at the conclusion of this procedure is 2.5–3.0 mM in protomer. The presence of metals was monitored at this point by using atomic absorption spectroscopy. Zn and Mn were in the range of 8–16 μM, and Ca was in the range of 9–20 μM in the protein samples, or less than 1% of the protein concentration in each case.

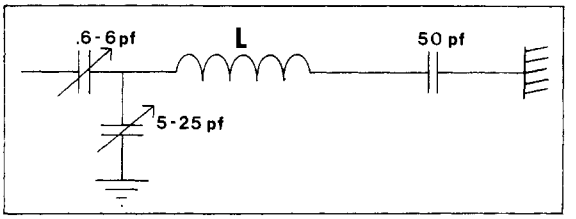

FIGURE 1: Schematic diagram of the transverse solenoid coil probe. This coil was constructed of 12 turns of 14 AWG solid tinned copper wire. Its diameter is 12 mm, and it is 41 mm in length. The coil has 0.68-μH inductance, and its Q is about 120. The sample is contained in a 10 mm diameter NMR tube about 5 cm long and is placed directly on the coil.

Locking time and other time-dependent NMR experiments on [^{113}Cd]Con A were performed on a Bruker WP-200 spectrometer by using a home-built transverse coil probe. This probe, which contains no lock or decoupling capability, is described in Figure 1. All other ^{113}Cd NMR experiments were performed on a highly modified Varian XL-100-15 spectrometer by using a frequency synthesizer mode of operation described elsewhere (Jakobsen et al., 1980) in a home-built multinuclear 18-mm probe described earlier (Byrd & Ellis, 1977). All ^{113}Cd chemical shifts were referenced to an external standard of 0.1 M $Cd(ClO_4)_2$ in 50:50 H_2O/D_2O, using positive chemical shifts to denote resonances to lower shielding. The experimental conditions used to collect data on the WP-200 were flip angle = 12°, recycle time = 0.0512 s, spectral window = 20 000 Hz, and line broadening for sensitivity enhancement = 30 Hz, and 200 000 scans were taken representing approximately 4 h total time for each experiment. The experimental conditions used to collect data on the Varian XL-100-15 were flip angle = 45°, recycle time = 0.4 s (unless specifically stated otherwise), spectral window = 10 kHz, and line broadening for sensitivity enhancement = 16 Hz, and 80 000 transients were taken requiring approximately 9 h of instrument time per experiment. In some experiments values for T_1 of each resonance were needed. The values used were 3.1 s for the resonance at 68 ppm, 5.5 s for the resonance at 46 ppm, and 5.0 s for the resonance at −125 ppm. These values have been determined in our laboratory; a detailed report on relaxation in Con A will be published later. All spectra within any one figure have been taken by using

[1] M_1:M_2 Con A will be used to represent concanavalin A with $M_1(II)$ at the S1 site and $M_2(II)$ at the S2 site. The unlocked form of Con A is characterized by rapid exchange of the metals. Therefore, when referring to the unlocked form, the expression M_1:M_2 Con A will simply indicate unlocked Con A with these metals present in the solution.

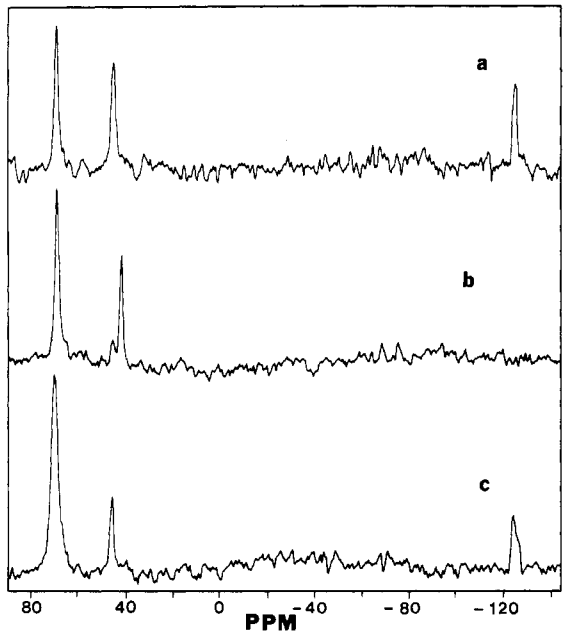

FIGURE 2: ^{113}Cd NMR spectra of locked Con A taken on the Varian XL-100-15 NMR spectrometer. For the NMR acquisition parameters on this instrument, see Materials and Methods. Three parameters were used for Figures 2, 6, 7, and 8. (a) Con A containing 2.2 equiv of $^{113}CdCl_2$ per monomer. Resonances occur at 68, 46, and −125 ppm. (b) The sample in (a) to which 1 equiv of Ca(II) has been added. The large resonances are at 68 and 43 ppm. The small resonance is at 46 ppm. (c) The sample in (a) to which 1 equiv of Mn(II) has been added. Resonances are at 68, 46, and −125 ppm.

identical spectrometer parameters and vertical scales, so the intensities of the resonance within any figure are directly comparable.

