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Activation of Saccharide Binding in Demetalized Concanavalin A by Transition Metal Ions†

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ABSTRACT: Saccharide-binding activity is induced when apoconcanavalin A binds only one Mn^{2+} ion near pH 6.5 at 5 or 23 °C. The affinity of Mn^{2+}–concanavalin A for 4-methylumbelliferyl α-D-mannopyranoside is about one-half that of Ca^{2+}–concanavalin A under similar conditions. Equilibrium dialysis studies with $^{54}Mn^{2+}$ show that a single Mn^{2+} binds per concanavalin A monomer in the presence and absence of saccharide. One Mn^{2+} is sufficient to activate sugar binding by each monomer. This result is similar to our earlier finding which showed that a single Ca^{2+} ion per protein monomer is most likely responsible for sugar-binding activation of Ca^{2+}–concanavalin A near physiological pH [Christie, D. J., Alter, G. M., & Magnuson, J. A. (1978) *Biochemistry 17*, 4425]. Water proton relaxation rates for Mn^{2+}–concanavalin A measured at 20.5 MHz, pH 6.4, and 23 °C decrease as the Mn^{2+} concentration is increased from 1 to 2 equiv of Mn^{2+} per monomer. This confirms similar observations made recently by others [Brown, R. D., III, Brewer, C. F., & Koenig, S. H. (1977) *Biochemistry 16*, 3883]. No decrease in relaxation rates occurs when concanavalin A is incubated with Mn^{2+} at 5 °C. At least two different conformational states exist, one near 5 °C and one near 23 °C. The form at 5 °C converts, upon incubation at 23 °C, to the higher temperature form. Both have full saccharide-binding activity, which only requires one bound Mn^{2+} per monomer. Mn^{2+} interaction with apoconcanavalin A is different than Ni^{2+}, Co^{2+}, and Zn^{2+} interaction. While binding with a stoichiometry of one metal ion per monomer, these other three transition metal ions do not induce saccharide-binding activity. Inactive Ni^{2+}–concanavalin A, however, is activated in the presence of Mn^{2+}, and no Ni^{2+} is displaced. This suggests that Mn^{2+} is not binding at exactly the same site as Ni^{2+}, Co^{2+}, and Zn^{2+}, but may be at a hybrid site which overlaps in some way with both the specific S1 transition metal ion site and the S2 Ca^{2+} ion site.

Concanavalin A[1] (Con A), the lectin isolated from jack bean (*Conavalia ensiformis*), has been the subject of major biochemical investigations for nearly a decade. Con A has been used in affinity purification of glycoproteins, glycopeptides, and polysaccharides, and a similar protocol has been used to separate viruses and bacteria (Bittiger & Schnebli, 1976). The mitogenic response elicited by Con A in cells such as lymphocytes (Wecksler et al., 1968; Powell & Leon, 1970; Beckert & Sharkey, 1970) is studied extensively. The lectin agglutinates or binds to many types of cells (Nicolson, 1974) and is widely used as a probe for studying membrane structure and function (Bittiger & Schnebli, 1976). Transformed cells are agglutinated more readily than normal cells (Inbar & Sachs, 1969a,b). The property of Con A which makes it useful as a probe is its ability to bind to carbohydrates which possess the D-arabinopyranoside ring structure (Goldstein et al., 1965, 1973).

The nature of the binding of divalent metal ions to Con A is of interest because the ions are necessary for saccharide-binding activity. It is generally accepted that near pH 5 each Con A subunit first binds a transition metal ion such as Mn^{2+} at a specific binding site S1 and then sequentially binds a Ca^{2+} ion at site S2 (Kalb & Levitzki, 1968; Shoham et al., 1973; Sherry et al., 1975). The sites have been identified in X-ray studies (Hardman & Ainsworth, 1976; Becker et al., 1975), and both the X-ray and NMR studies (Alter & Magnuson, 1974; Brewer et al., 1973a,b; Villafranca & Viola, 1974) show that the distance between a bound Mn^{2+} ion and the specific carbohydrate-binding site is ~10–14 Å. We recently demonstrated that near pH 7 Ca^{2+} ion binding to apo-Con A does not require the prior binding of a transition metal ion and that Ca^{2+} induces a cooperative effect on the binding of Mn^{2+} to Con A (Alter et al., 1977). Further studies have shown that

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[1] Abbreviations used: Con A, concanavalin A; apo-Con A, demetalized concanavalin A; Mn^{2+}–, Ni^{2+}–, Co^{2+}–, Zn^{2+}–, and Ca^{2+}–Con A, demetalized concanavalin A remetalized with the respective metal ion; MUM, 4-methylumbelliferyl α-D-mannopyranoside; Mops, 3-(*N*-morpholino)propanesulfonate; EDTA, ethylenediaminetetraacetate; NMR, nuclear magnetic resonance; ESR, electron spin resonance.

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Ca^{2+} also influences the rate of Mn^{2+} binding to Con A (Alter & Magnuson, 1979).

Near pH 5 the carbohydrate-binding activity of Con A is dependent on the specific binding of both a transition metal ion and a Ca^{2+} ion for each Con A subunit. Recently, we reported that this criterion for saccharide-binding activity in Con A is different at physiological pH (Christie et al., 1978). Near pH 7 Ca^{2+} ions alone will activate apo-Con A, and the affinity for a number of sugars was not affected by the presence of a transition metal ion. We demonstrated that Ca^{2+}–Con A has full saccharide-binding activity. Other studies on carbohydrate binding to Ca^{2+}–Con A have been reported (Harrington & Wilkins, 1978; Koenig et al., 1978). These workers have also examined the effect of Mn^{2+} ions alone on the activation of saccharide binding.

