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Role of Second Metal Ion in Establishing Active Conformations of Concanavalin A†

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ABSTRACT: The stoichiometry of Mn^{2+} binding to concanavalin A was found to be influenced by temperature, pH, and the presence or absence of saccharide. Demetalized concanavalin A binds one Mn^{2+} (S1 site) at 5 °C, pH 6.5, and two Mn^{2+} at 25 °C (S1 and S2 sites). The association constants for Mn^{2+} are 6.2×10^5 and 3.7×10^4 M^{-1} for the S1 and S2 sites, respectively, at 25 °C. Concanavalin A with one Mn^{2+} bound per monomer remains in an open conformation and exhibits a relatively high water proton relaxation rate. Concanavalin A with two Mn^{2+} ions remains in a closed conformation characterized by a lower relaxation rate. The rate of binding of the second Mn^{2+} to concanavalin A as determined by ESR and the rate of conversion of open form to closed form (folding over) as determined by proton relaxation rate measurements gave an identical rate constant of 80.0 ± 5.8 M^{-1} h^{-1} at 17 °C. Ca^{2+}, Sr^{2+}, and high levels of methyl α-D-mannopyranoside also induce folding of concanavalin A. Ca^{2+} is not catalytic but stoichiometric in causing the folding. Mn^{2+} in the S1 site can be displaced by Ni^{2+}, Co^{2+}, and Zn^{2+}, and Mn^{2+} in the S2 site can be displaced by Ca^{2+} and Sr^{2+}. Concanavalin A with Ni^{2+}, Co^{2+}, Zn^{2+}, or Mn^{2+} in the S1 site and Ca^{2+} or Sr^{2+} in the S2 site has a higher affinity for methylumbelliferyl α-D-mannopyranoside than Ni–Mn–, Co–Mn–, Zn–Mn–, and Cd–Cd–concanavalin A.

Concanavalin A (Con A),[1] the lectin isolated from jack bean (*Canavalia ensiformis*) (Sumner & Howell, 1936), has been widely used in the affinity purification of glycoproteins, glycopeptides, and polysaccharides and in the separation of viruses and bacteria (Bittinger & Schnebli, 1976). Con A has been used extensively to study cell surface architecture; differences occurring during cell growth and division and following transformation are commonly monitored by Con A binding (Lis & Sharon, 1973; Krach et al., 1974; Ruddon, 1983). The mitogenic response of lymphocytes elicited by Con A is used extensively as a model for antigen stimulation (Powell & Leon, 1970; Yahara & Edelman, 1973; Rosenberg et al., 1982; Fathman & Frelinger, 1983; Sharon, 1983). The interaction of Con A with cell surface components occurs because the lectin binds specifically to sugar moieties with the α-D-arabinopyranoside configuration at the C-3, C-4, and C-6 positions (Goldstein et al., 1973). Because of the usefulness of this lectin, it has been the subject of many structural studies.

Con A exists in two pH-dependent forms, each composed of identical subunits of molecular weight 25 500 (Wang et al., 1971; Edmundson et al., 1971). The form found near physiological pH is predominantly tetrameric (McKenzie et al., 1972; Kalb & Lustig, 1968). Each subunit possesses one specific carbohydrate binding site and two metal ion binding sites; the S1 site is usually occupied by a transition metal ion, and the S2 site is occupied by a Ca^{2+} ion. X-ray crystallographic studies of the protein (Mn–Ca–Con A) show that the S1 and S2 sites are only 4.25 Å apart (Hardman et al., 1982) and that the two metal ions are bridged by the same carboxyl groups of two aspartic acid residues (Hardman & Ainsworth, 1972; Edelman et al., 1972). A variety of divalent metal ions such as Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} can bind to the S1 site, whereas only Ca^{2+} and Cd^{2+} have been shown to bind to the S2 site (Shoham et al., 1973).

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[1] Abbreviations: Con A, concanavalin A; ESR, electron spin resonance; Mops, 3-(*N*-morpholino)propanesulfonate; α-MDM, methyl α-D-mannopyranoside; MUM, 4-methylumbelliferyl α-D-mannopyranoside; apo-Con A, demetalized Con A; Mn–, Ni–, Co–, Zn–, Ni–Mn–, Co–Mn–, Zn–Mn–, Mn–Sr–, Mn–Ca–, Mn–Mn–Con A, apo-Con A remetalized with one or both of the respective metal ions. In M–Con A, M is in the S1 site, and in M_1–M_2–Con A, M_1 is in the S1 site and M_2 is in the S2 site.

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Christie et al. (1979) demonstrated by equilibrium dialysis that one Mn^{2+} binds per monomer of apo-Con A at pH 6.5, 5 °C. Using NMR dispersion measurements, Brown et al. (1977) have shown that Mn^{2+} binds to both the S1 and S2 sites. Investigations in our laboratory (Christie et al., 1978) as well as other laboratories (Harrington & Wilkins, 1978; Koenig et al., 1978) have demonstrated that Ca^{2+} can bind to apo-Con A and produce an active conformation. Cd^{2+} can also bind to both the S1 and S2 sites and produce an active conformation (Pandolfino et al., 1980b).

In the present study the stoichiometry of Mn^{2+} ion binding to apo-Con A was determined under a variety of conditions. The stoichiometry of binding of the transition metal ions Ni^{2+}, Co^{2+}, and Zn^{2+} was determined at 5 and 25 °C. When Mn^{2+} is added to apo-Con A, the ion binds to the protein, restoring its saccharide binding activity. Simultaneously, a conformational change occurs at the metal binding region of the protein. This conformational change has been suggested by Brewer et al. (1983c) to accompany the cis–trans isomerization of an Ala–Asp peptide bond in the backbone of the protein, near one of the two metal ion binding sites. The conformational change following Mn^{2+} binding to apo-Con A can be monitored by measuring solvent water proton relaxation rates. When Mn^{2+} binds to apo-Con A at 5 °C, the spin–lattice relaxation rate ($1/T_1$) of water protons is greatly enhanced. The Con A–manganese complex at 5 °C remains open, solvent molecules have free access to the bound manganese, and the protons exchange freely with the protons of water ligands. Brown and et al. (1977) and later Christie et al. (1979) demonstrated an interesting concentration effect when Mn^{2+} was added to demetalized Con A at 25 °C. At equilibrium, with low stoichiometric ratios of Mn^{2+} to Con A, the enhancement in relaxation rates was observed to first increase and then decrease as the ratio increased. In contrast to the studies at 5 °C, apo-Con A appears to bind two Mn^{2+} ions per Con A monomer at 25 °C and to convert to a closed conformational state where solvent molecules have relatively restricted access to the bound Mn^{2+} ions. We report herein studies on the transition from the low-temperature open form to the high-temperature closed form. Kinetics of the binding of Mn^{2+} to apo-Con A were followed by monitoring the ESR signal of free Mn^{2+}, and kinetics of the folding process, open to closed, were followed by monitoring changes in the water proton relaxation rate.

