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Saccharide Binding to Transition Metal Ion Free Concanavalin A†

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ABSTRACT: Saccharide binding has been observed with demetallized concanavalin A in the presence of Ca^{2+} only, using the fluorescent sugar 4-methylumbelliferyl α-D-mannopyranoside. At pH 7.2 both the nicked and intact forms of concanavalin A bound 4-methylumbelliferyl α-D-mannopyranoside with similar affinities. Competitive binding with methyl α-D-mannopyranoside was demonstrated. The association constants at 5 °C were $9.6 \pm 0.6 \times 10^4$ M^{-1} for 4-methylumbelliferyl α-D-mannopyranoside and $1.1 \pm 0.3 \times 10^4$ M^{-1} for methyl α-D-mannopyranoside. 4-Methylumbelliferyl α-D-mannopyranoside binding was also observed if demetallized concanavalin A was remetallized with less than stoichiometric amounts of Ca^{2+}. The association constants with low Ca^{2+} concentrations were similar to those determined with saturating Ca^{2+}. With less than stoichiometric levels of Ca^{2+}, the number of sugar molecules bound per protein subunit was a reflection of the fraction of activated lectin subunits. These results show that saccharide binding activity of concanavalin A does not require a transition metal ion at pH 7.2; only Ca^{2+} is required. At pH values near 5, where most previous studies have been carried out, both a transition metal ion and Ca^{2+} are necessary.

Concanavalin A[1] (Con A), a protein isolated from jack bean (*Canavalia ensiformis*) (Sumner & Howell, 1936), is the most extensively studied lectin. Properties associated with Con A include stimulation of a mitogenic response in lymphocytes (Wecksler et al., 1968; Powell & Leon, 1970; Beckert & Sharkey, 1970) and differential agglutination of malignant and normal cells (Inbar & Sachs, 1969a,b). The induction of these activities is primarily due to the ability of Con A to bind specific carbohydrate moieties on cell surfaces (Lis & Sharon, 1973). Carbohydrates having the D-arabinopyranoside configuration at C-3, C-4, and C-6 positions possess the minimum structural characteristics for binding to Con A (Goldstein et al., 1965, 1973).

Con A exists in two pH-dependent forms each composed of identical subunits. The subunit molecular weight is 25 500 (Wang et al., 1971; Edmundson et al., 1971). The form below pH 5.5 is predominantly dimeric and that found near physiological pH is mostly tetrameric (Kalb & Lustig, 1968; McKenzie et al., 1972). Each subunit possesses one specific carbohydrate binding site (Kalb & Levitzki, 1968; Becker et al., 1975). Con A also possesses a naturally occurring cleavage site (nicked form) in some of the protein molecules (Wang et al., 1971).

Considerable work has been undertaken to investigate the nature of saccharide binding and divalent metal ion interaction

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[1] Abbreviations used: Con A, concanavalin A; Con A_{int}, intact polypeptide chains of concanavalin A; Mops, morpholinopropanesulfonate; Con A_D, demetallized concanavalin A; Ca^{2+}-Con A (Ca^{2+}-Con A_{int}), demetallized concanavalin A remetallized with Ca^{2+} only; Ca^{2+}-Mn^{2+}-Con A (Ca^{2+}-Mn^{2+}-Con A_{int}), demetallized concanavalin A remetallized with Mn^{2+} and Ca^{2+}; MUM, 4-methylumbelliferyl α-D-manopyranoside; MUG, 4-methylumbelliferyl α-D-glucopyranoside; α-MM, methyl α-D-mannopyranoside; α-MG, methyl α-D-glucopyranoside; $[Ca^{2+}]/[Con\ A]$, total molar Ca^{2+} concentration to the total molar Con A (Con A_{int}) subunit concentration.

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with the Con A molecule. Three-dimensional structures of crystallized Con A have been determined by X-ray techniques (Hardman & Ainsworth, 1973, 1976; Hardman, 1973; Becker et al., 1971, 1975; Edelman et al., 1972). The complex of Con A and methyl α-D-mannopyranoside has been studied by Hardman & Ainsworth (1976) and Becker & co-workers (1975). It shows a close proximity between the transition metal ion binding site and the saccharide-binding site. In these studies the Mn^{2+}–carbohydrate distance was found to be 10–14 Å. This is in good agreement with NMR studies which indicate the Mn^{2+}–carbohydrate distance to be on the order of 10–12 Å (Alter & Magnuson, 1974; Brewer et al., 1973a,b; Villafranca & Viola, 1974).