In the present work we have used equilibrium dialysis techniques to examine the affinities and stoichiometries of various transition metal ions to apo-Con A at pH 6.5. In conjunction with these experiments, the saccharide-binding activity of various transition metal ion–Con A complexes has been analyzed by using 4-methylumbelliferyl α-D-mannopyranoside (MUM). Our conclusions differ from those recently published (Brown et al., 1977; Koenig et al., 1978; Harrington & Wilkins, 1978). In the present paper we present evidence which strongly supports a different mechanism for Mn^{2+} activation of apo-Con A.

Materials and Methods

Chemicals. The 4-methylumbelliferyl α-D-mannopyranoside used in this work was purchased from Pierce. Jack beans, methyl α-D-mannopyranoside, and Mops were purchased from Sigma. We obtained $^{63}Ni^{2+}$, $^{57}Co^{2+}$, and $^{65}Zn^{2+}$ from New England Nuclear and $^{54}Mn^{2+}$ from ICN Pharmaceuticals. The transition metal ion salts $MnSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, and $CoSO_4 \cdot 7H_2O$ were obtained from Specpure Chemicals Limited, London; $NiSO_4 \cdot 6H_2O$ came from J. T. Baker Chemical Co. Chelex-100 was purchased from Bio-Rad Laboratories.

Con A Preparation. Con A was isolated by affinity chromatography from jack bean meal as previously described (Alter et al., 1977). Protein concentrations were determined spectrophotometrically at pH 6.4–7.0 by using an absorbance $A_{280nm}^{1\%,1cm} = 13.7$ (Yariv et al., 1968). All protein solutions were made up in NaCl or KCl which had twice been passed over an ion-exchange column of Chelex-100 to remove contaminating divalent metal ions. Unless specifically stated otherwise, solutions buffered between pH 6.4 and 7.0 contained 1.0 M NaCl and 0.05 M Mops. Protein samples used for NMR experiments were buffered at pH 6.4 in 0.9 M KCl and 0.05 M Mops or 0.1 M potassium acetate.

Preparation of apo-Con A and Various Metalized Forms of Con A. apo-Con A was prepared from freshly isolated protein by dialysis in a 0.1 N HCl bath as previously described (Christie et al., 1978). Atomic absorption was used to monitor the presence of Ca^{2+} contamination at the onset and conclusion of all experiments. Freshly prepared apo-Con A consistently contained less than 0.01 mol total of Ca^{2+} per mol of Con A subunits. Calcium contamination usually increased to 0.04–0.05 mol of Ca^{2+} per mol of 25 500-dalton subunits at the end of an experiment. Only data are reported where final Ca^{2+} contamination was less than 0.05 mol total of Ca^{2+} per mol of Con A subunits. Testing for the presence of other divalent metal ions in the apo-Con A samples was performed periodically for Mn^{2+} and Zn^{2+}, and less than 0.01 mol total of these metal ions was present per mol of Con A monomer. Previously, we found that Mn^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} together do not contribute significantly (<3%) to metal ion contamination of our apo-Con A samples (Christie et al., 1978).

Metalized forms of Con A used for MUM-binding experiments were prepared by preincubating apo-Con A (usually 1.0×10^{-4} M) at 5 °C (unless otherwise stated) with MUM and either Mn^{2+}, Ni^{2+}, Co^{2+}, or Zn^{2+} for at least 3 days prior to making the MUM-binding measurements.

Equilibrium Dialysis. Equilibrium dialysis experiments to measure transition metal ion binding to apo-Con A were performed by using dialysis membranes and Lucite cells which had been exhaustively boiled in 1 mM EDTA and washed in 1.0 N HCl followed by several rinses in distilled water to remove any exogenous divalent metal ions. Into one-half of a dialysis cell was placed 1.0 mL of 1.0×10^{-4} M apo-Con A buffered at pH 6.5. Into the other half of the cell was placed 1.0 mL of the same buffer solution containing the desired concentration of a particular transition metal ion. The concentration of metal ion in the buffer was varied so that the range of 0.2–0.8 mol of metal ion was bound per mol of Con A subunits. The cells were incubated for 5 days at 5 °C without agitation, and, except for some slight turbidity in protein samples containing ≤0.2 equiv of bound Mn^{2+} ion per Con A monomer, the solutions remained clear (agitation tended to cause turbidity in protein solutions containing less than 0.5 equiv of metal ion per Con A subunit). Control experiments showed that equilibrium was reached in ~5 h. Binding of $^{54}Mn^{2+}$, $^{57}Co^{2+}$, and $^{65}Zn^{2+}$ to apo-Con A was measured with a Beckman γ 4000 counting system. Levels of $^{63}Ni^{2+}$ were determined in Bray's solution (1960) with a Beckman LS-230 liquid scintillation system. Binding of Zn^{2+} was also measured by atomic absorption methods using a Perkin-Elmer 360 atomic absorption spectrophotometer.

To determine if saccharide binding influenced the nature of Mn^{2+} ion binding, we conducted equilibrium dialysis studies with Mn^{2+} and MUM. Either 1.60 or 2.00 mL of 1.0×10^{-4} apo-Con A was placed in a dialysis bag treated with EDTA as described above. Each dialysis bag of apo-Con A was dialyzed in a separate 5.0-mL polypropylene tube against 2.00 mL of a $^{54}Mn^{2+}$–buffer solution at pH 6.5 and 5 °C. Several experiments were performed by using a different concentration of $^{54}Mn^{2+}$ for each experiment so that a range of $(8–110) \times 10^{-5}$ M was covered. Within any given experiment, variable concentrations of MUM were added to different tubes so that the sugar-binding scheme covered a range of 0.2–0.8 mol of MUM bound per mol of active Con A subunits. The incubation period was 5 days.