Results and Discussion

Brown et al. (1977) and Koenig et al. (1978) have recently investigated the various conformational states of Con A by the method of solvent proton nuclear magnetic relaxation dispersion. They found that Con A exists in two conformational states in solution: an unlocked form or a locked form. The unlocked form is characterized by rapid exchange of any metals present with the S1 and S2 sites and by relatively large metal dissociation constants for these sites. The locked form is characterized by high binding constants for the metals and is the active form of Con A. Further, it appears that the ground-state energies of both conformations are approximately equal, but with a relatively high (22 kcal/mol) barrier separating the two states. This barrier is sufficient to allow the conformations to be studied separately. The unlocked conformation is prepared by removing the metals from the Con A system at room temperature. If apo Con A is cooled to 5 °C, it will remain in the unlocked conformation for several hours after the addition of Mn(II) and Ca(II) or for days in the presence of only Cd(II). At 25 °C and in the presence of these metals, the system will rapidly convert to the locked form.

Locked Conformation of Con A. Figure 2a depicts the ^{113}Cd NMR spectrum of locked Con A containing 2.2 equiv of $^{113}Cd(II)$ and no other divalent metals. The slight excess was used in order to ensure that cadmium would be available to sites other than S1 or S2. The spectrum consists of three resonances, at 68, 46, and −125 ppm, indicating that ^{113}Cd is present in three separate environments and that exchange between these environments is slow as compared to the ^{113}Cd NMR chemical shift time scale (no faster than 100/s).[2]

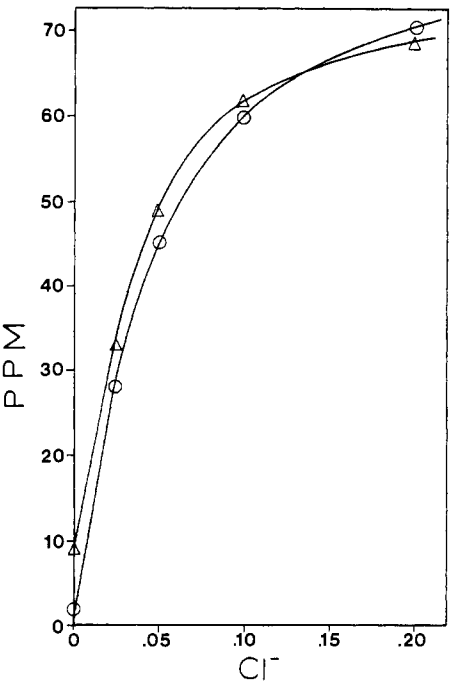

FIGURE 3: (△) Chemical shift of the free $^{113}Cd(II)$ resonance in Cd:Cd Con A vs. chloride ion concentration. The sample contained 2.2 mM Con A protomer, 5.0 mM $Cd(NO_3)_2$, and 0.05 M sodium acetate buffer, pH 5.2. Sodium chloride was added to give the desired Cl^- concentration. (○) Chemical shift of 4 mM $^{113}Cd(NO_3)_2$ in the absence of protein but in the same buffer as above.

The first step in assigning these resonances was to make certain that all cadmium could be accounted for; i.e., no cadmium resonances were hidden due to chemical dynamic processes. To assess this, we prepared a sample of Con A containing 2.0 equiv of 96% enriched ^{113}Cd. A capillary tube containing the same number of spins of ^{113}Cd [in the form of enriched $^{113}Cd(NO_3)_2$] was inserted into this sample. This capillary also contained a small amount of Mn(II), a paramagnetic ion which was added in order to increase relaxation rates and thus cause the resonance from the capillary to have approximately the same line width as the resonances from the protein solution. The sample sizes were such that both solutions were entirely inside the receiver coil of the spectrometer. In the resulting spectrum, which was taken by using a 20-s pulse delay and a 90° flip angle, the three resonances from the protein solution had a total intensity identical with the intensity of the resonance from the capillary tube (within a signal/noise error of about 10%). From this it was concluded that (a) all cadmium was accounted for and (b) any dynamic processes taking place around the metal binding sites of the protein were not causing extreme broadening of any resonances.