Currently accepted views hold that, for carbohydrate-binding activity, Con A requires one bound transition metal ion and one bound Ca^{2+} for each lectin subunit. In support of this, metal binding studies with Con A have shown that a specific Ca^{2+}-binding site S2 is induced by the prior binding of a transition metal ion at site S1 (Kalb & Levitzki, 1968; Shoham et al., 1973; Sherry et al., 1975). In contrast, Brewer and co-workers (1974), who studied Mn^{2+} binding by electron spin resonance and carbohydrate binding by ^{13}C magnetic resonance, suggest saccharide binding to Con A is independent of Ca^{2+} as long as S1 is saturated by a transition metal ion. It is emphasized here that all determinations made by the above mentioned workers were made in the vicinity of pH 5. This is significantly below the physiological pH range necessary for studies on viable cells.

Recently we demonstrated that demetallized Con A free of all transition metal ions binds Ca^{2+} with reasonable affinity in the pH range of 6.5–7.0 (Alter et al., 1977). This observation showed that near the physiological pH range the binding of a transition metal ion is not essential for the formation of a Ca^{2+} binding site. The question is then raised as to whether Con A_D reconstituted with only Ca^{2+} (Ca^{2+}–Con A) is capable of binding saccharides near pH 7. We chose as our major saccharide for this binding investigation the fluorescently modified sugar, 4-methylumbelliferyl α-D-mannopyranoside (MUM). MUM is well suited for binding studies because its fluorescence is completely quenched upon association with Con A (Dean & Homer, 1973; Loontiens et al., 1977). In this paper we demonstrate that MUM and other monosaccharides do bind to Ca^{2+}–Con A at pH 7.2. The saccharide-binding activity of Con A appears to be independent of transition metal ions within the physiological pH range.

Materials and Methods

Chemicals. The 4-methylumbelliferyl α-D-mannopyranoside and 4-methylumbelliferyl α-D-glucopyranoside used in this study were purchased from Koch-Light Limited. Jack beans, methyl α-D-mannopyranoside, and methyl α-D-glucopyranoside were purchased from Sigma. Melting points indicated that the commercially obtained sugars were pure.

Con A Preparation. Con A was isolated from jack bean meal as previously described (Alter et al., 1977). The intact subunits of Con A (Con A_{int}) were prepared using 1% ammonium bicarbonate according to the method described by Cunningham & co-workers (1972). Concentrations of protein solutions were determined spectrophotometrically at pH 7.2 and 5.2 using an absorbance $A_{280nm}^{1\%,1cm} = 13.7$ and 12.4, respectively (Yariv et al., 1968).

Preparation of Con A_D, Ca^{2+}–Con A, Ca^{2+}–Mn^{2+}–Con A. Demetallized Con A was prepared by first dialyzing solutions of Con A or Con A_{int} against 0.1 N HCl (ca. pH 1) for 30–60 min at room temperature (Kalb & Levitzki, 1968) and then dialyzing against glass-distilled water at 4 °C until the pH was close to 6. Con A, buffer, and sugar solutions were tested for the presence of Ca^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Ni^{2+}, Co^{2+}, and Cu^{2+} by atomic absorption. Con A_D solutions were shown to contain less than 0.03 mol total of the transition metal ions and less than 0.04 mol total Ca^{2+} per mol of Con A subunits. None of the divalent metal ions being monitored was detectable in other solutions. To prepare Ca^{2+}–Con A, Con A_D solutions were incubated at 4 °C for 24–48 h in solutions containing a tenfold molar excess of calcium chloride with respect to the Con A monomer concentration. Ca^{2+}–Mn^{2+}–Con A was similarly prepared except that Con A_D solutions were preincubated with a tenfold molar excess of manganese chloride for 24 h prior to incubation with Ca^{2+}. The same procedure was followed in remetallizing Con A_{int} solutions.