Materials and Methods

Chemicals. Jack bean meal, methyl α-D-mannopyranoside, 4-methylumbelliferyl α-D-mannopyranoside, and Mops were purchased from Sigma. $MnSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, and $CoSO_4 \cdot 7H_2O$ were obtained from Specpure Chemicals Ltd., London; $NiCl_2 \cdot 6H_2O$ was from Chemalog Chemical Dynamics Corp. Analytical grade $CaCl_2 \cdot 2H_2O$, $SrCl_2 \cdot 6H_2O$, and potassium acetate came from J. T. Baker Chemical Co. Chelex 100 was purchased from Bio-Rad Laboratories.

Con A Preparations. Con A was prepared from jack bean meal as previously described (Agrawal & Goldstein, 1967), and activity was measured by the quenching of MUM fluorescence (Christie et al., 1978). Con A concentrations were determined spectrophotometrically at pH 6.5 by using an absorbance $A_{280}^{1\%,1cm}$ of 13.7 (Yariv et al., 1968). All protein solutions were prepared in 1.0 M KCl that had twice been passed through an ion-exchange column of Chelex 100 to remove contaminating divalent metal ions. Solutions were buffered with 0.1 M potassium acetate or 0.05 M Mops. Demetalized Con A was prepared by dialyzing Con A against 0.1 M HCl as described earlier (Christie et al., 1978). Atomic absorption analysis (Perkin-Elmer 360) of the apoprotein showed that contamination by Ca^{2+} was less than 0.03 mol/mol of 25 500-Da subunit.

Metalized forms of Con A, Mn–, Ni–, Co–, or Zn-Con A, were prepared by incubating apo-Con A (usually 3×10^{-4} M) with 2 mM solutions of Mn^{2+}, Ni^{2+}, Co^{2+}, or Zn^{2+} at 25 °C. Ni–Mn–, Co–Mn–, Zn–Mn–, or Mn–Sr–Con A were prepared by adding small aliquots of 0.1 M solutions of Ni^{2+}, Co^{2+}, Zn^{2+}, or Sr^{2+} to apo-Con A preincubated with 2.0 mM Mn^{2+} at 25 °C.

NMR Measurements. Water proton relaxation rates of Con A samples were measured at 20.5 MHz by using a Bruker SXP NMR spectrometer. The solutions were buffered in 1.0 M KCl solution at pH 6.5 with 0.05 M Mops or 0.1 M potassium acetate. Spin–lattice relaxation rates ($1/T_1$) were determined by the inversion recovery method with a sample volume of 0.3 mL. Proton relaxation rates of known concentrations of Mn^{2+} were determined at 20.5 MHz in the same buffer and at the same temperature but in the absence of protein.

For kinetic experiments, apo-Con A buffered at pH 6.5 was mixed with Mn^{2+} in an NMR tube at 17 °C, and T_1 values at 20.5 MHz were determined for a period of 5–6 h. Changes following Ca^{2+} addition to Mn–Con A were followed by adding Ca^{2+} to apo-Con A that had been equilibrated with 1.4 equiv of Mn^{2+} for 4 days at 5 °C.

ESR Measurements. The stoichiometry of Mn^{2+} binding to Con A was determined by ESR spectroscopy using a Varian E-9 spectrometer. Samples of apo-Con A were incubated with 4 equiv of Mn^{2+} with or without 0.1 M α-MDM at 5 and 25 °C in buffer solutions of varying pH values. Solutions were buffered by using a mixture of 0.05 M Mops and 0.05 M potassium acetate in 1.0 M KCl solution. The amplitude of the ESR signal in the low-field extremum was measured in each case, and the concentration of free Mn^{2+} in solution was calculated by comparing the signal intensities with signals produced by known concentrations of Mn^{2+} in the same buffer but in the absence of protein. The signal from bound Mn^{2+} was negligible compared to that from free Mn^{2+} in solution as shown by the observation that the ESR spectra of samples from the inside and the outside of a dialysis bag in which apo-Con A was dialyzed against excess of Mn^{2+} were superimposable. Time courses of Mn^{2+} association to Con A were monitored by ESR spectroscopy (Alter & Magnuson, 1979). For displacement studies aliquots of 0.1 M solutions of appropriate metal ions were added to apo-Con A that had been equilibrated with 4 equiv of Mn^{2+}, with or without α-MDM, at 5 or 25 °C.

Equilibrium Dialysis. Samples of apo-Con A buffered at pH 6.5 were dialyzed against buffer solutions containing Ca^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, or Zn^{2+}. Metal ion concentrations on both sides of the dialysis bag were measured by atomic absorption methods using a Perkin-Elmer 360 atomic absorption spectrometer. Dialysis tubing was treated with EDTA and rinsed extensively with glass-distilled water before use.

Fluorescence Measurements. Fluorescence measurements were carried out at 5 °C on 2.0-mL protein samples that had been prepared as described above and diluted to a final concentration of 5×10^{-5} M. Aliquots (20 μL) of a 1.0×10^{-3} M solution of MUM were added, and the relative fluorescence was measured after each addition. Binding of MUM to Con A samples was monitored by fluorescence quenching at an excitation wavelength of 350 nm and an emission wavelength of 375 nm as described earlier (Christie et al., 1979) by using a Perkin-Elmer MPF-3L fluorescence spectrophotometer

Table I: Stoichiometry of Binding of Different Metal Ions to Con A at pH 6.5[a]

metal ion	[ion bound]/[Con A] 5 °C	25 °C
Mn^{2+}	1.1 ± 0.1	1.9 ± 0.1
Ni^{2+}	1.0 ± 0.05	1.05 ± 0.05
Co^{2+}	1.03 ± 0.04	1.1 ± 0.05
Zn^{2+}	1.04 ± 0.06	1.15 ± 0.1

[a] All the experiments were conducted in 1.0 M KCl, 0.05 M Mops, at pH 6.5. Stoichiometry was determined by equilibrium dialysis of apo-Con A (3.1×10^{-4} M) against 10 volumes of buffer solutions containing 3–4 equiv of the respective metal ions. Errors shown are standard deviations from at least three separate experiments.

equipped with a thermostated cuvette holder. Concentrations of MUM solutions were determined spectrophotometrically at 318 nm by using $\epsilon = 1.36 \times 10^4$ M^{-1} cm^{-1} (Loontiens et al., 1977).