All experiments were carried out in triplicate, and some were repeated with Con A prepared from independent batches of jack bean meal to insure against possible artifacts being introduced by any single protein preparation. Solutions used in these experiments were stored in polyethylene containers which did not contaminate solutions with calcium.

Fluorescence Measurements. Fluorescence measurements were performed on 2.00-mL protein samples which had been prepared as described above and then incubated with microliter aliquots of MUM. Con A concentrations were typically 1.0×10^{-4} M, and MUM concentrations ranged between 2×10^{-5} and 2×10^{-4} M to insure saturation of the total active subunit population. Measurements were carried out at 5 °C, although some were performed at 23 °C where noted. All 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 previously (Christie et al., 1978). Concentrations of MUM solutions in buffer were determined spectrophotometrically at 318 nm by

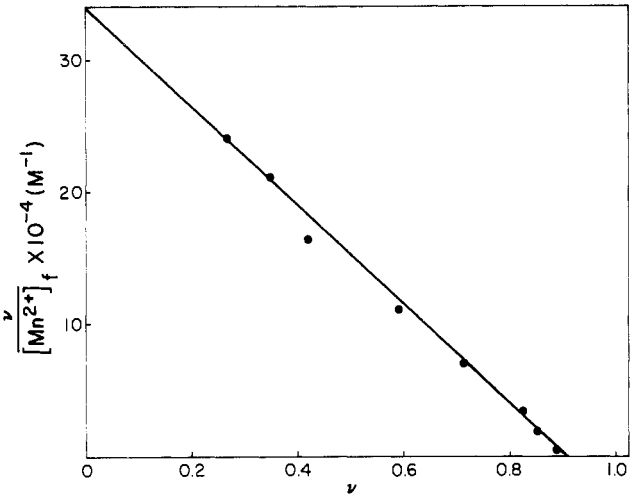

FIGURE 1: Scatchard plot of Mn^{2+} binding by 7.6×10^{-5} M apo-Con A at 5 °C, pH 6.5. The fraction of bound Mn^{2+} per total protein subunits is represented by ν, and $[Mn^{2+}]_f$ is the free Mn^{2+} concentration. The buffer was 1.0 M NaCl and 0.05 M Mops.

using $\epsilon_M = 1.36 \times 10^4$ M^{-1} cm^{-1} (Loontiens et al., 1977). Fluorescence spectra were measured by using a Perkin-Elmer MPF-3L fluorescence spectrophotometer equipped with a thermostated cuvette holder.

NMR Measurements. Relaxation rates of water protons were measured at 20.5 MHz for apo-Con A samples which had individually received an increasing increment of Mn^{2+}. Samples were buffered at pH 6.4 and incubated at either 5 or 23 °C. Several relaxation measurements were made over a period of 1–5 days. Samples incubated at 5 °C were warmed to 23 °C just before taking the relaxation measurements (~5 min). Within 24 h a slight precipitate of protein was visible in all tubes incubated at 23 °C (room temperature) except those containing more than 1 equiv of Mn^{2+}. No precipitate was observed in samples incubated at 5 °C. Different preparations of apo-Con A isolated from separate batches of jack bean meal were used in these experiments to avoid introduction of artifacts due to any single Con A preparation. Experiments were conducted with both 3×10^{-4} and 4×10^{-4} M Con A. All measurements were taken at 23 °C with a sample volume of 0.3 mL, using a Bruker SXP NMR spectrometer. Spin–lattice relaxation rates were determined by the inversion recovery method.

Results

When Mn^{2+} is added to apo-Con A, saccharide-binding activity is observed. No other divalent cations are required. The experiments described below were designed to determine the affinity and stoichiometry of Mn^{2+} binding to apo-Con A in the presence and absence of saccharide and to correlate the metal ion binding parameters with those observed for saccharide binding. Other transition metal ions were also evaluated for their ability to bind to apo-Con A and activate saccharide binding.

Mn^{2+} Binding to apo-Con A. Manganese binding to apo-Con A was studied by equilibrium dialysis using $^{54}Mn^{2+}$. Figure 1 is a Scatchard plot (1949) showing representative results of Mn^{2+} binding to apo-Con A at pH 6.5, 5 °C. The association constant K_a obtained from this analysis was $(4.0 \pm 1.0) \times 10^5$ M^{-1} with $n = 0.94 \pm 0.15$ Mn^{2+} bound per subunit of Con A, where n is the total number of ions binding per 25 500-dalton subunit of protein. Even with a 10-fold molar excess of Mn^{2+} we observed only one metal ion bound per subunit of protein. Binding data were obtained from 5 h to 5 days after dialysis was commenced with no significant differences being detected. When a 25-fold excess of methyl α-D-mannopyranoside was included, the binding data were not altered. Saccharide binding did not influence the affinity of Mn^{2+} for Con A. Under all conditions examined only a single Mn^{2+} ion was bound per Con A subunit at 5 °C (see Table I). Binding of Mn^{2+} to apo-Con A was also studied at 23 °C again by using equilibrium dialysis methods with $^{54}Mn^{2+}$. apo-Con A at 1.0×10^{-4} M was buffered at pH 6.4 in 0.9 M KCl and 0.1 M potassium acetate in order to repeat conditions used by Brown et al. (1977). Scatchard analysis (1949) was not feasible at this temperature since protein precipitation occurred for samples of apo-Con A dialyzed in the presence of less than 1 equiv of Mn^{2+} ion. Therefore, the experiment was performed with increasing equivalents of Mn^{2+} so that six data points in triplicate covered a range of 1.5–10.0 equiv of Mn^{2+} per Con A monomer. Dialysis cells were allowed to equilibrate for 72 h. With ratios of 1.5–5.0 for total moles of Mn^{2+} per total moles of Con A, we found a range of (0.93 ± 0.1)–(1.1 ± 0.04) Mn^{2+} bound per Con A monomer. Higher ratios of Mn^{2+} to Con A seemed to produce some nonspecific binding as evidenced by a stoichiometry of ~1.3 Mn^{2+} ions bound per subunit at a ratio of 10. These data clearly demonstrate that apo-Con A binds only one Mn^{2+} ion per subunit at 23 °C.