Fluorescence Titrations. Binding of MUM and MUG to Con A in all solutions prepared as described above was monitored by fluorescence quenching. Linear standard curves were obtained for these sugars in buffered protein-free solutions. In these standard curves, MUM and MUG concentrations typically covered ranges of 5–50 and 14–100 μM, respectively. Titrations were performed in quartz cuvettes by additions of microliter aliquots of MUM or MUG to 2.00 mL of a Con A or Con A_{int} solution at pH 7.2 and 5 °C (±0.5). For a typical titration the protein concentration was 25 μM in terms of Con A monomers and the MUM or MUG concentrations were within the limits of the standard curves. Competitive binding assays with methyl α-D-mannopyranoside and methyl α-D-glucopyranoside were carried out in the following manner. Con A solutions were preincubated with a known amount of MUM and then titrated with aliquots of a solution of competing sugar. Concentrations of methyl α-D-mannopyranoside and methyl α-D-glucopyranoside ranged between 30–500 μM and 0.2–3 mM, respectively. Binding of the competing sugar was deduced by observing the increase in fluorescence resulting from the release of MUM from the Con A–MUM complex.

Con A solutions titrated at pH 7.2 were buffered in 1.0 M NaCl and 0.05 M Mops. Buffer at pH 5.2 contained 1.0 M NaCl and 0.05 M sodium acetate. Sugar solutions were similarly buffered and appropriate concentrations of divalent metal ions were added corresponding to the divalent metal ion concentrations of the particular Con A solution being titrated. In the titrations involving less than stoichiometric amounts of Ca^{2+} no metal ions were added to the sugar solutions. Concentrations of MUM and MUG solutions were determined spectrophotometrically at 318 nm using $\epsilon_M = 1.36 \times 10^4\ M^{-1}$ (Loontiens et al., 1977).

All fluorescence measurements were made using a Perkin-Elmer MPF-3L fluorescence spectrophotometer with a thermostated cuvette holder. The excitation wavelength was 350 nm and emission wavelength was 375; excitation and emission slit widths were both 5 nm. The excitation wavelength of 350 nm was necessary to overcome an inner filter effect observed when the excitation maximum of 320 nm was used. With the conditions reported here, linear standard curves for MUM and MUG throughout the titration ranges were obtained.

Results

Validity of Fluorescence Measurements. The fluorescence of MUM was completely quenched upon binding by Ca^{2+}–Con A as shown from an equilibrium dialysis experiment. At equilibrium the same level of fluorescence was observed on both sides of the dialysis cell. Since MUM fluorescence was completely quenched when the sugar was bound by Ca^{2+}–Con A, all binding parameters could be determined directly with the observed fluorescence being a direct measure of the free

TABLE I: Parameters for Sugar Binding to Ca^{2+}-Concanavalin A.[a]

type of Con A	sugar	K_a (M^{-1})[b]	n[b]
Ca^{2+}-Con A	MUM	9.3 ± 0.4[c] $\times 10^4$	1.02 ± 0.03[c]
Ca^{2+}-Con A_{int}	MUM	$9.6 \pm 0.8 \times 10^4$	1.07 ± 0.03
Ca^{2+}-Mn^{2+}-Con A	MUM	$9.3 \pm 0.1 \times 10^4$	1.10 ± 0.01
Ca^{2+}-Mn^{2+}-Con A_{int}	MUM	$9.3 \pm 0.3 \times 10^4$	1.09 ± 0.03
Ca^{2+}-Con A_{int}	MUG	$2.2 \pm 0.2 \times 10^4$	0.93 ± 0.07
Ca^{2+}-Con A[d]	α-MM[d]	$1.1 \pm 0.3 \times 10^4$	
Ca^{2+}-Con A_{int}	α-MG	$4.7 \pm 0.3 \times 10^3$	

[a] All experiments were carried out at pH 7.2 and 5 °C. [b] 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 the x intercept, respectively, by Scatchard analysis (Scatchard, 1949). [c] Errors shown are standard deviations. [d] Similar binding was observed for Ca^{2+}-Con A_{int}.

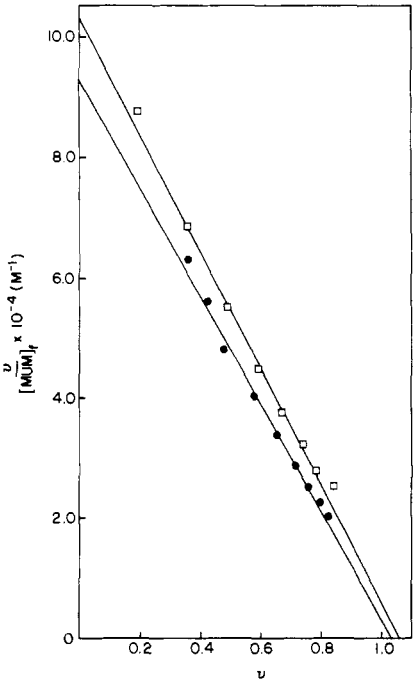

FIGURE 1: Scatchard plots of MUM binding by Ca^{2+}-Con A (●) and Ca^{2+}-Con A_{int} (□) at 5 °C, pH 7.2. The fraction of bound MUM per total protein subunits is represented by ν. $[MUM]_f$ is the free MUM concentration.