The second step in the assignment of the resonances was to determine if one of the resonances was due to ^{113}Cd which was free in the solution. Chloride ions in aqueous solutions have a very high affinity for Cd(II) ions (Reilly & Stokes, 1970) and have a large deshielding effect of the $^{113}Cd(II)$ chemical shift. A spectrum of Cd:Cd Con A in a chloride-free buffer contains three resonances, at 46, 8, and −125 ppm. The peaks at 46 and −125 ppm were independent of the chloride

[2] When chemical exchange is slow, the rate of exchange κ may be represented by $\kappa = \pi\nu$, where ν represents the chemical exchange contribution to the width of the width of the resonance at half-height. As the spectra presented in this paper have line widths of around 30 Hz, the maximum exchange rate can be no faster than 100 s^{-1}.

concentration, but the peak at 8 ppm did show chloride ion dependence. The chemical shift of this resonance and the chemical shift of 4 mM $^{113}Cd(NO_3)_2$ in an identical buffer were plotted as a function of chloride ion concentration (Figure 3). The chemical shift chloride ion dependences of these two resonances were very similar. Hence, the resonance at 68 ppm was assigned to Cd(II) either free in solution or in very weak association with the protein.

Assignment of the resonances from ^{113}Cd bound to the protein was now possible. A sample of Con A was prepared containing 2.2 equiv of $^{113}Cd(II)$ (Figure 2a). One equivalent of Ca(II) was then added. In the resultant spectrum (Figure 2b), the resonance at 68 ppm, representing free cadmium, gained intensity and the resonance at −125 ppm was absent or below the noise level. The resonance at 46 ppm gained intensity by about 10% and shifted slightly to 43 ppm, leaving a small resonance in the original (46 ppm) position. This added intensity was attributed to the mass action effect resulting from the greater amount of free cadmium available for the site. (This binding constant is discussed later in this paper.) Integration of these resonances (after scaling for relaxation effects)[3] indicated that they have the same total intensity as the three peaks in the Cd:Cd Con A spectrum (Figure 2a). After addition of an excess of calcium, the small resonance at 46 ppm was gone entirely, leaving only the resonance at 43 ppm and the resonance at 68 ppm due to free cadmium in evidence. Clearly, Ca(II) successfully completed with the cadmium which gave rise to the resonance at −125 ppm, so this resonance was assigned the S2 site.

By a process of elimination, the resonance at 46 ppm must be due to cadmium at the S1 site. A sample of Con A containing 2.2 equiv of ^{113}Cd and 1.0 equiv of Mn(II) was prepared to confirm this. Divalent manganese is a paramagnetic ion which will enhance the relaxation (T_1 and T_2) of nearby nuclei and thus cause line broadening, but due to a lack of spin pairing it cannot cause electron-mediated chemical shifts of the resonances. The chemical shifts of the Mn(II)–Cd(II)-containing Con A sample (Figure 2c) are identical with the chemical shifts of Cd:Cd Con A (Figure 2a), but their line shapes and intensities are different. The free cadmium resonance at 68 ppm showed greatly increased intensity and line width. The S2 resonance at −125 ppm has been broadened slightly, and the resonance at 46 ppm has no line shape change but has a reduced intensity. Several observations may be made from these data. First, the increased intensity of the free ^{113}Cd resonance at 68 ppm is greater than can be explained by cadmium driven out of any site by Mn(II). The increased intensity and line width can be explained only by efficient paramagnetic relaxation of $^{113}Cd(II)$ which originally had a relaxation time much greater than the 0.4-s recycle time used for the data acquisition. This indicates that a significant amount of the Mn(II) is free from the protein. Second, the change in the line shape of the S2 resonance at −125 ppm suggests that this resonance is being perturbed by Mn(II) binding elsewhere on the protein. This could be due to paramagnetic relaxation at that site or due to a slight chemical nonequivalence of this site in the Mn:Cd protein as compared to the Cd:Cd protein. This observation is, however, not definitive as we cannot rule out the possibility that cadmium in the S2 site is affected by exogenous Mn(II). Finally, the reduction in intensity at 46 ppm suggests that Mn(II) is competing for that site. This experiment alone is not absolutely conclusive, but when all data are considered the resonance at 46 ppm can be unambiguously assigned to the S1 site.