Table I: Transition Metal Ion Binding to Apoconcanavalin A[a]

metal ion	K_a (M^{-1})[b]	n[b]
Mn^{2+}[c]	$(3.5 \pm 1.0) \times 10^5$[d]	1.0 ± 0.19[d]
Ni^{2+}	$(6.9 \pm 0.2) \times 10^6$	1.06 ± 0.01
Co^{2+}	$(7.9 \pm 0.2) \times 10^6$	0.86 ± 0.01
Zn^{2+}[e]	$(4.0 \pm 2.4) \times 10^6$	0.85 ± 0.2

[a] All experiments were performed at pH 6.4–6.5 and 5 °C. [b] The association constants (K_a) and the number of metal ion binding sites occupied per 25 500 molecular weight subunit (n) were determined from the slope and abscissa intercept, respectively, by the method of Scatchard (1949). [c] The affinity and stoichiometry of Mn^{2+} binding to apo-Con A was determined in the presence and absence of methyl α-D-mannopyranoside. [d] Errors shown are standard deviations. [e] The Zn^{2+}-binding results are an average from both $^{65}Zn^{2+}$ measurements and atomic absorption techniques.

Effects of Temperature on Mn^{2+}–Con A. Figure 2 shows the results of an NMR experiment in which water proton relaxation rates were measured at 20.5 MHz for samples of apo-Con A in the presence of varying levels of Mn^{2+} ions. Samples were incubated at either 23 (triangles) or 5 °C (circles) and measured periodically between 1 and 5 days. The two curves show that there is a distinct difference in relaxation rates between the cases of Mn^{2+}–Con A initially incubated at these two temperatures. In the time course of this experiment only minimal changes in relaxation rates for individual samples were observed. Samples incubated at 5 °C for more than 5 days with 2.0 equiv of Mn^{2+} were observed to have relaxation times which very slowly decreased. Data were not corrected for contributions from free Mn^{2+} ion, as the relaxation rates for water protons in buffer with Mn^{2+} concentrations covering the range shown in Figure 2 were linear and insignificant compared with those for Mn^{2+} in the presence of apo-Con A. Subtracting out the free Mn^{2+} contribution did not noticeably alter the curves shown. Relaxation rates were measured for several increments of Mn^{2+} above 2 equiv. The relaxation rates increased almost linearly above 2.5 equiv of Mn^{2+} per Con A monomer and indicated the appearance of free Mn^{2+} upon saturation of the protein. This is seen as the upward trend in the relaxation rates at higher Mn^{2+} levels

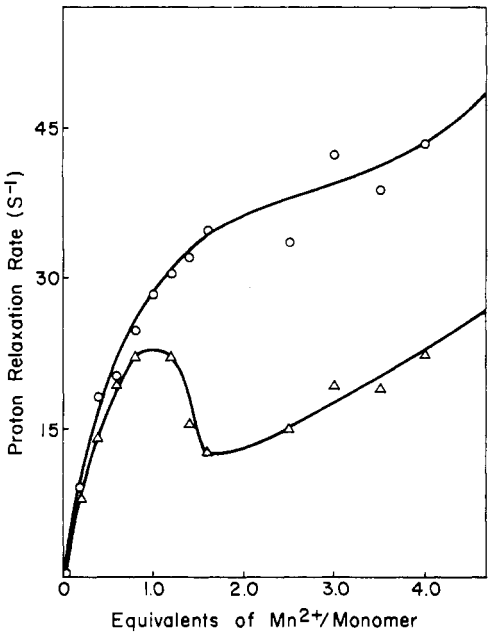

FIGURE 2: Water proton relaxation rates measured at 20.5 MHz and 23 °C for samples of 3.7×10^{-4} M apo-Con A monomers with different concentrations of Mn^{2+} ions and no Ca^{2+} ions. Protein samples were prepared in 0.9 M KCl and 0.05 M Mops, pH 6.4, 5 °C (○), and in 0.9 M KCl and 0.1 M potassium acetate, pH 6.4, 23 °C (△). Samples were incubated at 5 °C for 72 h and warmed to 23 °C just prior to making the NMR measurements. These same samples were then incubated for an additional 72 h at 23 °C and measured again. Similar results were obtained for an incubation period of 7 days. When samples were measured at different times between 1 and 7 days, the relaxation rates for individual samples at either 5 or 23 °C remained constant. Even samples incubated at 5 °C for 15 days showed no tendency toward a marked decrease in relaxation rates near 2.0 equiv of Mn^{2+}. Lines through the points shown indicate the general trend of the data.

in Figure 2. Our results obtained with Mn^{2+}–Con A initially incubated at 23 °C confirm the recently published observations of Brown et al. (1977) which showed a marked decrease in the relaxation rate near 2 equiv of Mn^{2+}. However, they did not carry out experiments with Mn^{2+} binding to apo-Con A incubated at 5 °C. From Figure 2 it can be seen that protein prepared at 5 °C exhibits no decrease in water proton relaxation rates near 2 equiv of Mn^{2+} per Con A subunit. This suggests the existence of two different temperature-dependent states of Mn^{2+} ion bound by apo-Con A, one near 23 °C and one near 5 °C. Our equilibrium dialysis binding studies with $^{54}Mn^{2+}$ have further shown that for each of these states only one Mn^{2+} ion is bound per Con A monomer.

