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Metal ion binding to concanavalin A

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Abstract. Metal ion activation of saccharide binding has been studied for concanavalin A near pH 7.0. Although two metal ions, a transition metal ion and a Ca^{2+} ion, can bind, both are not required. Ca^{2+} alone, Mn^{2+} alone, or Ca^{2+} with other transition metal ions can activate this lectin. Only one Ca^{2+} ion per subunit or only one Mn^{2+} per subunit is sufficient. Metal ion binding was studied by magnetic resonance techniques and direct binding assays. Saccharide binding activity was monitored by following the fluorescence of 4-methylumbelliferyl α-D-mannopyranoside. When Ca^{2+} binds to demetalized concanavalin A, the transition metal ion site is hindered. When Mn^{2+} alone binds to demetalized concanavalin A, saccharide binding activity is induced. A subsequent conformational change, not necessary for carbohydrate binding activity, covers the Mn^{2+}.

Keywords. Concanavalin A; metal ions; lectin; saccharide binding.

Introduction

Concanavalin A, the most extensively studied of all lectins, possesses two metal ion binding sites per 35,500-dalton subunit. Although the metal ions are required for activity, the ions do not coordinate with atoms of the sugars which are bound by concanavalin A (Con A) (Brewer *et al.,* 1973a, b; Alter and Magnuson, 1974; Villafranca and Viola; 1974; Becker *et al.,* 1976; Hardman and Ainsworth, 1976). Each subunit has one sugar binding site and one binding site for a transition metal ion designated *S*1*,* ion and one site for a Ca^{2+} ion, designated *S*2 (Shoham *et al.,* 1973). A variety of transition metal ions bind to *S*1 but requirements at *S*2 are more rigid with only a limited number of ions such as Cd^{2+} being able to replace Ca^{2+}.

In our initial work we were interested in repeating studies on the metal ion requirements for sugar binding activity. In control experiments near pH 7.0 where Con A is tetrameric, we noticed that when Ca^{2+} alone was added to demetallized Con A, glycogen agglutination would occur. From this point we were forced to question the standard idea that first a transition metal ion binds to Con A with subsequent formation of the *S*2 site. This results in formation or activation of the sugar binding site. This hypothesis is based primarily on work done near pH 5.0 where Con A is dimeric.

Abbreviations used: Con A, Concanavalin A; MUM, 4-methylumbelliferyl α-D-mannopyranoside Mops, morpholinopropanesulphonate.

Dean and Homer (1973) reported that the fluorescence of 4-methylumbelliferyl α-D-mannopyranoside (MUM) was completely quenched upon binding to Con A. This provided a very sensitive assay for studying the activity of Con A containing a variety of metal ions. The fluorescence properties of this compound have also been used extensively to study kinetics of saccharide binding to Con A (Clegg *et al*., 1981). In the work reported below we have utilized the quenching of the umbelliferyl derivative as a tool for measuring how many and what kind of metal ions are required for conferring sugar binding activity on demetalized Con A.

Materials and methods

Concanavalin A

Concanavalin A was isolated from jack bean meal by affinity chromatography as described by Agrawal and Goldstein (1967). Protein concentrations were determined spectrophotometrically at pH 6.4 to 7.0 by using an absorbance $A^{1\%cm}_{280nm} = 13.7$ (Yariv *et al.*, 1968). Demetalized Con A (or apoCon A) was prepared by dialysis against 0.1 N HCl. Metal ion contamination was monitored using atomic absorption spectroscopy. Removal of transition metal ions was relatively simple, but plastic glassware (usually polypropylene) was used to keep Ca^{2+} ion contamination to a minimum. Ca^{2+} contamination was always checked before and after experiments and only experiments where Ca^{2+} contamination was less than 5% on a molar basis with Con A subunits are reported.

Fluorescence measurements

Fluorescence measurements were carried out on 2ml samples containing protein, MUM, and the appropriate metal ion or ions. The cuvette holder of the Perkin-Elmer MPF-3L fluorescence spectrophotometer was thermostated at the appropriate temperature. Fluorescence was monitored with an excitation wavelength of 350 nm and an emission wavelength of 375 nm. Protein concentrations were typically 0.1 mM in Con A subunits and MUM concentrations ranged between 0.02 and 0.2 mM.