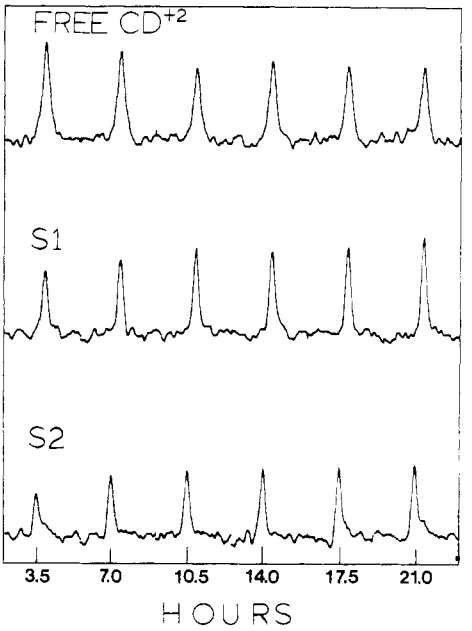

FIGURE 4: Time dependence of each resonance in a Con A solution to which 2.0 equiv of $Cd(Cl)_2$ was added to the previously metal-free preparation of 0 h. For acquisition parameters on the Bruker WP-200, see Materials and Methods. These parameters were used for Figures 4 and 5.

There are several possible explanations for the presence of a large resonance from free cadmium ions in Cd:Cd Con A. First, there could be a significant fraction of unlocked protein in the presence of stoichiometric amounts of Cd(II). Second, the resonance could be due to damaged or otherwise metal-deficient forms (Williams et al., 1978a; Cunningham et al., 1972). Finally, the resonance could be present because the Cd(II) binding constant is small enough to allow a significant fraction of the Cd(II) ions to remain free in the solution. The first explanation may be ruled out by observing the $^{113}Cd(II)$ NMR spectra of locked and unlocked Cd:Cd Con A in a chloride-free buffer (vida supra). The resonance from unlocked Con A (−12 ppm) is significantly shielded with respect to the free resonance from locked Cd:Cd Con A (4–6 ppm).

In order to examine the second possibility, that of a metal-deficient form of Con A, we prepared Con A conventionally (Agrawal & Goldstein, 1967) and by the procedures of Cunningham et al. (1972) and Williams et al. (1978a). These preparations were examined, both during the locking process and after equilibrium had been reached, for differences in Cd(II) binding. Figure 4 represents spectra of Con A at 25 °C after the addition of 2 equiv of $^{113}Cd(II)$ to the apoprotein. Each spectrum represents 3.5 h of data acquisition. The data reveal a locking rate constant (assuming first-order kinetics) on the order of 0.7 h^{-1}. Similar spectra were taken at 25 °C on Cd:Ca preparations of Con A, although the locking rate in this case was too fast to measure by ^{113}Cd NMR. These experiments were performed on the different preparations of Con A, and the data revealed no differences, either in kinetics or final stoichiometry, between the different preparations. Heterogeneity in the metal binding of Con A would thus be

[3] The intensities of the resonance were scaled for integration by using the formula $M_t = M_0 [1 - (e^{-\tau}/T_1)]$, where M_t is the observed intensity, M_0 is the equilibrium magnetization, T_1 is the longitudinal relaxation time, and τ represents the recycle time between pulses (0.4 s in this case). This scaling is important because the solutions of the calcium-containing protein contain much more cadmium in the efficiently relaxing exogenous metal peak. However, the effect of the scaling changes the ratio of the areas of the two spectra only by 10%.

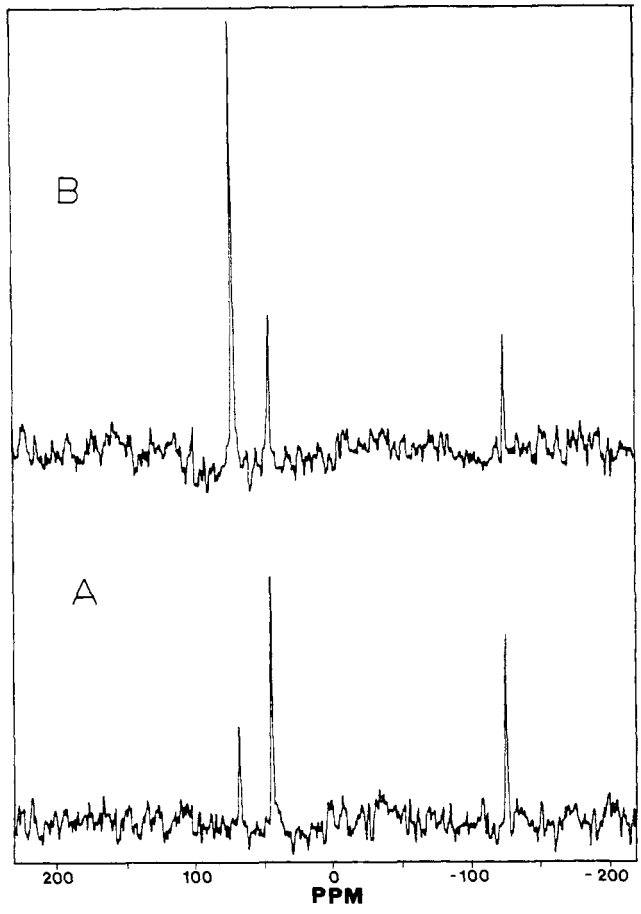

FIGURE 5: (A) Spectrum of Con A containing 1 equiv of enriched ^{113}Cd. (B) Spectrum of above sample after addition of 2.5 equiv of isotopically normal Cd(II).

an unlikely explanation for the presence of free Cd(II) ions.