To further verify that only one Mn^{2+} ion was bound per subunit in the NMR experiments, we measured the free Mn^{2+} concentration in some of the samples used for the data shown in Figure 2 by ESR spectroscopy (Alter et al., 1977). Differences between total and free Mn^{2+} always demonstrated that no more than one Mn^{2+} was bound per Con A monomer.

Binding of Other Transition Metal Ions to apo-Con A. Table I lists the results from equilibrium dialysis of transition metal ion binding to apo-Con A at pH 6.5, 5 °C. Data were evaluated by Scatchard analysis (1949). The affinities of the metal ions for apo-Con A increase in the following order: $Mn^{2+} \ll Zn^{2+} \leq Ni^{2+} \leq Co^{2+}$. These results are similar to previously published work in which metal ion binding to Con A was studied at pH 5.2, 4 °C (Shoham et al., 1973). Our results agree with those of Shoham et al. (1973) and show that Mn^{2+} binds with a significantly lower affinity to apo-Con A than the other transition metal ions studied. The absolute values of the affinities are 1 to 2 orders of magnitude greater

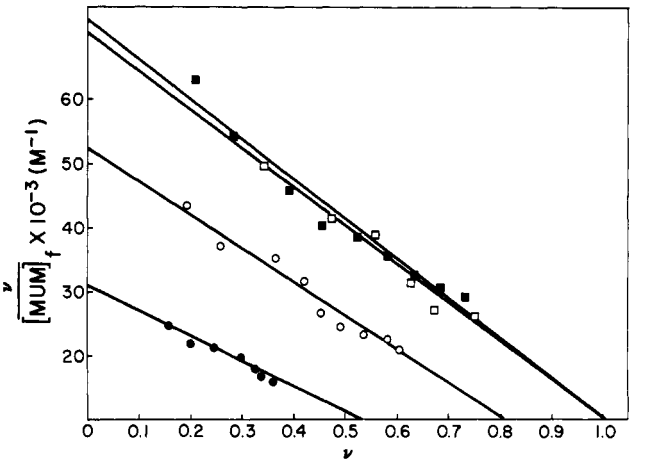

FIGURE 3: Scatchard plots of MUM binding by Mn^{2+}–Con A with different ratios of total Mn^{2+} ions per Con A monomer at 5 °C, pH 6.5, in 1.0 M NaCl and 0.05 M Mops. The fraction of bound MUM per total protein subunits is represented by ν, and $[MUM]_f$ is the free MUM concentration. Ratios of total Mn^{2+} ions per Con A monomer: 1.0 with [Con A] = 7.5×10^{-5} M; 2.0 with [Con A] = 1.3×10^{-4} M; 5.0 with [Con A] = 9.9×10^{-5} M; and 10.0 with [Con A] = 1.3×10^{-4} M.

Table II: Comparison of Bound Mn^{2+} with MUM-Binding Activity in Mn^{2+}–Concanavalin A[a]

$\frac{Mn}{Con\ A}$[b]	Mn_{bound}[c]	n[d]	$K_a \times 10^{-4}$ (M^{-1})[d]
0.8	0.54 ± 0.05[e]	0.50 ± 0.05[e]	2.9 ± 0.4[e]
1.0	0.66 ± 0.02	0.63 ± 0.10	3.6 ± 0.3
2.0	0.81 ± 0.01	0.80 ± 0.03	5.2 ± 0.1
3.0	0.91 ± 0.03	1.02 ± 0.02	5.3 ± 0.2
5.0	0.97 ± 0.01	0.99 ± 0.02	6.4 ± 0.3
11.0	1.14 ± 0.16	1.01 ± 0.01	5.0 ± 1.2

[a] All experiments were performed at pH 6.5 and 5 °C. [b] This is the ratio of total moles of Mn^{2+} to total moles of apo-Con A subunits. [c] Mn_{bound} is the number of moles of bound Mn^{2+} per mole of Con A subunits. [d] The association constants (K_a) and the number of sugar-binding sites occupied per 25 500 molecular weight subunit (n) were determined from the slope and abscissa intercept, respectively, by the method of Scatchard (1949). [e] Errors shown are standard deviations.

near pH 7 than near pH 5. Each transition metal ion shown has a stoichiometry of one bound metal ion per Con A subunit.

Saccharide-Binding Activity of Mn^{2+}–Con A. Saccharide-binding activity with Mn^{2+}–Con A was measured by following the quenching of MUM fluorescence which occurs when this monosaccharide is bound by Con A (Dean & Homer, 1973). The Scatchard analysis (1949) in Figure 3 is representative of results obtained for MUM binding to preparations of apo-Con A containing different levels of total Mn^{2+}. MUM-binding experiments were performed by incubating Mn^{2+}–Con A and MUM in polypropylene tubes. As the total number of equivalents of Mn^{2+} per Con A monomer was increased over a range of 1–10 equiv, the extent of MUM binding increased from about 50 to 100%. Activation allowing MUM binding to all the Con A subunits occurred when protein was incubated with ~3 equiv of Mn^{2+} ions per apo-Con A subunit.

To determine the extent of saccharide-binding activity induced by the binding of Mn^{2+}, we measured the binding of MUM for apo-Con A activated with given amounts of Mn^{2+}. Scatchard analysis (1949) for MUM binding in equilibrium dialysis experiments was used to measure the affinity and fraction of activated sites. The extent of Mn^{2+} binding was determined in the same experiments by measuring free and

bound $^{54}Mn^{2+}$. Table II summarizes these results. The data clearly show a direct one-to-one correlation between the moles of bound Mn^{2+} and the moles of bound MUM per Con A subunit (designated n in Table II). At very high ratios of total Mn^{2+} to Con A some nonspecific Mn^{2+} association may occur. Values slightly larger than 1.0 Mn^{2+} per Con A subunit could be measured, but this value never approached 2.0.