MUM concentration. Previous studies on the complete quenching of fluorescence of MUM bound by Con A have been conducted in the presence of transition metal ions and Ca^{2+} (Dean & Homer, 1973; Loontiens et al., 1977). To demonstrate that the Ca^{2+}-Con A was active during the dialysis experiment, an excess of methyl α-D-mannopyranoside was added to a solution of Ca^{2+}-Con A and MUM that had reached equilibrium. The fluorescence level increased by an amount corresponding to release of the bound MUM.

Saccharide Binding of Ca^{2+}-Con A and Ca^{2+}-Con A_{int}. Analysis of the MUM-binding data was performed according to the method of Scatchard (1949). Figure 1 shows plots of representative data for Ca^{2+}-Con A and Ca^{2+}-Con A_{int} titrated with MUM. The association constants K_a for the two Con A types were the same within experimental error. At pH 7.2 the K_a value for Ca^{2+}-Con A was found to be $9.3 \pm 0.4 \times 10^4$ M^{-1}, while that for Ca^{2+}-Con A_{int} was $9.6 \pm 0.8 \times 10^4$ M^{-1}. The number of saccharide binding sites (n) per lectin monomer was 1.02 ± 0.03 for Ca^{2+}-Con A and 1.07 ± 0.03 for Ca^{2+}-Con A_{int}. These results were in close agreement with the K_a for MUM binding by Con A in the presence of Ca^{2+} and Ni^{2+} reported by Loontiens & co-workers (1977) at pH 7.2 ($8.9 \pm 0.6 \times 10^4$ M^{-1} and $n = 1.06 \pm 0.04$).

Table I summarizes the results of binding experiments with various sugars under similar conditions. We found that reconstitution of Con A_D in the presence of both Mn^{2+} and Ca^{2+} (Ca^{2+}-Mn^{2+}-Con A) gave the same K_a for MUM binding as did Ca^{2+}-Con A. Similar results were obtained when the K_a's for MUM binding by Ca^{2+}-Con A_{int} and Ca^{2+}-Mn^{2+}-Con A_{int} were compared. In these experiments virtually no difference between K_a's in the presence or absence of Mn^{2+} was detected. Similarly, no differences in K_a's were observed between the intact form of Con A and the native form which contained fragmented polypeptide chains. Con A_D in the presence of Ca^{2+} showed no appreciable affinity for MUM when titrations were performed at pH 5.2.[2] This is in agreement with previous findings for other saccharides (Yariv et al., 1968; Kalb & Levitzki, 1968). Similarly, titrations with only Con A_D and MUM carried out at pH 7.2 showed no significant sugar-binding activity. Some fluorescence quenching was detected at high levels of MUM. This would be expected for activation of a small amount of Con A due to a 3-4% contamination by transition metal ions and Ca^{2+}. The possibility that Con A_D might bind MUM but be in a conformation that does not allow for fluorescence quenching was ruled out by equilibrium dialysis experiments. The same level of fluorescence was observed on both sides of the dialysis membrane indicating that no unquenched MUM was bound by the Con A_D on the protein side of the membrane.

The binding of a sugar L such as methyl α-D-mannopyranoside by Ca^{2+}-Con A or Ca^{2+}-Con A_{int} in the presence of MUM was determined assuming the following reaction scheme (Bessler et al., 1974; where the association constants are given by their respective arrows). Con A as shown below is representative of either the nicked or intact protein subunits.

$$\begin{array}{ccc} \text{L + Con A + MUM} & \xrightleftharpoons{K_{MUM}} & \text{Con A–MUM + L} \\ \Big\downarrow K_L & & \Big\downarrow K_{MUM-L} \\ \text{Con A–L + MUM} & \xrightleftharpoons{K_{L-MUM}} & \text{Con A–MUM–L} \end{array} \quad (1)$$

Con A–MUM, Con A–L, and Con A–MUM–L represent the three most likely protein-sugar complexes, where Con A–MUM–L is the complex when both sugars are bound by Con A concomitantly. We have shown from equilibrium dialysis that methyl α-D-mannopyranoside releases bound MUM from the Con A–MUM complex. Dean & Homer (1973) have

[2] MUM binding was investigated at pH 5.2 with Con A_D which had first been remetallized with Ca^{2+} at pH 7.2. Fluorescence quenching was observed immediately following the dropping of the pH from 7.2 to 5.2, but reappearance of fluorescence, reduction of MUM binding, occurred with time. These results are currently being investigated in this laboratory. The authors thank the reviewer for suggesting this experiment.