Equilibrium dialysis

Three different methods were used for these experiments. Polycarbonate cells with approximately 2-ml volume on each side of the dialysis membrane were filled with a total of 1.0 ml of the appropriate Con A solution on one side and 1.0 ml of buffer on the other. Metal ions were added to appropriate concentrations. The whole cell was agitated slowly at the desired temperature. Other dialysis experiments were carried out in polypropylene test tubes. Con A solutions in small dialysis tubing were placed into the test tubes and the tubing was covered with appropriate buffer. Test tubes were agitated slowly at the desired temperature. In some cases, dialysis bags with Con A were placed in Erlenmeyer flasks and the dialysate was changed many times with buffer containing metal ion of the desired free concentration. In these experiments, free metal ion concentrations were determined by the choice of buffer. For example, dialysis against repeated changes of buffer containing 10 μM Mn^{2+} gave a free Mn^{2+} concentration of 10 μM. In other cases metal ion concentrations were monitored with radioactive nuclides or atomic absorption spectrophotometry.

Magnetic resonance experiments

Electron spin resonance spectra of Mn^{2+} were obtained on a Varian E-3 or E-9 ESR spectrometer. In following Mn^{2+} binding to Con A, the low field extremum of the Mn^{2+} signal was observed as a function of time.

Nuclear magnetic resonance experiments were carried out on a Bruker WH-90/ SXP NMR spectrometer, or Varian A-60 NMR spectrometer. Spin-lattice relaxation times T_1 of water protons were determined at 20.5 MHz. T_2 values were determined from water proton line widths.

Results

In early experiments in our laboratory using demetalized Con A, we tested for activity by glycogen precipitation. Total activity was determined either by measureing the amount of protein in the Con A-glycogen precipitate or by observing scat tered light in a spectrophotometer. As the Con A-glycogen precipitate increased, the light scattered would increase. In these experiments metal ion requirements were tested by adding metal ions to demetalized Con A in solution with glycogen. Our experiments showed that a precipitate not only formed when a transition metal ion and Ca^{2+} were added, but that glycogen precipitates formed near pH 7.0 when Ca^{2+} was the only divalent metal ion present. The experiments presented below were designed to re-examine the requirements for transition metal ions and Ca^{2+} ions.

The x-ray studies identifying the metal sites (Hardman, 1973; Becker *et al.,* 1975) suggests that if Ca^{2+} alone activates Con A, Ca^{2+} should inhibit transition metal ion binding. The time course of Mn^{2+} binding to demetallized Con A in the presence of various amounts of Ca^{2+} was examined by following the amplitude of the Mn^{2+} ESR signal. The signal of free Mn^{2+} in solution is approximately 15 times that of an equivalent amount of Mn^{2+} bound to Con A. The amplitude of the ESR signal observed is a combination of both bound and free Mn^{2+}. The bound Mn^{2+} contributes so little to the intensity at a given point that the intensity measured is a good measure of the free Mn^{2+}. If Mn^{2+} is added to demetallized Con A, the resonance of free Mn^{2+} decreases with time as Mn^{2+} binds to the protein. As shown in figure 1, the amount of free Mn^{2+} rapidly decreases to a relatively low concentration when Mn^{2+} is added to demetallized Con A. When Ca^{2+} is present before Mn^{2+} is added, the rate of disappearance of Mn^{2+} is markedly reduced.

When Mn^{2+} binds to demetalized Con A, the relaxation rate of the solvent water protons are greatly enhanced. This can be observed in proton magnetic resonance experiments examining either T_1, the spin-lattice, or T_2, the spin-spin relaxation time. Early studies by Carver and Barber (1973) and Brown *et al*. (1977), among others, used water relaxation to study metal ion complexation to Con A. We found (figure 2) that the water proton T_2 relaxation enhancement $\varepsilon^{\star}$ (Dwek, 1973) observed in the presence of Mn^{2+} bound to demetallized Con A decreased slowly with time and more slowly than the disappearance of Mn^{2+} (figure 1). The enhancement value decreased slowly over a period of 1 to 2 h when no Ca^{2+} was present. In the presence of Ca^{2+} (figure 2), values decreased in a time course almost identical to the disappearance of Mn^{2+} in the presence of Ca^{2+} (figure 1). If