Results

Stoichiometry and Affinity of Mn^{2+} Binding to Apo-Con A. Manganese binding to apo-Con A was followed by monitoring the ESR signal of free Mn^{2+} in solution. The extent of binding of Mn^{2+} to Con A was pH and temperature dependent. The optimum binding of Mn^{2+} to apo-Con A was found to be at pH 6.0–7.0, at 5 or 25 °C in the presence or absence of α-MDM.

Binding of Mn^{2+} and other transition-metal ions at pH 6.5 was studied at 5 and 25 °C by equilibrium dialysis experiments (Table I). At 5 and 25 °C only one Ni^{2+}, Co^{2+}, or Zn^{2+} ion was bound per Con A monomer. For Mn^{2+} the stoichiometry was 1 at 5 °C and 2 at 25 °C (Christie et al., 1979; Brown et al., 1977). A stoichiometry of 2 was found for Mn^{2+} at 5 or 25 °C in the presence of α-MDM. The results obtained from ESR measurements and atomic absorption measurements of Mn^{2+} under equilibrium conditions at pH 6.5 were in agreement. The results were found to be identical in 1.0 M KCl, 0.05 M Mops, and in 1.0 M KCl, 0.1 M potassium acetate (pH 6.5). The stoichiometry of Ca^{2+} binding to apo-Con A at 25 °C, pH 6.5, was found to be 2.0 by equilibrium dialysis and atomic absorption measurements. The values were in agreement with the reported values (Brewer et al., 1983b).

The affinity of Con A-manganese binding was determined by Scatchard analysis (1949). Figure 1 is a Scatchard plot showing representative results of Mn^{2+} binding to apo-Con A at pH 6.5, 25 °C. The data were analyzed by the method described by Cantor and Schimmel (1980). There are two types of Mn^{2+} binding sites, and the total number of binding sites per Con A monomer is two. The association constant for the higher affinity site was 6.2×10^5 M^{-1} at 25 °C compared to 3.5×10^5 M^{-1} at 5 °C (Christie et al., 1979) and that for the second site or lower affinity site was found to be 3.7×10^4 M^{-1} at 25 °C. In the presence of 0.1 M α-MDM at 25 °C, the association constants of Mn^{2+} for both the sites were increased. However, the amount of free Mn^{2+} was too low to be measured by ESR spectroscopy when the ratio of Mn to Con A was less than unity. The Scatchard plot for the binding of Mn^{2+} to the S2 site at 25 °C in the presence of α-MDM is given in Figure 2. The association constant of Mn^{2+} for the S2 site in the presence of sugar at 25 °C was found to be 2.6×10^5 M^{-1}. The results were identical in KCl–potassium acetate and in KCl–Mops buffer systems at pH 6.5.

Factors Influencing Folding of Con A. Changes in the relaxation rate of water protons following binding of Mn^{2+} to a protein reflect changes in the environment of Mn^{2+} (Barber & Carver, 1973). The addition of Mn^{2+} to apo-Con A resulted in an immediate enhancement of longitudinal relaxation rate ($1/T_1$) of solvent water protons. Following the enhancement, the $1/T_1$ value changed little after 7 days at 5 °C. At 25 °C, however, the $1/T_1$ value slowly decreased and reached an equilibrium value within several hours. The water proton relaxation rates of samples equilibrated at 25 °C did not change on incubation at 5 °C even after 7 days. ESR measurements showed that Mn–Mn–Con A formed at 25 °C did not revert to Mn–Con A in 7 days on incubation at 5 °C.

Figure 3 shows the results of an NMR experiment in which water proton relaxation rates were measured for samples of apo-Con A incubated with varying amounts of Mn^{2+} in the presence or absence of α-MDM at 5 or 25 °C. Measurements were made periodically between 1 and 5 days, and during this time no changes in relaxation rates for individual samples were

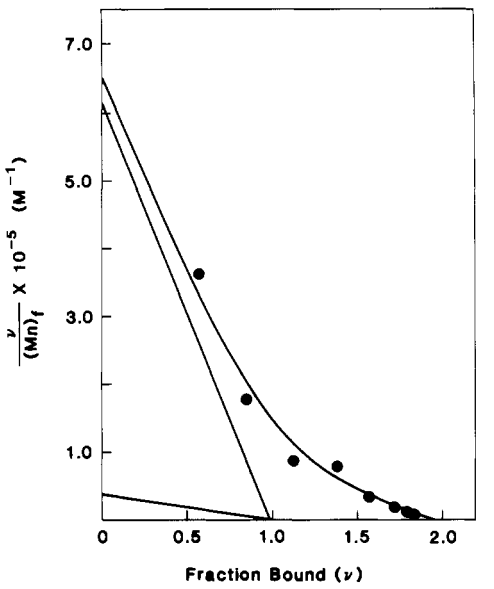

FIGURE 1: Scatchard plot of Mn^{2+} binding to apo-Con A (3.0×10^{-4} M) at pH 6.5, 25 °C. The fraction of bound Mn^{2+} per total Con A subunits is represented by ν. $[Mn]_f$ is the free Mn^{2+} concentration. The solid line through the data points is the theoretical sum of the binding curves for the S1 and S2 sites.

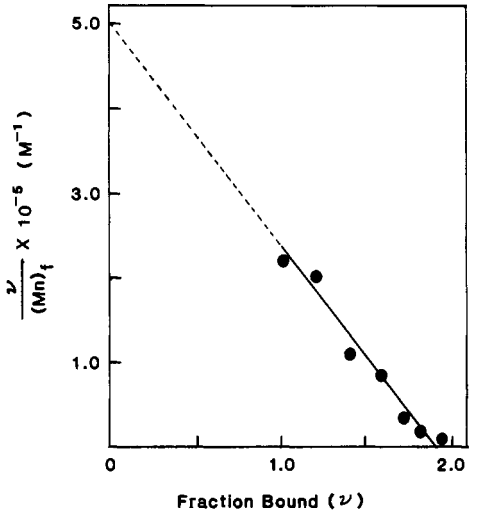

FIGURE 2: Scatchard plot of Mn^{2+} binding to apo-Con A (5×10^{-4} M) at pH 6.5 and 25 °C in the presence of 0.1 M α-MDM. The fraction of bound Mn^{2+} per total Con A subunits is represented by ν. $(Mn)_f$ is the free Mn^{2+} concentration. When Mn^{2+}/Con A was less than 1.0, the free Mn^{2+} was too low to be measured by ESR spectroscopy.