These experiments suggest that the final explanation, that of a small binding constant, is the cause of the free ^{113}Cd resonance in 2Cd Con A. Two experiments were performed to test the feasibility of this hypothesis. First, it was necessary to demonstrate that an equilibrium and chemical exchange exist at both sites. To accomplish this, we took a spectrum of Con A which contained 1 equiv of $^{113}Cd(II)$, 96% enriched in this isotope (Figure 5A). Prior to obtaining this spectrum, the sample was locked for 36 h at 25 °C. To this solution was added 2.5 equiv of isotopically normal Cd(II) (12% ^{113}Cd), and a second spectrum was immediately taken (Figure 5B). This second spectrum contains a sharply increased resonance at 68 ppm and sharply decreased resonances at 46 and −125 ppm. Clearly, the isotopically normal, "invisible" cadmium is freely exchanging and displacing the labeled cadmium at the S1 and S2 sites, indicating that an equilibrium condition does exist.

As previously noted, the observable resonances in Cd:Cd Con A account for all cadmium present in the system. Further, the relaxation times for each resonance are known. Therefore, these data may be utilized quantitatively to calculate the binding constants for cadmium at the individual sites within Con A. To make this calculation, we took a spectrum of 3.0 mM Con A containing 6.0 mM $^{113}Cd(II)$, using a 20-s recycle time between pulses. This recycle time is much greater than the relaxation times involved and allows for accurate integrations of the resonances without scaling. The intensities, normalized to 6 mM, of the free resonance, the S1 resonance, and the S2 resonance respectively were 1.39, 2.43, and 2.18 mM. From these data, the apparent binding constants for the

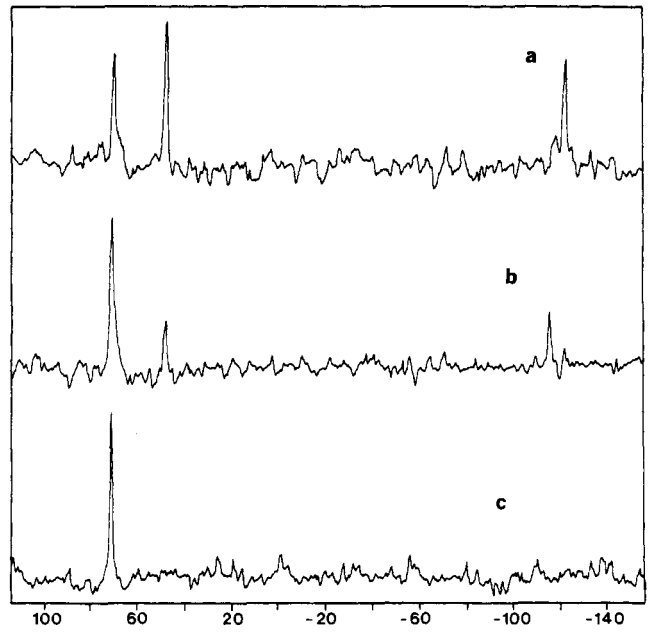

FIGURE 6: (a) ^{113}Cd spectrum of Cd:Cd Con A for reference. The resonances are at 68, 46, and −125 ppm. (b) ^{113}Cd spectrum of Cd:Cd Con A to which 1.0 equiv of Zn(II) has been added. The resonances are at 68, 46, and −118 ppm. (c) The same sample as in (a) but with 2.0 equiv of Zn(II) added. The resonance is at 68 ppm.

S1 and S2 sites are 3.1×10^3 and 2.1×10^3 M^{-1}, respectively.

Our value for the binding constant for Cd(II) at the S2 site is in good agreement with that of Shoham et al. (1973), who found that binding constant to be 1.5×10^3 M^{-1}. Their S1 binding constant of 6.2×10^4 M^{-1} is in fair agreement with our value of 3.1×10^3 M^{-1}. The time dependence of metal binding in Con A was not known at the time of the Shoham et al. (1973) report.