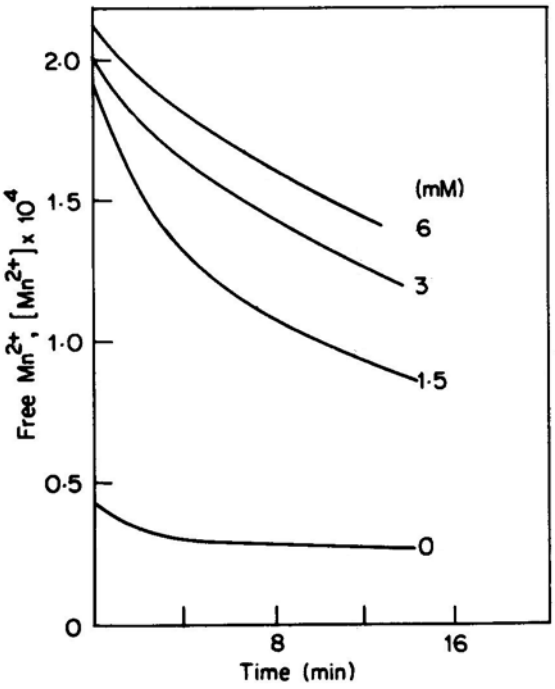

Figure 1. Amplitude of the lowfield extremum of the Mn^{2+} ESR signal as a function of time. At time zero 0.34 mM, Con A monomers were incubated with 0.32 mM, Mn^{2+} and the amount of Ca^{2+} given on the figure. The buffer was 0.05 M Mops, pH 6.5,0.2 M NaCl; temperature was 18° C.

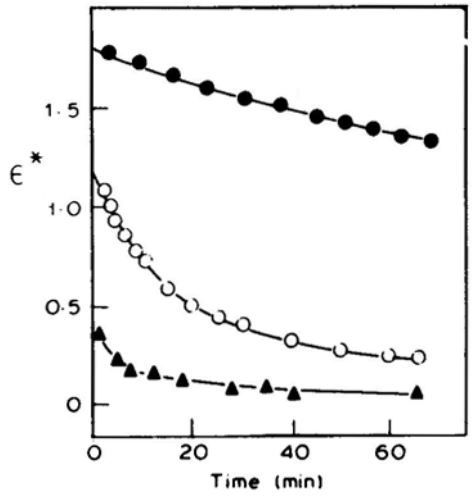

Figure 2. Enhancement for T_2 relaxation rates following addition of Mn^{2+} to Con A. Individual points were calculated from line widths at times given. The Con A subunits were 0.33 mM; Mn^{2+} was added to a total concentration of 0.26 mM. Mn^{2+} was added to demetallized Con A, but no Ca^{2+} present (●) Mn^{2}+ added to demetallized Con A, preincubated with 2.8 mM Ca^{2+} for 30 min, (O). Ca^{2+},2.8 mM, added to Con A incubated for 80 min with 0.26 mM Mn^{2+}, (▲) . Buffer was 0.05 Mops, pH 6.45, 0.2 M NaCl; temperature was 28.5°C.

Ca^{2+} were added to Con A containing only Mn^{2+}, a rapid decrease in , $\varepsilon^{\star}$ the relaxation rate enhancement, would occur. Ca^{2+} binding to Con A, therefore, produced two effects. One is detected in the ESR experiment and the other is detected in the water proton magnetic resonance experiment. Ca^{2+} inhibits the rate at which Mn^{2+} binds to Con A, but Ca^{2+} also will promote a conformational change which covers the bound Mn^{2+} ion so that interaction of water with the bound Mn^{2+} is blocked, at least as detected in the NMR experiment.