The K_a values in Table II for MUM binding have a range of (3–6) $\times 10^4$ M^{-1} with an overall average $K_a = (4.7 \pm 1.3) \times 10^4$ M^{-1}. This value for the K_a suggests that MUM binds less strongly to Mn^{2+}–Con A than to Ca^{2+}–Con A or Ca^{2+}–Mn^{2+}–Con A. In the case of the latter two types of Con A, we have shown that the K_a for MUM binding is 1×10^5 M^{-1} at 5 °C and pH 7.2 (Christie et al., 1978). Addition of methyl α-D-mannopyranoside to a solution of Mn^{2+}–Con A and MUM caused the reappearance of the quenched fluorescence. This is indicative of MUM interaction with the specific saccharide-binding site in Mn^{2+}–Con A and not simply nonspecific binding on the lectin. This observation is consistent with our previous findings which showed competition between MUM and methyl α-D-mannopyranoside for the specific saccharide-binding site in Ca^{2+}–Con A (Christie et al., 1978). Other active divalent metal ion complexes of Con A also show similar behavior (Dean & Homer, 1973; Loontiens et al., 1977). Our results demonstrate that MUM-binding activity induced when Mn^{2+} binds to apo-Con A has a requirement of only one bound Mn^{2+} ion per Con A subunit. These findings are similar to results we recently reported suggesting that a single Ca^{2+} ion per Con A subunit produced saccharide-binding activity near pH 7 (Christie et al., 1978).

The effect of methyl α-D-mannopyranoside binding on the water proton relaxation rate was examined at 20.5 MHz in Mn^{2+}–Con A. Two samples of 4.2×10^{-4} M apo-Con A were incubated with 5 equiv of Mn^{2+} each for 4 days at 5 °C. One sample was incubated with 10 equiv (a saturating level) of methyl α-D-mannopyranoside at 23 °C for 1 h. The resulting proton relaxation rates with and without sugar present were 55.0 and 59.8 s^{-1}, respectively. Correcting for dilution from addition of the saccharide, this difference represents only a 4% change in relaxation rates. It suggests that water exchange is insensitive to changes in protein conformation upon saccharide binding to Mn^{2+}–Con A.

Manganese Activation of Other Transition Metal Ion–Con A Complexes. Figure 4 compares the MUM-binding activity of Mn^{2+}–Con A with that of Ni^{2+}–Con A at pH 6.5 and 23 °C. Similar results were obtained at 5 °C. Lines in the figure are actual tracings of the MUM fluorescence patterns resulting from the various treatments indicated. The upper panel shows the rather slow process of saccharide-binding activation as seen by the gradual quenching curve which results when Mn^{2+} alone is added to a solution of apo-Con A and MUM. Upon addition of Ca^{2+} the rate of quenching is strongly accelerated and the binding goes essentially to completion in less than 30 min. In all of these experiments complete quenching represents ≥0.5 mol of bound MUM per mol of Con A monomer. When only Ca^{2+} was added to apo-Con A and MUM (not shown), the rate of quenching closely paralleled that seen with Mn^{2+} addition alone. The extent of quenching with Ca^{2+} was greater than with Mn^{2+}. This is in agreement with our observation that MUM has a greater affinity for Ca^{2+}–Con A than for Mn^{2+}–Con A. In both cases complete quenching of MUM in the presence of either Ca^{2+} or Mn^{2+} alone was not achieved for several hours (~72 h at 5 °C).

In the middle panel MUM was added to Ni^{2+}–Con A which was prepared by preincubating apo-Con A with Ni^{2+} for 48 h. The level at 100% fluorescence, A, is apo-Con A and MUM. No quenching of MUM fluorescence occurred even after more than 7 days. The level of fluorescence marked B is Ni^{2+}–Con A and MUM. Less than 2% quenching of MUM was observed even when the sugar was allowed to incubate for an additional period of 10 days. This slight quenching was most likely due to both a low level of Ca^{2+} contamination and the probability that in extremely high concentrations of Ni^{2+} it is possible to force the equilibrium of apo-Con A and Ni^{2+} toward active Con A. We used a 10- or 20-fold molar excess of Ni^{2+} compared to the apo-Con A subunit concentration in all of these experiments. The results show that Ni^{2+}, unlike Mn^{2+}, does not activate apo-Con A for MUM binding. Only after the addition of Ca^{2+} does fluorescence quenching of MUM result. Similar results were observed if the experiment was carried out with Co^{2+}– or Zn^{2+}–Con A. Even when a 10-fold molar excess of Co^{2+} was incubated with apo-Con A at pH 6.5 and 23 or 5 °C for more than 30 days, no quenching