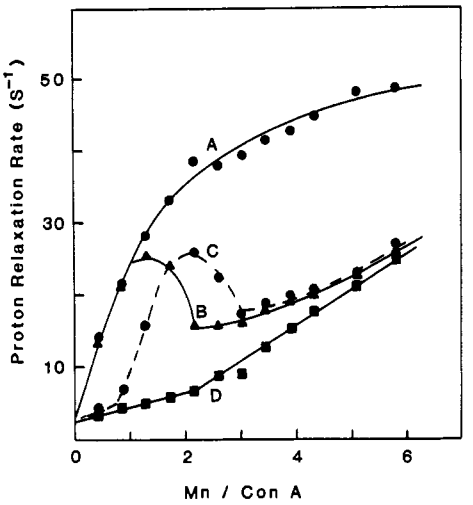

FIGURE 3: Water proton relaxation rates measured at 20.5 MHz for samples of apo-Con A (5.0×10^{-4} M) monomers incubated with different concentrations of Mn^{2+} ions in the presence or absence of 0.1 M α-MDM at 5 or 25 °C. The protein samples were buffered at pH 6.5 in 1.0 M KCl and 0.05 M Mops. (A) Apo-Con A and Mn^{2+} incubated at 5 °C (●); (B) apo-Con A and Mn^{2+} incubated at 25 °C (▲); (C) apo-Con A, Mn^{2+}, and 0.1 M α-MDM incubated at 5 °C (●); (D) apo-Con A, Mn^{2+}, and 0.1 M α-MDM incubated at 25 °C (■). Water proton relaxation rates of samples incubated at 5 °C were measured at approximately 10 °C, and those incubated at 25 °C were measured at room temperature.

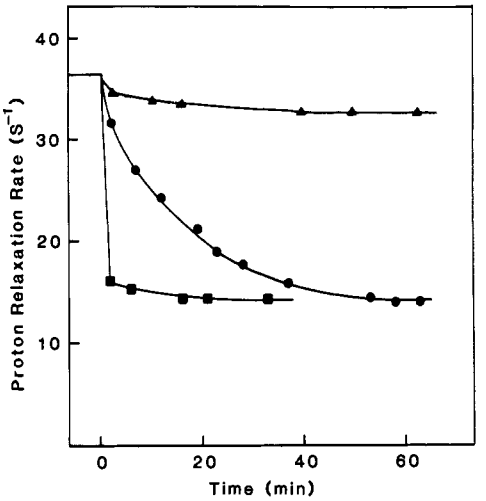

FIGURE 4: Time course for water proton relaxation rate following addition of Ca^{2+} or excess of α-MDM to apo-Con A preincubated with approximately 5 equiv of Mn^{2+} at pH 6.5, 5 °C. Apo-Con A (3.2×10^{-4} M) was incubated with 1.5 mM Mn^{2+} for 5 days. Water proton relaxation rates at 20.5 MHz of Mn–Con A: (A) in the absence of Ca^{2+} or α-MDM (▲); (B) following addition of Ca^{2+} to a final concentration of 2.0 mM (■); (C) following addition of 0.1 M α-MDM (●) at time zero.

observed. The curves at 25 °C in the presence or absence of α-MDM are identical with those reported by Brewer et al. (1983b).

The curves presented in Figure 3 are most easily interpreted in terms of two conformational states of Mn–Con A complex, an open form characterized by a high proton relaxation rate and a closed form characterized by a low proton relaxation rate. At 5 °C, in the absence of α-MDM, only one Mn^{2+} was bound to apo-Con A monomer, and the Mn–Con A was in the open form with a relatively large proton relaxation rate (curve A in Figure 3). For the samples of apo-Con A incubated with Mn^{2+} at 25 °C (curve B), the proton relaxation rate increased as the Mn^{2+}/Con A ratio increased from 0 to 1.0 but then decreased, reached a minimum at approximately 2.0 equiv, and then increased linearly with increase in free Mn^{2+} following saturation of the protein. When Mn^{2+}/Con A was less than 1.0, only the open form was present, but at higher ratios the second Mn^{2+} was bound and the protein folded over. A similar trend in the relaxation rates was observed for Con A samples incubated with Mn^{2+} in the presence of α-MDM at 5 °C (curve C), even though there was a slight shift in the curve toward high Mn^{2+}/Con A ratio. For Con A samples with less than 1 equiv of Mn^{2+}, enhancement of proton relaxation rate was not observed, indicating that in the presence of high levels of α-MDM (0.1 M) Mn^{2+} in the S1 site was inaccessible to water protons. As the Mn^{2+}/Con A ratio was increased above 1.0, the proton relaxation rate markedly increased until approximately 2.0 (curve C). At this point the rate decreased, reaching a minimum at approximately 3.0. ESR measurements showed that approximately two Mn^{2+} were bound at a Mn^{2+}/Con A ratio of 3.0 and approximately 1.5 were bound at a Mn^{2+}/Con A ratio of 2.0. The increase in rate (Mn^{2+}/Con A = 1.0–2.0) must be from a new, as yet unidentified, species. As Mn^{2+} binds to Con A with one Mn^{2+} per monomer, relaxation enhancement was observed, indicating that a conformational change to the closed form did not occur until Mn^{2+} filled almost all the S2 sites. We suggest at this point (see also Figure 6 later) that Mn^{2+} in half of the S2 sites of a dimer causes rapid relaxation; when the second S2 site is filled, the protein folds to the closed form. It is important to note that the rapidly relaxing form is only long-lived in the cold (5 °C). At warm temperatures, folding of the monomer with two Mn^{2+} occurred. An enhancement of the proton relaxation rate was not observed in the presence of α-MDM at 25 °C. A biphasic curve was obtained for Con A samples incubated with Mn^{2+} and high levels of α-MDM at 25 °C, with a sharp break at approximately 2.0 equiv of Mn^{2+} (curve D). The transition from the open form to the closed form of Con A occurred at 25 °C even at less than 1 equiv of Mn^{2+} and in the presence of 0.1 M α-MDM.