Water proton interaction was also studied by examining the time dependence of the spin lattice relaxation time T_1. Brown *et al.* (1977) had demonstrated an interesting concentration effect when Mn^{2+} was added to demetallized Con A. At 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. We repeated this experiment at 23°C with longer-times for the Mn^{2+} Con A interactions to reach equilibrium, but also noted that at 5° C with little delay after mixing metal ion and protein no marked reduction in relaxation enhancement occurred (figure 3), unless the T_1 measurement were made after very long incubation periods. The T_1

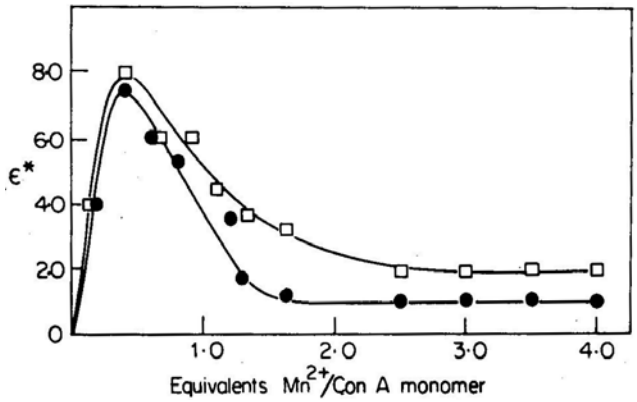

Figure 3. Enhancement for T_1 relaxation rates following addition of Mn^{2+} at various levels to demetallized Con A. The buffer was 0.05 M Mops, pH 6.5, 0.9 M KCl (the same results were obtained in acetate buffered at 6.5 to reproduce other reported values, Brown *et al.,* 1977). Sample prepared at 5°C, (□); sample prepared at 23°, (●). Note that the kinetic analysis in figure 4 is following conversion from (□) to (●) at a Mn^{2+}/Con A ratio of 3.0.

experiment is detecting two species. One is a Con A conformation with Mn^{2+} open to solvent water. This conformation changes to one where water is not as freely exchanging with waters of hydration on the Mn^{2+}. We were able to measure the changes in T_1 as a function of time which really monitored the change from the open to the closed conformation. Standard kinetic analyses (Daniels *et al.,* 1962) presented in figure 4 shows that the conversion is a simple first order process. The activation energy for this process which occurs even when Ca^{2+} is not present was 22.3 kcal mol^{-1}.

The above experiments show that Con A can bind Ca^{2+}, near pH 7.0, without first binding a transition metal ion. When a transition metal ion such as Mn^{2+} binds, there is a slow conformational change which covers the Mn^{2+} ion. Ca^{2+} bound to demetalized Con A inhibits the rate at which Mn^{2+} binds but, when pre-

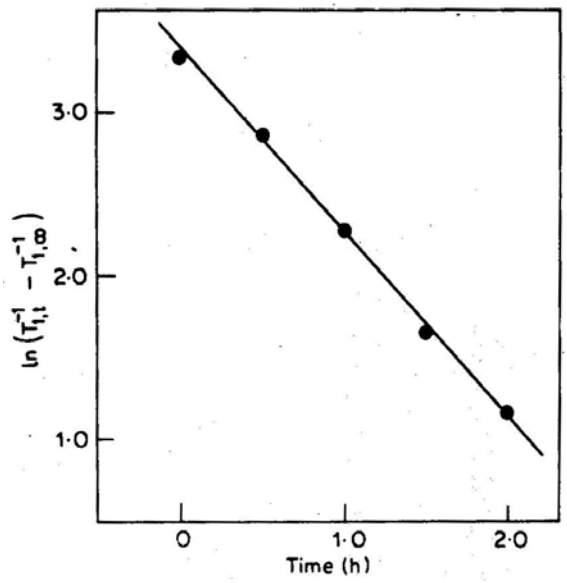

Figure 4. Kinetic analysis of conversion of unstable Con A to stable Con A. Experiment carried out by measuring T_1 relaxation times following Mn^{2+} addition. Mn^{2+} was added at 0-5°C and solution was warmed to 32°C. $T_{1,t}$ is the observed T_1 at various times (in h) and $T_{1,\infty}$ is the spin-lattice relaxation time at very long time periods when no further change with time is detected. The first-order rate constant is 0.96 h^{-1}.

sent, Ca^{2+} increases the rate of the conformational change covering the bound Mn^{2+}. The following experiments were designed to determine which metalized states have saccharide binding activity.