The presence of poorly binding Con A forms or inadvertently present competing metals would imply higher binding constants than are given here, so our binding constants should be considered to be minimum. Further, it is apparent (from Figure 2c) that 0.9 equiv of Ca(II) added to 2Cd Con A is at least 90% bound at the S2 site. This implies a Ca(II) binding constant at least 100 times as large as the binding constant for Cd(II) at that site, i.e., at least 2×10^5 M^{-1}. This figure is in poor agreement with that of Shoham et al. (1973) and Kalb & Levitski (1968); each of those papers reports Ca(II) binding constants around 3×10^3 M^{-1}.

Clear-cut criteria have not been established as to why a particular metal will bind to the S1 and S2 site. We have investigated the binding of several diamagnetic metals to Con A by allowing them to compete with the Cd:Cd form of the protein and observing the ^{113}Cd spectrum.

Figure 6b is a spectrum of Cd:Cd Con A to which 1 equiv of Zn(II) was added. (Figure 6a is Cd:Cd Con A for reference.) The spectrum contains a free cadmium resonance at 68 ppm with almost twice the intensity of the corresponding peak in Figure 6a and two small resonances at 46 and −118 ppm. Addition of a second equivalent of Zn(II) leaves only the resonance at 68 ppm above the noise level (Figure 6c). It is clear that Zn(II) is competing strongly at both the S1 and S2 sites. The resonance at −118 ppm in Figure 6b is presumably due to protein molecules containing Cd(II) at S2 and Zn(II) at S1. The absence of a resonance at −125 ppm in this spectrum (due to Cd:Cd Con A) is not easily explained. It is suggestive that both the Zn:Cd form and the Cd:Zn form are preferred over the Cd:Cd form. Further experiments are currently being performed to verify this. Shoham et al. (1973)

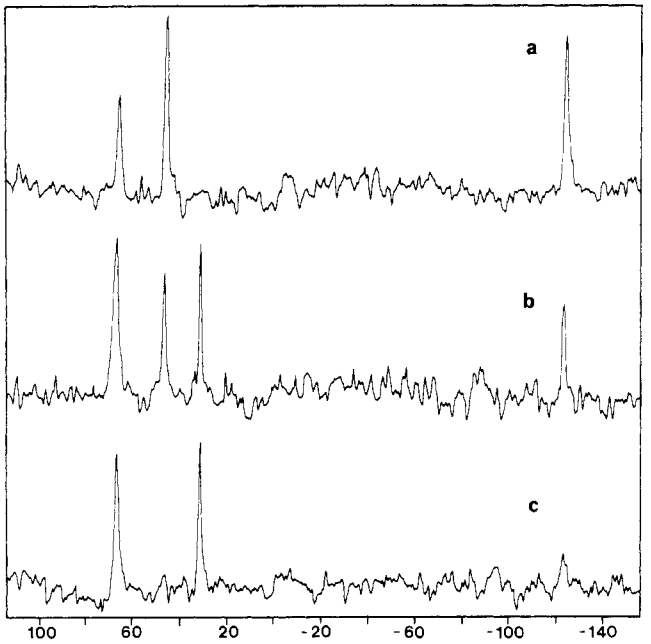

FIGURE 7: (a) ^{113}Cd spectrum of Cd:Cd Con A for reference. The resonances are at 68, 46, and −125 ppm. (b) The same sample after addition of 1.0 equiv of Pb(II). The resonances are at 68, 46, 32, and −125 ppm. (c) The same sample as (a) but with 2.0 equiv of Pb(II) added. The resonances are at 68 and 32 ppm.

did not directly investigate the binding of Zn(II) at the S2 site of Con A, although their report seems to have assumed that Zn(II) would be similar to other transition metals in not binding at that site.

Another metal competition experiment that could be studied by ^{113}Cd NMR was the binding of Pb(II) to Cd:Cd Con A. A sample of 3.0 mM Cd:Cd Con A was prepared (Figure 7a), to which 1.0 equiv of Pb(II) has been added (Figure 7b). This spectrum contained four resonances, at 68, 46, 32, and −125 ppm. The S2 resonance (−125 ppm) was reduced to about half of its original intensity, most likely due to Pb competition at that site. The S1 resonance appears to have been split, the Cd:Cd Con A molecules giving rise to an S1 resonance at 46 ppm and the Cd:Pb Con A molecules giving rise to an S1 resonance at 32 ppm. Figure 7c is the ^{113}Cd spectrum of the same sample after addition of a second equivalent of Pb. The resonances at −125 and 46 ppm have decreased below the noise level, leaving only the resonances at 68 and 32 ppm visible.