That sugar promoted the conformational change to the closed state (Figure 3, curve D) suggests that rates of folding should be enhanced by sugar. When α-MDM was added to apo-Con A that had been preincubated with Mn^{2+} at 5 °C, conversion from the open to the closed form was markedly enhanced (Figure 4). The folding was not as fast, however, as that induced by Ca^{2+} or Sr^{2+}, both of which induce folding at a rate too fast to be measured in our apparatus.

To establish whether or not folding is necessary for saccharide binding activity, water proton relaxation rates and binding activity were measured simultaneously on apo-Con A samples to which Mn^{2+} had been added. Apo-Con A was incubated with 3 equiv of Mn^{2+} at 25 °C for periods of 0–2 h and then immediately transferred to an ice bath to prevent further folding. Proton relaxation rate and MUM binding activity were then measured. The fraction of active Con A at early time points was always greater than the fraction folded over.

Kinetics of Metal Ion Binding and Folding of Con A. To determine if Ca^{2+} is catalytic in inducing the folding of Mn–Con A, less than stoichiometric amounts (0.3, 0.5, 0.7, or 1.0 equiv) of Ca^{2+} were added to apo-Con A preincubated with Mn^{2+}. Results obtained are shown in Figure 5. The maximum change in proton relaxation rates at 20.5 MHz of Mn–Con A correlated directly with the amount of Ca^{2+} added. Even after 14 days at 5 °C, the extent of reduction of proton relaxation rates was directly related to the amount of Ca^{2+} added. The same samples were then incubated at 25 °C for 2 days, and $1/T_1$ were measured. Even though the $1/T_1$ values

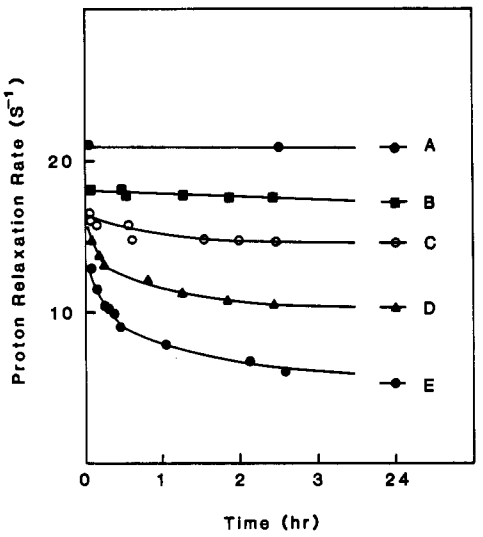

FIGURE 5: Changes in proton relaxation rates following addition of varying amounts of Ca^{2+} to apo-Con A (2.7×10^{-4} M) preincubated with 1.4 equiv of Mn^{2+} for 4 days at pH 6.5, 5 °C. The protein samples were buffered at pH 6.5 by using 0.05 M Mops in 1.0 M KCl solution. Changes in proton relaxation rate at 20.5 MHz of Mn-Con A: (A) without any Ca^{2+} added (●); (B) after addition of 0.3 equiv of Ca^{2+} (■); (C) after addition of 0.5 equiv of Ca^{2+} (○); (D) after addition of 0.7 equiv of Ca^{2+} (▲); (E) after addition of 1.0 equiv of Ca^{2+} (●) at zero time. All measurements were made at approximately 5–7 °C.

were further decreased, the extent of reduction was proportional to the amount of total metal ions (Mn^{2+} and Ca^{2+}) present. These results confirmed that Ca^{2+} was not catalytic but was stoichiometric in causing the folding of Con A (Christie et al., 1980; Koenig et al., 1982) and also further demonstrated that the amount of folded conformation was proportional to Con A with metals at both S1 and S2.

Mn^{2+} association rates could be monitored in stopped-flow ESR experiments, and rates of folding from open to closed conformations could be easily obtained by following changes in the water proton relaxation rates. When apo-Con A was mixed with a stoichiometric excess of Mn^{2+}, the ESR signal of free Mn^{2+} decreased with time. One Mn^{2+} per monomer associated rapidly at 25 °C in a step too fast to monitor with our apparatus, but a second Mn^{2+} bound slowly over a period of hours. At equilibrium, 2.0 Mn^{2+} bound per Con A monomer. It was also found that the association rate of the second Mn^{2+} to apo-Con A was greatly enhanced in presence of 0.1 M α-MDM.

When a stoichiometric excess of Mn^{2+} was added to an apo-Con A solution, the spin–lattice relaxation rate ($1/T_1$) increased quickly, as the open conformation of Mn–Con A was produced, and then decreased slowly, as the second Mn^{2+} was bound and the closed conformation of Mn–Mn–Con A was produced. The equilibrium was reached after several hours at 17 °C. The equilibrium state obtained at 17 °C was the same as that at 25 or 32 °C. The experiment was done at 17 °C, rather than at 25 °C, because the rate of folding was slower at 17 °C than at 25 °C. At 5 °C, however, the relaxation rate increased with added Mn^{2+} but did not decrease even after 5 days; no conversion to the closed conformation occurred.

Rates of transition from the open to the closed form were followed by measuring proton relaxation rates at appropriate times. The rate of change of $1/T_1$ was found to be dependent on concentrations of both Mn^{2+} and Con A monomers. A rate law consistent with our findings is

$$-d(1/T_1)/dt = k[\text{Mn–Con A}][Mn^{2+}] \quad (1)$$

Table II: Displacement of Bound Mn from Mn–Mn–Con A[a]

metal ion used for displacement	Mn^{2+} bound per subunit after displacement	metal ion used for displacement	Mn^{2+} bound per subunit after displacement
none	1.9 ± 0.1	Sr^{2+}, Ni^{2+}	1.0
Ni^{2+}	0.9 ● 0.1	Zn^{2+}	0.9 ± 0.1
Ni^{2+}, Ca^{2+}	0.0	Zn^{2+}, Ca^{2+}	0.0
Ca^{2+}	1.0	Ni^{2+}, Zn^{2+}	0.9 ± 0.1
Ca^{2+}, Ni^{2+}	1.0	Ni^{2+}, Cd^{2+}	0.0
Sr^{2+}	1.0	Co^{2+}	0.9 ± 0.1
Ni^{2+}, Sr^{2+}	0.0	Co^{2+}, Ca^{2+}	0.0