The fluorescence of 4-methylumbelliferyl α-D-mannopyranoside was used to monitor saccharide binding activity. Activity was generally determined through Scatchard analyses (Scatchard, 1949) in order to demonstrate the extent of active Con A in addition to the binding affinity. To demetalized Con A was added the appropriate amount of metal ion. Binding analyses using the quenching of the MUM fluorescence could then be carried out by titrating the Con A with MUM in the fluorescence cuvette. Results for Con A with only Ca^{2+} and for Con A with only Mn^{2+} are shown in figure 5. These data show that the amount of active Con A is approximately equal to the total amount of divalent ion supplied. For example, when the bound metal ion to Con A monomer ratio is 0.5 the amount of active Con A was about 50%. The slopes of the plots were the same for different concentrations of a given metal ion, although the abscissa intercept demonstrated that all the Con A was not activated. The similarity of the slopes showed that no intermediate binding states were produced as all active species had the same affinity for MUM. In these studies Zn^{2+}, Ni^{2+} and Co^{2+} alone could not activate Con A for saccharide binding, although Scatchard analyses of metal binding using radioactive nuclides showed that one, and only one, of these ions or Mn^{2+} bound per Con A monomer. In addition, Scatchard plots for binding of these four ions did not deviate from linearity, suggesting that only one type of binding site is present.

Thus far our results have shown that Ca^{2+} and a transition metal, Ca^{2+} alone, or Mn^{2+} alone could induce the saccharide binding activity. Distance measurements between Mn^{2+} and the bound saccharide were made on the two forms to obtain infor-

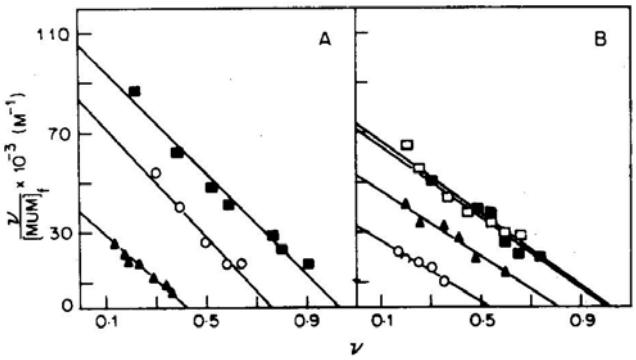

Figure 5. Scatchard analysis of MUM binding activity for various metal additions to demetallized Con A. Panel A: Ca^{2+} only added to demetallized Con A. The ratio of Ca^{2+} to Con A monomers is 0.58, (▲); 0.87 (O),< 1, (■). Experiment carried out at pH 7.2, 5°C. Panel B: Mn^{2+} only added to demetallized Con A at Mn^{2+} to Con A ratios of 1.0 (O); 2.0, (▲); 5.0, (□); 10, (■). The ratio of Mn^{2+} bound to Con A subunits at these values are 0.5, 0.8, 1.0, and 1.0, respectively (Christie *et al.,* 1979). Experiment carried out at pH 6.5, 5°C.

mation about the location of the Mn^{2+} ion. Distance measurements between Mn^{2+} and the fluorine on N-trifluoroacetyl-D-glucosamine were made in NMR experiments similar to those reported previously (Alter and Magnuson, 1974). Fluorine T_1 and T_2 values were measured at 84.7 and 254.0 MHz. T_1's were measured by inversion-recovery and T_2's were calculated from line widths. Con A containing both Mn^{2+} and Ca^{2+} and Con A with just Mn^{2+} were examined. The Mn^{2+}-F distances were found to be identical (Pandolfino *et al.,* 1980), suggesting that Mn^{2+} in each form is the same site.

The water proton relaxation experiments clearly demonstrated that when Mn^{2+} alone is bound to Con A, two conformations can exist. The initial one slowly converts to the more stable form. At 5° C, the conversion takes several days. Binding of MUM by both conformers was examined. Both species bound MUM with the same affinity (Christie *et al*., 1980). In addition, no time dependence for induction of saccharide binding activity was detected. These results have shown that Mn^{2+} binding at pH values near 7 is enough to activate the sugar binding site. The conformational change, subsequent to Mn^{2+} binding and studied so extensively by various workers, is not necessary for activity. With Ca^{2+} activated Con A in the absence of a transition metal ion, the Con A appears to have a similar conformation to Con A with two metal ions, as the affinities for saccharides are essentially identical.