It is also possible to explain the above data with a model in which Pb(II) competes at the S1 site and the S2 site is split at −125 and 32 ppm. This possibility was eliminated by adding a large excess of Ca^{2+} to the sample containing 2 equiv of lead. This caused the resonance at 32 ppm to shift to 43 ppm, its normal position in Cd:Ca Con A (Figure 2b). This result was only possible if Ca^{2+} and Pb^{2+} were competing for the same site, so the conclusion that Pb binds at the S2 site is confirmed.

It is interesting to note that the addition of 1 equiv of Pb(II) reduces the S2 resonance to half of its former intensity while the addition of 2 equiv of Pb(II) removes it entirely. If the binding constant for lead were about 3.5×10^3 M^{-1}, the 1 equiv of lead would bind to the extent of 50%. By use of this same equilibrium constant, however, 2 equiv of lead should bind to about 70% of the protein, leaving 30% in the Cd:Cd form. The total absence of Cd:Cd Con A in Figure 6c indicates that this is not the case. These results suggest that the first equivalent of lead was partitioned equally between two specific binding sites with binding constants in excess of 10^4 M^{-1} rather than between the S2 site and the free ionic form. A separate lead

Table I: ^{113}Cd Chemical Shifts of Unlocked Con A and Other Model Systems

sample	^{113}Cd chemical shift (ppm) 0.2 M NaCl	0.2 M $NaNO_3$
unlocked Con A + 3 mM $Cd(NO_3)_2$	68	12
"free" resonance in locked Con A	69	8
6.0% lysozyme + 3 mM $Cd(NO_3)_2$	68	6
6.0% bovine serum albumin + 3 mM $Cd(NO_3)_2$	68	6
3 mM $Cd(NO_3)_2$, no protein present	68	2

site has been described previously by Becker et al. (1975) and Sherry et al. (1973).

Several reports of a third binding site exist [Shoham et al. (1973) report a Hg site; Barber et al. (1975) and Richardson & Behnke (1978) report a La(II) site]. We have observed that the addition of Hg to a sample of 2Cd(II) Con A causes no change in the ^{113}Cd NMR spectrum of the protein. It is feasible to observe lead (^{207}Pb) and mercury (^{199}Hg) directly by using NMR; a future paper will report their use to probe for a third metal site in Con A.

Unlocked Conformation of Con A. The unlocked form is easily obtained by adding the desired metals to cold apo Con A by keeping the solution below 5 °C. As described earlier, unlocked Con A is characterized by rapid exchange of the metals between the metal sites and the surrounding solution.

The ^{113}Cd NMR spectrum of Cd:Cd Con A in the unlocked form consists of one resonance, with a chemical shift which is highly dependent on the buffer. In an acetate buffer, pH 5.2, containing 0.2 M NaCl, this resonance appears at 68 ppm. If the NaCl is replaced by $NaNO_3$, the resonance appears at −12 ppm. These unlocked samples may be converted to the locked form by allowing them to stand at room temperature for several hours. The resulting ^{113}Cd NMR spectra of these samples are then identical with the spectra of locked Cd:Cd Con A in the appropriate buffer.

The ^{113}Cd resonance in the unlocked form may be due to free cadmium ions, Cd(II) in rapid exchange with the protein metal binding sites, or Cd(II) associating nonspecifically with the protein. Table I compares the chemical shift of the ^{113}Cd resonance in unlocked Con A with the chemical shift of the free ^{113}Cd resonance in locked Con A, ^{113}Cd resonances in the presence of proteins lacking specific metal binding sites, and ^{113}Cd resonances in the absence of protein. In buffers containing 0.2 M NaCl, the ^{113}Cd resonance from unlocked Con A has exactly the same chemical shift as in all of the above systems. This suggests that the strongest interaction with Cd(II) in all of these systems is the interaction with chloride ion and that binding with Con A protein either specifically or nonspecifically is minimal. In buffers containing NO_3^- but not containing Cl^-, the ^{113}Cd resonance from unlocked Con A is 14–18 ppm to higher shielding from the resonances of the systems in Table I. These observations suggest that under the given conditions, the Cd(II) undergoes some chemical exchange with specific metal binding sites on unlocked Con A. Independent experimentation in our laboratory using activity assays (Goldstein et al., 1965) has shown that the locking process is much slower in chloride-containing buffers than in chloride-free buffers. These experiments suggest that the unlocked form of Con A contains specific binding sites for

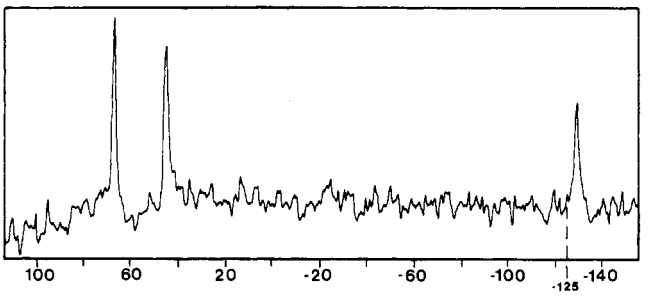

FIGURE 8: ^{113}Cd spectrum of Cd:Cd Con A containing 2 equiv of α-methyl D-mannopyranoside. The resonances are at 68, 46, and −133 ppm.