[a] All displacements were done at pH 6.5, 25 °C, by adding 8-fold excess of the metal ions with respect to Con A monomers. Mn–Mn–Con A was prepared by incubating 3.1×10^{-4} M apo-Con A with 1.5 mM Mn^{2+} at 25 °C for 2 days. In cases where two metal ions are shown, the second metal ion was added 50–60 min after addition of the first metal ion. Concentration of displaced Mn^{2+} was determined by measuring the amplitude of ESR signal of free Mn^{2+} in solution.

where Mn–Con A represents a Con A monomer with one bound Mn^{2+}. The integrated form of the rate law is

$$1/([Mn^{2+}]_{t=i} - [\text{Mn–ConA}]_{t=i}) \ln ([\text{Mn–Con A}]_{t=i} \times [Mn^{2+}]_t/[\text{Mn–Con A}]_t[Mn^{2+}]_{t=i}) = F = kt \quad (2)$$

where $t = i$ refers to the time after an initial equilibrium between Mn^{2+} and Con A is established to form Mn–Con A. Because Mn^{2+} binds rapidly to S1, $t = i$ is approximately the time of mixing. Concentrations at later times are indicated by the subscript t. The observed $1/T_1$ at time t is the sum of contributions from three species: (1) free Mn^{2+} in solution; (2) Mn–Con A complex in the open form; and (3) Mn–Mn–Con A complex in the closed form. The molar relaxivities of open and closed forms were calculated from $(1/T_1)_{t=i}$ and $(1/T_1)_{t=\infty}$, knowing the concentration of free Mn^{2+} in solution and assuming that all Con A molecules are in the open form at $t = i$ and in the closed form at $t = \infty$ with one or two Mn^{2+} bound to them. $[\text{Mn–Con A}]_t$ and $[Mn^{2+}]_t$ were calculated from $(1/T_1)_t$ and molar relaxivities of free Mn^{2+}, Mn–Con A in the open form, and Mn–Mn–Con A in the closed form, knowing the concentrations of Con A and Mn^{2+} used in the experiment. The time courses were well described by the integrated rate law given by eq 2; the slopes in the linear plots obtained are equal to the rate constant for the folding process. The rate constants were pH and temperature dependent. The rate constant at 17 °C, pH 6.5, was found to be 80.0 ± 5.8 M^{-1} h^{-1}. The rate of association of the second Mn^{2+} to Mn–Con A as determined by ESR spectroscopy and the rate of folding of Mn–Mn–Con A complex were found to be identical.

Displacement of Bound Mn^{2+} from Con A–Manganese Complex. The stoichiometry of Mn^{2+} binding to Con A was observed to be 2.0 at 25 °C both by ESR spectroscopy and by equilibrium dialysis. Displacement of Mn^{2+} from Mn–Mn–Con A was followed by monitoring the Mn^{2+} ESR signal after addition of competing metal ions. The results obtained at equilibrium are given in Table II. The transition-metal ions, Ni^{2+}, Co^{2+}, and Zn^{2+}, are specific for binding to the S1 site. They displaced 1 equiv of Mn^{2+} from Mn–Mn–Con A at 25 °C. The remaining 1 equiv of Mn^{2+} could be displaced by Ca^{2+}, Sr^{2+}, or Cd^{2+} when added 1 h after Ni^{2+}, Co^{2+}, or Zn^{2+} was added to Mn–Mn–Con A. If Ca^{2+} or Sr^{2+} was added before Ni^{2+}, Co^{2+}, or Zn^{2+} ions, one Mn^{2+} was displaced and the remaining Mn^{2+} could not be displaced by Ni^{2+}, Co^{2+}, or Zn^{2+}. Mn^{2+}, therefore, was able to occupy both S1 and S2 sites; Mn^{2+} at S1 could be displaced by Ni^{2+}, Co^{2+}, or Zn^{2+}, and the Mn^{2+} at S2 could be displaced by Ca^{2+}, Sr^{2+}, or Cd^{2+}.

Table III: Comparison of Activity and Binding Constant of Various Metalized Forms of Con A toward MUM[a]

metalized forms of Con A	activity	K_a (10^{-4} M^{-1})
Mn–Ca–Con A	+	9.5 ± 0.5
Mn–Mn–Con A	+	5.0 ± 0.2
Ni–Mn–Con A	+	5.0 ± 0.2
Co–Mn–Con A	+	4.9 ± 0.3
Zn–Mn–Con A	+	4.8 ± 0.3
Mn–Sr–Con A	+	9.5 ± 0.5
Cd–Cd–Con A	+	4.8 ± 0.6[b]
Sr–Con A	–	
Ni–Con A	–	
Co–Con A	–	
Zn–Con A	–	

[a] All experiments were carried out at pH 6.5, 5 °C. Mn–Ca, Ni–Mn, Co–Mn–, Zn–Mn, and Mn–Sr–Con A were prepared by adding 10-fold excess of Ca^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, or Sr^{2+}, respectively, to Mn–Mn–Con A at room temperature Mn–Mn–, Ni–, Co–, Zn–, and Sr–Con A were prepared by incubating apo-Con A with 10-fold excess of Mn^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, or Sr^{2+}, respectively, at room temperature in 1.0 M KCl, 0.05 M Mops pH 6.5 buffer. Errors shown are standard deviations from at least three separate experiments. [b] Pandolfino et al. (1980b).

Once Ca^{2+} or Sr^{2+} occupied the S2 site, however, Mn^{2+} in the S1 site could not be displaced. A similar behavior was observed for Mn–Mn–Con A in the presence of α-MDM; displacement of Mn^{2+} from either S1 or S2 sites was not detected within several hours.

MUM Binding Activity of Various Metalized Forms of Con A. MUM binding activity of various metal derivatives of Con A was monitored at 5 °C in 1.0 M KCl, 0.05 M Mops buffer, pH 6.5. The stoichiometry and association constant for MUM binding to various metalized forms were determined by Scatchard analysis as described by Christie et al. (1978). The number of saccharide binding sites per Con A monomer was 1.0 in all the cases, although the binding constant was found to be dependent on the metal ion in the S2 site. The binding constant of various metalized forms of Con A for MUM is given in Table III.