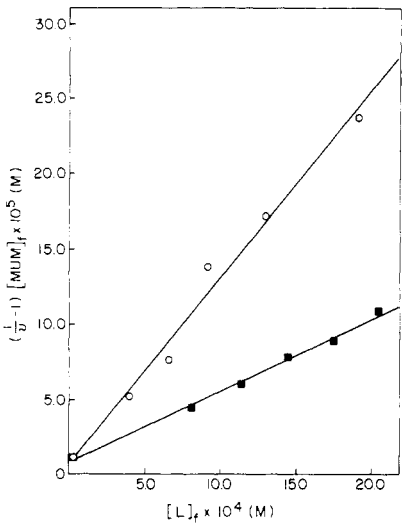

FIGURE 2: Competitive binding with Ca^{2+}-Con A (similar results were obtained with Ca^{2+}-Con A_{int}) at 5 °C, pH 7.2. $[L]_f$ is the free concentration of competing sugar; $[MUM]_f$ is the free concentration of MUM. The fraction of bound MUM per total protein subunits is represented by ν. α-MM (○); α-MG (■).

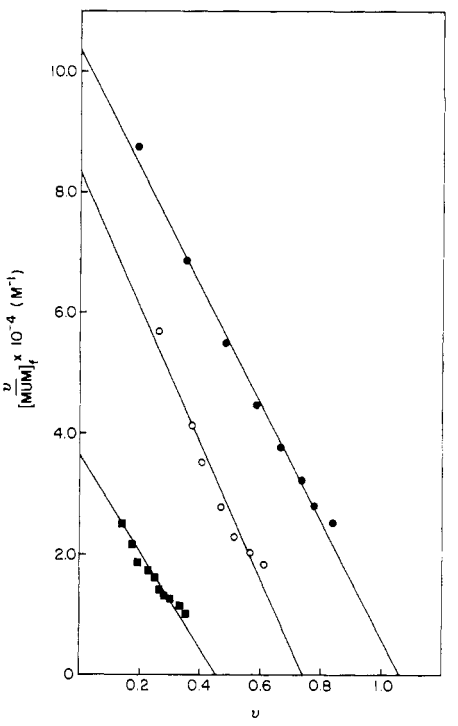

FIGURE 3: Scatchard plots of MUM binding by Ca^{2+}-Con A (similar results were obtained with Ca^{2+}-Con A_{int}) at 5 °C, pH 7.2. The fraction of bound MUM per total protein subunits is represented by ν. $[MUM]_f$ is the free MUM concentration. $[Ca^{2+}]/[Con\ A] > 1$ (●); $[Ca^{2+}]/[Con\ A] = 0.87$ (○); $[Ca^{2+}]/[Con\ A] = 0.58$ (■).

demonstrated similar competition. We may then assume that both ligands compete for the same saccharide-binding site on the Con A molecule. From this it follows that [Con A-MUM-L] = 0 and $K_{MUM-L} = 0$. Equation 1 is then characterized by the following:

$$\left(\frac{[\text{Con A}]_{total}}{[\text{Con A-MUM}]} - 1\right)[MUM]_f = \frac{K_L}{K_{MUM}}[L]_f + \frac{1}{K_{MUM}} \quad (2)$$

A plot of $(1/\nu - 1)[MUM]_f$ vs. $[L]_f$, where $\nu = [\text{Con A-MUM}]/[\text{Con A}]_{total}$, yields a straight line (Figure 2). From the slope and intercept of the resulting linear relationships K_a's for the competing sugars were determined (Table I). The $K_{\alpha\text{-MM}}$ of 1.1×10^4 M^{-1} reported here is in reasonable agreement with that reported by Bessler & coworkers (1974) (1.65×10^4 M^{-1} at pH 7, 8 °C).