Cd(II), but cadmium–chloride complexes bind poorly to them.

Interaction of Saccharides with Con A. This section of this paper is concerned with the consequences of saccharides binding on the metal sites. It has been suggested that the binding site of Con A involves more than one residue when binding to a polysaccharide (So & Goldstein, 1968; Williams et al., 1978b). Others have suggested separate (but related) binding sites for saccharides and cell membranes (Cuatrecassas, 1973; Richardson & Behnke, 1976). Recently, arguments have been advanced in favor of a single saccharide binding site (Brewer & Brown, 1979). In addition to these reports, Williams et al. (1978a) have discussed inhomogeneity in the binding of oligosaccharides (but not monosaccharides) to Con A.

The addition of a saccharide to unlocked Cd:Cd Con A has no effect on the protein ^{113}Cd NMR spectrum. This indicates that either the sugars do not bind or if binding does occur the properties of the metals are not significantly altered. Several authors have postulated the latter possibility on the basis of stopped-flow NMR (Grimaldi & Sykes, 1975) and on the basis of proton relaxation NMR data (Koenig et al., 1978).

Figure 8 is the ^{113}Cd spectrum of locked Cd:Cd Con A to which 2 equiv of α-methyl D-mannopyranoside (αMM) has been added. This spectrum contains three resonances at 68, 46, and −133 ppm. These resonances are identical with the resonances in the Cd:Cd Con A except for a slight shift in the resonance at −125 ppm which corresponds to the resonance at −133 ppm in Figure 8 and a slight increase in the intensity of the resonance at 46 ppm. The 8-ppm shift in the S2 resonance suggests that this site is sensitive to the binding of αMM, but the S1 site is relatively insensitive. Becker et al. (1976) have demonstrated a conformational change in the metal binding region induced by saccharides. Sherry et al. (1973) and Hardman & Ainsworth (1976) have presented data consistent with a more direct involvement of the S2 site than the S1 site. Our data are clearly in agreement with all of these observations.

^{113}Cd NMR spectra were obtained for Cd:Cd Con A in the presence of 1 and 2 equiv of a variety on mono-, di-, and trisaccharides (Table II). Each sugar known to inhibit the mannan precipitation assay for Con A produced a ^{113}Cd NMR spectrum indistinguishable from the spectrum depicted in Figure 8. Hence, it is apparent that these sugars perturb the S2 site of Con A to similar extents. The only sugar used in this study that does not inhibit Con A activity is galactose. Addition of 5 equiv of galactose to Cd:Cd Con A solutions causes no observable change in the resulting ^{113}Cd spectrum.

The preceding experiments were repeated by using the various preparations of Con A. Again, all spectra taken were identical with the spectrum in Figure 8, so the data were independent of the presence of nicked subunits or other possible heterogeneity in the Con A preparations. Hence, whatever

Table II: Sugars Used in Saccharide Binding Comparisons

monosaccharides	disaccharides	trisaccharides
glucose	maltose	maltotriose
α-methyl D-mannopyranoside	sucrose	melezitose
α-methyl D-glucopyranoside		
β-methyl D-glucopyranoside		
galactose (noninhibitor)		

the origin of the heterogeneity observations of Williams et al. (1978a), these heterogeneities appear to be either absent or greatly reduced in Cd:Cd Con A. These data, however, are not proof of the hypothesis that all sugars bind to Con A by a common mechanism. However, they do indicate that all saccharide binding mechanisms cause similar perturbations at the S2 site and have little if any effect on the S1 site.

Conclusions

It is clear that ^{113}Cd NMR provides a unique method for the study of metal binding in metalloproteins with multiple metal sites. In this paper we demonstrated metal competition equilibrium data and limits on the exchange rate for each site individually. We also demonstrated the presence of weakly binding metal sites in the unlocked form of Con A. One can examine slow kinetic processes (as in the locking dynamics) or fast processes by line shape analysis. One can examine the substrate binding of metalloproteins with respect to individual metal sites. It is apparent that spin $^1/_2$ metal nuclide NMR spectroscopy will be a helpful experimental tool in future studies involving metalloproteins.

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