Discussion

A model which accounts for metal ion binding data is presented in figure 6. Different states are represented by symbols and the metal ions within each symbol are used to indicate the binding to each subunit. All the circles represent monomers with saccharide binding activity. We suggest that it is necessary for the Ca^{2+} only species to lose Ca^{2+} before being able to bind a transition metal ion. The intermediate state could be the demetalized triangle or another unidentified conformation. Mn^{2+} alone must produce a different conformation, a circle, than other transition metal ions, a hexagon, but Ca^{2+} can convert the latter to the active species.

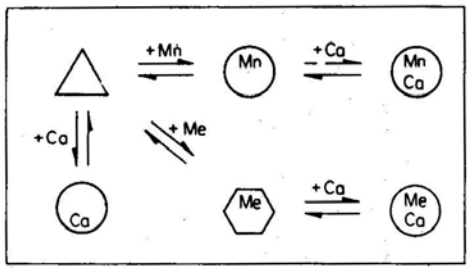

Figure 6. Model for metal binding to each subunit of Con A. Conformations when metalized have metal within symbol. Me represents transition metal ion not Mn^{2+}. Circles are active, saccharide binding conformations.

The two metal sites, *S*1 for the transition metal ion and *S*2 for the Ca^{2+} ion, do not both need to be occupied for activity near pH 7.0. One Mn^{2+} is sufficient with distance measurements showing that the ion is most likely in *S*1. We suggest that Ca^{2+} binds at *S*2 with or without the presence of a transition metal ion. We have no direct determination for this, but difference ultra-violet (UV) spectra for Con A with only Ca^{2+} versus Con A with Ca^{2+} and Mn^{2+} ion are essentially identical (Munske and Magnuson, unpublished observation). The saccharide binding affinity also appears to be identical for Con A with both Mn^{2+} and Ca^{2+} or with just Ca^{2+}.

In summary, demetalized Con A can bind either a transition metal or a Ca^{2+} ion. Activation of the sugar binding site probably occurs simultaneously with Mn^{2+} binding and a slow conformational change, unrelated to the sugar binding site, follows and covers the Mn^{2+} site: Only one Mn^{2+} binds per subunit (Christie *et al.,* 1979). When Ca^{2+} binds first, sugar binding activity is induced and the Ca^{2+}, as would be expected from crystallographic results, prevents Mn^{2+} from binding. The Ca^{2+} most probably must dissociate before Mn^{2+} can bind and the Ca^{2+} which is present then enhances the rate at which the Mn^{2+} is covered by other ligands. These steps, it should be noted, have led to much confusion in previous interpretations. The covering of Mn^{2+} by movement of a protein segment was suggested early by Barber and Carver (1973).

Early work on Con A assumed that Ca^{2+} could only bind after the transition metal ion had bound to *S*1 and induced the formation of the Ca^{2+} site *S*2. Kinetic studies of transition metal bindingin the presence of Ca^{2+} are confusing because Ca^{2+} markedly reduces binding rates. Indeed, care must be taken to prevent Ca^{2+} contamination.

In some cases MUM fluorescence has been used to measure the induction of saccharide binding activity. The technique is very sensitive and it is possible to measure easily the activity of a small percentage of the Con A present. For example, when Co^{2+} is added to demetallized Con A, a 5% contamination with Ca^{2+} would generate 5% active Con A. If Co^{2+} is added in large excess, the Co^{2+} solutions can contain relatively large amounts of Ca^{2+} which could activate a relatively large portion of the Con A. In all experiments it is necessary, we feel, to determine the relative amounts of active Con A.

The work presented here demonstrates that careful studies of metal ion requirements for Con A, and other lectins, must include two points. Metal ion contamina-

tion, especially by Ca^{2+}, must be checked, before and after experimentation, by using atomic absorption spectrophotometry or other suitable technique. Extent of activity must be checked. We prefer Scatchard analyses using the fluorescence of MUM but other quantitative methods may be necessary with other lectins.

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