Saccharide Binding in the Presence of Less than Stoichiometric Ca^{2+}. Con A_D in the absence of Ca^{2+} showed no appreciable binding of MUM at pH 7.2. If Con A_D solutions were allowed to incubate with less than stoichiometric amounts of Ca^{2+}, less than 1.0 Ca^{2+} per Con A monomer, the resulting protein solutions did bind MUM. Figure 3 represents the results of these kinds of binding studies using either Con A or Con A_{int}. K_a's obtained were slightly different from those observed when Ca^{2+} was present at saturating levels. These differences, however, do not appear to be significant. The observed K_a's when less than stoichiometric Ca^{2+} was present ranged between 8 and 11×10^4 M^{-1}. This is clearly in agreement with the overall K_a of 9.6×10^4 M^{-1} determined in the presence of saturating Ca^{2+}.

The n values determined from the x intercepts in Figure 3 corresponded to the less than stoichiometric levels of Ca^{2+} present. Under conditions in which the ratio of the total molar Ca^{2+} concentration to the total molar Con A (Con A_{int}) subunit concentration, $[Ca^{2+}]/[Con\ A]$, was 0.58, n was found to be 0.48 ± 0.02. When the $[Ca^{2+}]/[Con\ A] = 0.87$, n was found to be 0.75 ± 0.02. If the $[Ca^{2+}]/[Con\ A]$ was greater than 1, n was essentially equal to 1. This was true with $[Ca^{2+}]/[Con\ A]$ of 10. All the data for this work was fitted to plots by linear least-squares analysis. Linearity of all plots shown in Figures 1-3 and those used to produce the results listed in Table I were based on linear correlation coefficients r greater than 0.97. However, most r values were greater than 0.99. All determinations for K_a and n values were based on an average of at least three experiments.

Discussion

Concanavalin A interacts with a wide variety of cells presumably by association with carbohydrate residues on their surfaces (Lis & Sharon, 1973). Binding stoichiometry, affinity, and physiological effects of Con A are routinely studied at neutrality to ensure viability of the cells. Yet the characterization of the role of metal ions in lectin sugar-binding has been examined at a much lower pH (Kalb & Levitzki, 1968; Brewer et al., 1974; Sherry et al., 1975). Our results indicate that the accepted mechanism for sugar-binding activation of Con A determined at pH 5.0-5.5 does not apply at physiological pH. While Kalb & Levitzki (1968) found that very little [^{14}C]-methyl α-D-glucopyranoside was bound by Con A at pH 5.2 when only a single divalent metal ion was present, we find that at physiological pH Ca^{2+} is fully capable of activating Con A_D to bind saccharides. Further, Mn^{2+} has no effect on the observed K_a for MUM binding. These results show that saccharide binding by Con A at physiological pH does not require a transition metal ion.

In our studies we were unable to detect differences in binding of MUM and other sugars investigated to either nicked or intact Ca^{2+}-Con A. Williams & co-workers (1978) observed no difference in K_a for the binding of p-nitrophenyl α-D-mannopyranoside by Con A and Con A_{int} at pH 5 in the presence of Mn^{2+} and Ca^{2+}. Loontiens & co-workers (1977), using Ca^{2+} and Ni^{2+}-treated Con A_{int} in their MUM binding studies, determined a K_a similar to the one reported here. Differences in binding were seen, however, when studies were carried out using the disaccharide, p-nitrophenyl 2-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (Williams et al.,

1978). Our results and those of others, therefore, suggest that nicked and intact Con A bind MUM and other monosaccharides in the same manner.

The competitive binding assays demonstrate that Ca^{2+}-Con A was capable of binding a simple nonderivatized monosaccharide. This shows that MUM binding by Con A in the presence of Ca^{2+} but in the absence of any transition metal ions is not simply binding resulting from association with the umbelliferyl moiety. It is known that other saccharides possessing hydrophobic substituents show enhanced binding affinities over their unsubstituted counterparts (Poretz, 1968). MUM, MUG, methyl α-D-mannopyranoside, and methyl α-D-glucopyranoside are almost certainly competing for the same specific carbohydrate site. This competition is identical with both the nicked and intact forms of Con A. The competitive binding depicted in Figure 2 is indicative of competition between MUM and a second sugar for the same saccharide-binding site. Since linear relationships were obtained this further suggests that only one of the competing saccharides may associate with a Ca^{2+}-Con A subunit at any given time. It is known from the n values determined by Scatchard analysis that only one MUM molecule is bound by each Ca^{2+}-Con A or Ca^{2+}-Con A_{int} subunit (Figure 1). Therefore, at the very least, the derivatized and nonderivatized sugars bind at overlapping or strongly interacting sites. Similar results have been found for MUM and methyl α-D-mannopyranoside competition with Con A or Con A_{int} in the presence of both Ca^{2+} and a transition metal ion (Dean & Homer, 1973; Loontiens et al., 1977).