Apo-Con A incubated with an excess of Mn^{2+} at 25 °C was active, and the binding constant for MUM binding was approximately 5.0×10^4 M^{-1}. Ni^{2+}, Co^{2+}, or Zn^{2+} alone could not activate Con A. Ni–Mn–Con A, Co–Mn–Con A, and Zn–Mn–Con A prepared by adding a 10-fold excess of Ni^{2+}, Co^{2+}, or Zn^{2+} to Mn–Mn–Con A at 25 °C were found to be active. ESR measurements demonstrated that each of these transition-metal ions displaced 1 equiv of Mn^{2+} from Mn–Mn–Con A, most probably from the S1 site. Mn–Sr–Con A, prepared by adding Sr^{2+} to Mn–Mn–Con A at 25 °C, was found to be active, and the binding constant for MUM was found to be 9.4×10^4 M^{-1}, a value identical with that reported for Mn–Ca–Con A (Christie et al., 1978). Apo-Con A incubated with Sr^{2+} alone was not active. In contrast to metalized Con A prepared by adding Ni^{2+}, Co^{2+}, or Zn^{2+} to Mn–Mn–Con A, attempts to prepare Ni–Mn–, Co–Mn–, and Zn–Mn–Con A by adding Mn^{2+} to Ni–, Co–, or Zn–Con A were unsuccessful. Very little binding of Mn^{2+} to Zn–, Co–, or Ni–Con A was detected by ESR spectroscopy even after 48 h, and no saccharide binding could be detected during this period.

Discussion

Establishment of the stoichiometry and mechanism of metal ion binding to Con A has been a subject of intense interest. In spite of extensive studies, there is controversy in the literature regarding the stoichiometry of Mn^{2+} binding to Con A (Sophianopoulos et al., 1983; Sophianopoulos & Sophianopoulos, 1986; Brown et al., 1977; Brewer et al., 1983b; Christie et al., 1979). Previous studies in our laboratory were carried out predominantly at pH 6.5 and 5 °C. The stoichiometry of Mn^{2+} binding under these conditions was found to be 1.0 (Christie et al., 1979). Brewer and co-workers carried out most of their studies at 25 °C (Brown et al., 1977; Brewer et al., 1983b) and reported the stoichiometry of Mn^{2+} binding to Con A monomer to be 2.0 (Figure 1). In contrast to Brewer's studies, Sophianopoulos and co-workers obtained the stoichiometry of Mn^{2+} binding at 25 °C to be 1.0/Con A monomer (Sophianopoulos et al., 1983; Sophianopoulos & Sophianopoulos, 1986). Our present studies show that they obtained a value of 1.0 instead of 2.0 because they had determined the stoichiometry before equilibrium was reached. It takes several hours at 25 °C for the S2 site to be saturated. It is important to note that the stoichiometry of Mn^{2+} binding to Con A is influenced by a variety of factors, such as pH, temperature, and the presence of sugar. Our results agree with those of Brewer and co-workers (Brown et al., 1977; Brewer et al., 1983b) in that Mn^{2+} can bind to S1 and S2 sites under proper conditions. The affinities of Mn^{2+} for S1 and S2 sites are different, however, and are influenced by sugar (Figures 1 and 2).

At this point it is important to present a general summary of our experimental findings. Equilibrium dialysis and ESR experiments show that apo-Con A in the cold (5 °C) binds one Mn^{2+} and no conformational change from open to closed form occurs, but at higher temperatures (15–25 °C) a second Mn^{2+} can bind. This step induces the folding of protein. Ca^{2+} and Sr^{2+} have the same effect as the second Mn^{2+}, but these two ions will bind and induce folding in the cold. Displacement studies and sugar binding show that Mn–Mn–Con A is slightly different from Con A with Ca^{2+} or Sr^{2+} in S2. Sugar can bind to Mn–Con A that is in the open form and induce the conformational change to the closed form at 25 °C. The fact that Mn–Mn–Con A formed at 25 °C does not revert to Mn–Con A on incubation at 5 °C shows that Mn–Con A and Mn–Mn–Con A are two states that are not in equilibrium at 5 °C.

A model for Mn^{2+} and sugar binding consistent with our results is presented in Figure 6. Mn^{2+} binds to the S1 site of apo-Con A (A in figure) in a fast step at 5 or 25 °C to give Mn–Con A (B), which is in the open conformation (curve A in Figure 3). B binds a second Mn^{2+} at 25 °C in a slow step as determined by ESR spectroscopy. The Mn–Mn–Con A produced is immediately converted to the closed conformational state (C), as evidenced by proton relaxation rate measurements. In fact, Mn–Mn–Con A is detectable only in the closed form at 25 °C (curve B in Figure 3). Mn–Mn–Con A (C) binds sugar in a fast step at 5 or 25 °C to form the sugar complex F, as demonstrated in MUM binding experiments. Mn–Con A (B) binds sugar in a slow step (Christie et al., 1979) and simultaneously gets converted to the closed form D (curves C and D in Figure 3). A second Mn^{2+} binds to D in a fast step at 25 °C to produce F, as demonstrated by the enhanced rate of binding of Mn^{2+} to Mn–Con A in the presence of sugar. However, at 5 °C conversion of D to F goes through a hitherto unidentified intermediate E. This must be postulated to explain the increase in relaxation rate for Mn^{2+}/Con A = 1–2 in the presence of α-MDM at 5 °C (curve C in Figure 3). At 25 °C D might be going to F through the same intermediate E, but E is undetectable.