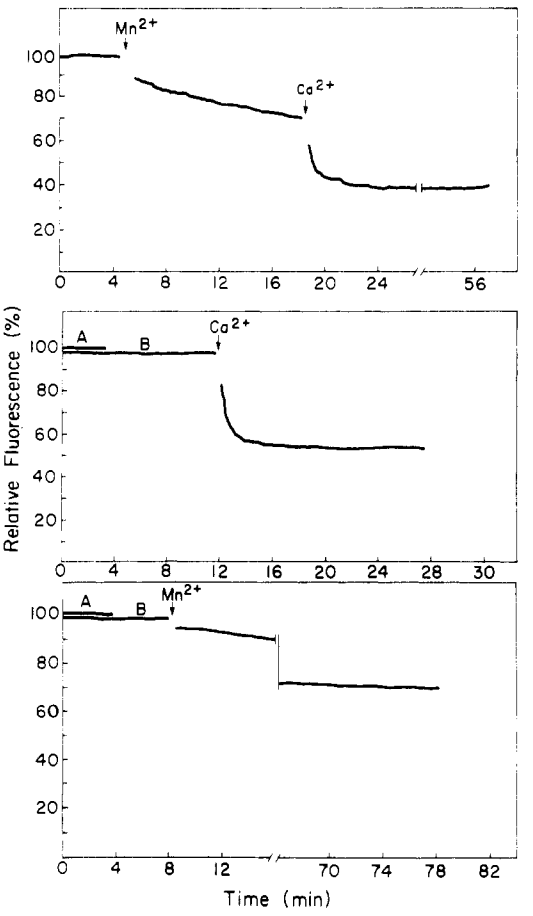

FIGURE 4: Fluorescence quenching of MUM by apo-Con A (top panel) and Ni^{2+}–Con A (middle and bottom panels) upon addition of Mn^{2+} and Ca^{2+} at the times indicated by the arrows. All Con A solutions were buffered in 1.0 M NaCl and 0.05 M Mops at pH 6.5 and 23 °C. Top panel: Mn^{2+} was added to a final concentration of 7.2×10^{-4} M. apo-Con A was 8.0×10^{-5} M, and MUM was 1.1×10^{-4}. The 100% fluorescence level in all panels represents no MUM binding with no fluorescence quenching. A slow process leading to activation of sugar binding occurs which is rapidly accelerated by the addition of Ca^{2+} to a final concentration of 7.1×10^{-4} M. Middle panel: Ni^{2+}–Con A was prepared by incubating 8.0×10^{-5} M apo-Con A in Ni^{2+} to a final concentration of 8.0×10^{-4} M for 3 days at 23 °C. MUM was then added to a final concentration of 9.1×10^{-5} M. (A) apo-Con; (B) Ni^{2+}–Con A. Essentially no quenching is observed until Ca^{2+} is added to a final concentration of 1.3×10^{-3} M. Bottom panel: (A) apo-Con A; (B) Ni^{2+}–Con A (prepared as described above). Activation of sugar binding occurs very slowly upon addition of Mn^{2+} to a final concentration of 1.6×10^{-3} M. Essentially no quenching occurs with Ni^{2+}–Con A alone.

of MUM fluorescence was observed until Ca^{2+} had been added. In every experiment inactive Con A–transition metal ion complexes were fully activated upon addition of Ca^{2+}. These experiments show that Mn^{2+} is not like Co^{2+}, Zn^{2+}, and Ni^{2+}. Only Mn^{2+} activates apo-Con A for MUM binding even though the others bind with similar stoichiometry and with higher relative affinity.

In the bottom panel of Figure 4 Mn^{2+} was added to a solution of Ni^{2+}–Con A and MUM. Note that quenching occurs, but at a greatly reduced rate relative to that observed when Ca^{2+} was added (middle panel). Even after the 78 min shown quenching was not yet complete. The time course for activation was not followed to completion, but as in the case of Mn^{2+} activation of apo-Con A (top panel) it was apparent that several hours were required. In this process of MUM-binding activation, it was unlikely that Mn^{2+} displaced Ni^{2+} from Con A since the K_a for Ni^{2+} binding is at least 20 times greater than the K_a for Mn^{2+} binding. Even so, to test this possibility we dialyzed 1.0×10^{-4} M $^{63}Ni^{2+}$–Con A at 5 °C, pH 6.5, against Mn^{2+} so that the total Mn^{2+} concentration exceeded the total Ni^{2+} concentration by a factor of 10. By measuring the free and bound $^{63}Ni^{2+}$, we found that one Ni^{2+} was bound per Con A subunit. This shows that under conditions where Mn^{2+} activates Ni^{2+}–Con A for MUM binding, Mn^{2+} was not displacing Ni^{2+}. We have also observed similar results for Mn^{2+} activation of Co^{2+}– and Zn^{2+}–Con A.

Discussion

Mn^{2+}-Binding and Saccharide-Binding Activation. The stoichiometry of Mn^{2+} binding to apo-Con A at pH 6.5 was found to be one Mn^{2+} ion bound per lectin subunit at 5 and 23 °C. These results are in contrast to the interpretation of Mn^{2+} binding to apo-Con A studied indirectly with NMR techniques by Brown et al. (1977), who proposed that the observed water proton relaxation rates near 2 equiv of Mn^{2+} ions (see Figure 2) are indicative of two bound Mn^{2+} ions per Con A subunit. Our direct metal ion binding studies show only one bound Mn^{2+} ion per subunit. The observed relaxation rate phenomena cannot be the result of two Mn^{2+} ions binding close together in a single Con A monomer as previously proposed (Brown et al., 1977). If a second Mn^{2+} ion were bound by Con A in the presence of 2 equiv of total Mn^{2+} per subunit, it should be detectable by the direct-binding techniques described in the present paper. A value for the K_a of a second bound Mn^{2+} ion per monomer was estimated from the previously published results of Brown et al. (1977). For 90% of the Con A subunits to have two bound Mn^{2+} ions per monomer in the presence of 2 equiv of total Mn^{2+} ions at pH 6.4, the association constant for the second Mn^{2+} ion would have to be greater than 2×10^5 M^{-1}. We have found a K_a of 4.0×10^5 M^{-1} (see Table I) for one bound Mn^{2+} ion per subunit. A second Mn^{2+} ion binding with an affinity of 2×10^5 M^{-1} or greater would easily have been detected in our equilibrium dialysis experiments. We observed no evidence for a second bound Mn^{2+}.