The terms unlocked and locked used by Brewer and co-workers are analogous to the terms open and closed used in this study. We use the terms open and closed in relation to the water proton relaxation rates of Con A–manganese complexes in different conformational states; in the open form

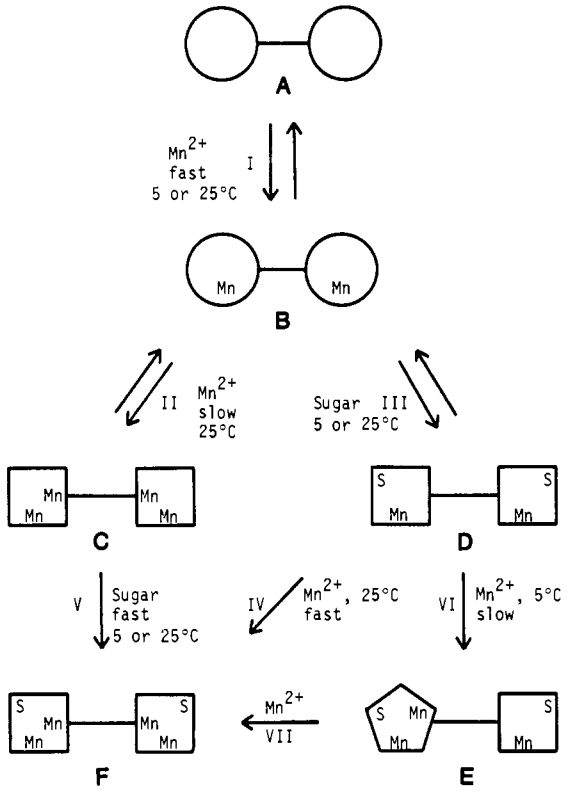

FIGURE 6: Model of the conformational changes occurring to concanavalin A upon binding of Mn^{2+} and sugar. The pairs of connected figures represent Con A dimers. The circles represent the open form, the squares represent the closed form, and the pentagon represents an intermediate form. The Mn and S shown inside the figures represent bound Mn^{2+} and sugar, respectively.

solvent molecules have free access to the bound Mn^{2+}, whereas in the closed state solvent molecules have only limited access.

Brewer et al. (1983c) have put forth a comprehensive model for association of Mn^{2+} and sugar and interconversions of locked and unlocked forms of different metal–Con A–sugar complexes. We agree with most of their concepts, but we have questions about a few. Our results agree with those of Brewer and co-workers (Brown et al., 1977; Brewer et al., 1983b) in that Mn^{2+} can bind to S1 and S2 sites under proper conditions. According to their concept, the linearity of $1/T_1$ values for Con A samples incubated with less than 2.0 equiv of Mn^{2+} in the presence of 0.1 M α-MDM (curve D in Figure 3) would be due to binding of Mn^{2+} to S1 and S2 sites simultaneously in the presence of α-MDM. We determined the association constant of Mn^{2+} for S1 and S2 sites in the absence of α-MDM and that for S2 in the presence of 0.1 M α-MDM at 25 °C (Figures 1 and 2). The affinity of Mn^{2+} for S1 and S2 sites is increased in the presence of 0.1 M α-MDM. However, we do observe a difference in association constants of Mn^{2+} to S1 and S2 sites in the presence as well as in the absence of α-MDM. The absolute value of K_a for S1 in the presence of α-MDM could not be determined because of the very low concentration of free Mn^{2+}, but it is definitely greater than that for S1 in the absence of α-MDM. Thus, the association constants are in the order K_a S1(α-MDM) > K_a S1 > K_a S2(α-MDM) > K_a S2, where K_a S1, K_a S2, K_a S1(α-MDM), and K_a S2(α-MDM) are the association constants of Mn^{2+} to S1 and S2 sites in the absence and presence of α-MDM. On the basis of Brewer's model, the absence of proton relaxation rate enhancement for Con A samples incubated with less than 1 equiv of Mn^{2+} in the presence of 0.1 M α-MDM at 5 °C (curve C, Figure 3) would be due to the conversion of apo-Con A in the unlocked form to the locked form in the presence of 0.1 M α-MDM and binding of Mn^{2+} to both S1 and S2 sites of Con A molecules that are in the locked form. In their scheme (Brewer et al., 1983c), it is given that apo-Con A exists in equilibrium between locked and unlocked forms and that sugar binds to all the forms irrespective of whether it is metalized or demetalized, locked or unlocked. Also, they suggest that α-MDM shifts the equilibrium between unlocked and locked apoprotein (P and PL, respectively) to the PL side.

We have never detected sugar binding in MUM fluorescence assays to demetalized Con A in which the metal ion contamination was strictly controlled. Although we routinely obtain apo-Con A with less than a 5% stoichiometric contamination of Ca^{2+}, Brewer et al. (1983b) have carried out studies with as much as 19% contamination. This high level of contamination could account for their reported saccharide binding activity of apo-Con A; Brewer and co-workers (Brewer et al., 1983a; Koenig et al., 1983), however, did not report the levels of Ca^{2+} contamination. Our model does not require that sugar bind to apo-Con A, but our model does require that sugar bind to Mn–Con A. Christie and co-workers have previously demonstrated this at 5 °C (Christie et al., 1979), and in this study also we have shown that this occurs. The binding constant analysis is consistent with sugar binding to Mn–Con A. The sugar-induced folding process at Mn/Con A ratios less than or equal to 1.0 (curves C and D in Figure 3) is consistent with this suggestion. In addition, kinetic studies show that folding of Mn–Con A is induced and accelerated by sugar.

On the basis of the displacement data (Table II), it appears that Mn^{2+} in Mn–Mn–Con A is much more labile than that in Mn–Ca–Con A, which suggests that there are two different conformations. α-MDM (0.1 M) when bound to Mn–Mn–Con A does prevent displacement of Mn^{2+}, suggesting that Mn–Mn–Con A with sugar is like Mn–Ca–Con A. Sr^{2+} behaves similarly to Ca^{2+} in causing rapid folding of Con A. Specific binding of various metal ions to S1 and S2 sites in Con A can be explained on the basis of their ionic radii. The ionic radii are in the order $Ni^{2+} \approx Zn^{2+} \leq Co^{2+} < Mn^{2+} < Cd^{2+} \approx Ca^{2+} < Sr^{2+}$. The transition-metal ions Ni^{2+}, Co^{2+}, and Zn^{2+}, at 5 and 25 °C, bind only to the S1 site and can displace Mn^{2+} from the S1 site, although they alone do not activate apo-Con A. Mn^{2+}, Cd^{2+}, or Ca^{2+} can bind to S1 and S2 sites and activate apo-Con A. Sr^{2+} binds only to the S2 site. Although Sr^{2+} can activate in combination with another metal occupying the S1 site, Sr^{2+} cannot by itself activate Con A. This implies that when Ca^{2+} alone activates Con A, it might be the Ca–Ca–Con A form that is active.

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Registry No. Con A, 11028-71-0; Mn, 7439-96-5; Ca, 7440-70-2; Sr, 7440-24-6; Ni, 7440-02-0; Co, 7440-48-4; Zn, 7440-66-6; Cd, 7440-43-9; methylumbelliferyl α-D-mannopyranoside, 28541-83-5; methyl α-D-mannopyranoside, 617-04-9.

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