The metal ion binding studies presented in this paper strongly argue against four Mn^{2+} ions being bound per dimer or eight per tetramer. Our experiments have not ruled out the possibility that two Mn^{2+} ions are bound to a single subunit of a Con A dimer, leaving the other monomer empty, although our Scatchard analysis strongly indicates the existence of only one type of site. The simplest explanation for these observations in conjunction with the MUM-binding studies shown in Table II is that the binding of one Mn^{2+} ion per Con A monomer is sufficient to fully activate that subunit. We have also recently shown that an apo-Con A subunit could be activated for MUM binding by one Ca^{2+} ion in the absence of transition metal ions at pH 7.2 and 5 °C (Christie et al., 1978). In light of these results, we suggest that, while both Ca^{2+} and Mn^{2+} activation processes can be independent of each other, they proceed by similar, though not necessarily identical, mechanisms. The Ca^{2+} ion binding site and Mn^{2+} ion binding site (in the absence of Ca^{2+}) may have common ligands which when coordinated to Ca^{2+} or Mn^{2+} induce saccharide-binding activity. Under experimental conditions used in this present study, the other transition metal ions, Ni^{2+}, Co^{2+}, and Zn^{2+}, cannot coordinate with the same ligands and induce activation. Other studies have recently appeared attempting to elucidate the role of divalent metal ions in the activation of saccharide binding with Con A (Koenig et al., 1978; Harrington & Wilkins, 1978; Sherry et al., 1978; Cardin & Behnke, 1978). Harrington & Wilkins (1978) conducted a kinetic study of Mn^{2+} and Ca^{2+} interaction with apo-Con A by following the fluorescence quenching of MUM. Their results demonstrated that Mn^{2+}–Con A possesses MUM-binding activity. However, because they used micromolar protein concentrations in the presence of millimolar Mn^{2+} concentrations, their experiments were not designed to determine the minimum requirement for the number of bound Mn^{2+} ions in sugar-binding activation of one Con A monomer. They concluded indirectly that their Ca^{2+}–Con A and Mn^{2+}–Con A species near pH 7.0 probably contain two bound Ca^{2+} and two bound Mn^{2+} ions per subunit, respectively. In view of the fact that extremely high levels of metal ions were used in their experiments, this possibility exists. It is clear from the work presented herein and our previous work with Ca^{2+}–Con A (Christie et al., 1978) that the binding of a second Mn^{2+} or a second Ca^{2+} per subunit is completely incidental to saccharide-binding activity. One metal ion per subunit is sufficient for activation. Interpretations proposed by Koenig et al. (1978) are unlikely because they are largely based on a mechanism for the induction of saccharide-binding activity which involves two bound metal ions per subunit, an assumption which was developed in their previous Mn^{2+}–Con A study (Brown et al., 1977).

Two States of Mn^{2+}–Con A. Samples from our water proton relaxation studies containing between 1 and 2 equiv of Mn^{2+} ions were tested for MUM-binding activity. We found similar binding activity for Con A samples incubated at 23 or 5 °C. The affinity of Mn^{2+}–Con A for MUM was lower at 23 °C than at 5 °C, as expected (Loontiens et al., 1977), but the percentage of total active subunits relative to total Mn^{2+} present correlated very well with the results shown in Table II. From these observations and those obtained from our water proton relaxation results (Figure 2), we suggest that at least two active states of Mn^{2+}–Con A exist which bind MUM. The form detected at 5 °C does slowly change into the form detected at 23 °C, but this form does not change back into that detected at 5 °C when the sample is equilibrated at the lower temperature. Because the process appears to be irreversible, it most certainly involves a conformational change and not the process of dimer–tetramer association known to occur for Con A (Huet & Claverie, 1978). The interconversion of the two detectable conformations is currently under study in our laboratory. Whether these two forms correspond to locked and unlocked Con A as originally proposed by Brown et al. (1977) is open to speculation. If the form at 23 °C corresponds to locked Con A and that at 5 °C to unlocked Con A, then it is unlikely that the locking mechanism is directly involved in changing inactive Con A to an active sugar-binding conformation. One other point about the low temperature or 5 °C conformation should be noted. Sugar binding to the

lower temperature conformation induces no detectable change at the Mn^{2+}-binding site. Water proton relaxation rate parameters are not altered by methyl α-D-mannopyranoside binding, and the affinity of Mn^{2+} for Con A is the same, within experimental limits, in the absence or presence of sugar.

Mn^{2+} Activation of Inactive Ni^{2+}-, Co^{2+}-, and Zn^{2+}-Con A. We have shown by the direct methods of this study that Mn^{2+} in the absence of Ca^{2+} will activate saccharide binding in apo-Con A at pH 6.5 (Figure 3 and Table II). The transition metal ions Co^{2+}, Ni^{2+}, and Zn^{2+}, under similar conditions, do not activate apo-Con A. However, when Mn^{2+} was added to these inactive Con A–transition metal ion complexes, MUM-binding activity was observed. In the case of Mn^{2+} ion activation of Ni^{2+}–Con A, Ni^{2+} ion was not displaced by the added Mn^{2+} ion. These results implicate a role for Mn^{2+} ion interaction with Con A that is slightly different from that of the other transition metal ions studied. We suggest from these results that activation of Con A by Mn^{2+} involves interaction of Mn^{2+} with a site different than the specific S1 transition metal ion site. The nature of this site and the binding of Mn^{2+} ion to inactive transition metal ion complexes of Con A are currently under investigation in our laboratory. Although the process by which transition metal and Ca^{2+} ions activate Con A is not yet completely understood, it is clear that a simple model in which transition metal ions have one function and Ca^{2+} ions have another is not a satisfactory description.

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