The binding affinity in the presence of less than a stoichiometric amount of Ca^{2+} was essentially the same as that observed with a tenfold molar excess of Ca^{2+} (Figure 3). The question then arises as to how many Ca^{2+} ions are required for activation of a Con A subunit. That is, one or more Ca^{2+} ions could be essential for activity. Alternatively, Ca^{2+} may be acting catalytically to induce a sugar-binding conformation at physiological pH. Our results show that n values for MUM binding correlate with the [Ca]/[Con A] ratios employed. As the [Ca]/[Con A] ratios became less than stoichiometric the n values were indicative of only partial activation of the total Con A (Figure 3). At a [Ca]/[Con A] of 0.58, the fraction of Con A subunits activated was 0.48, and a [Ca]/[Con A] of 0.87 produced activation of 75% of the Con A subunits. A very reasonable explanation for these findings is that only one Ca^{2+} ion is required for each protein subunit to possess saccharide-binding activity. The n value obtained is, therefore, the fraction of lectin subunits that possess sugar-binding activity. The nearly one-to-one correlation observed between n values of MUM and [Ca]/[Con A] ratios strongly suggests Ca^{2+} is required in stoichiometric and not catalytic amounts for carbohydrate-binding activation of the lectin. Because Ca^{2+} binding to Con A_D is a complicated process (Alter et al., 1977), our results cannot eliminate the possibility that two or more Ca^{2+} ions may bind to Con A_D. However, from our results with MUM binding at less than stoichiometric levels of Ca^{2+}, it is highly unlikely that two bound Ca^{2+} ions, one at S1 site and one at S2 site, are required for saccharide binding. Because of the many ways in which Ca^{2+} could bind randomly to Con A dimers and tetramers each having two possible Ca^{2+} binding sites per monomer other modes of Con A activation might be postulated. Much further work will be required to delineate the contributions, if any, from other modes. The simplest model at present is to propose that at physiological pH and in the absence of transition metal ions sugar-binding activation of Con A most likely occurs in the presence of one bound Ca^{2+} ion per monomer.

We have found that Con A prepared by affinity chromatography followed by dialysis to remove carbohydrate contained much less than stoichiometric amounts of Mn^{2+} and Zn^{2+} combined. However, our carbohydrate-free Con A always contained at least a stoichiometric amount of Ca^{2+} as determined by atomic absorption techniques (unpublished observations). It should be noted that we made no attempts to prevent Ca^{2+} contamination from sources such as glassware. Agrawal & Goldstein (1968) similarly observed low Mn^{2+} content in their own preparations of Con A. Furthermore, reports have appeared showing that far less than stoichiometric amounts of certain transition metal ions (notably Mn^{2+}) are contained in commercial preparations of Con A (Reed & Cohn, 1970; Barber & Carver, 1973). Certainly the majority of biological studies performed with Con A have used either commercial or affinity purified preparations without any remetallization. These preparations always appear to be adequate for inducing the biologic responses of interest. Our results show that a transition metal ion is not required for monosaccharide binding-activity with either intact or nicked Con A at physiological pH. This allows for the prediction that association of Con A with polysaccharide receptors on cell surfaces may only depend on calcium.

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Metal Ion Induced Conformational Transitions of Prothrombin and Prothrombin Fragment 1†

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ABSTRACT: Circular dichroism experiments indicate that prothrombin fragment 1 undergoes essentially the same secondary structural change whether in the presence of Ca^{2+}, Mg^{2+}, or Mn^{2+}. Titration with any of these metal ions results in a sigmoidal titration curve indicative of cooperative binding. Mg^{2+} and Ca^{2+} have nearly identical transition midpoints, while that for Mn^{2+} is an order of magnitude less. These results correlate well with the results of previous metal ion intrinsic fluorescence quenching experiments. Fragment 1 has previously been shown to undergo a second transition corresponding to dimerization at high calcium concentrations. The present circular dichroism experiments show that this transition does not result in a gross alteration of secondary structure in the fragment 1 molecule. Studies with prothrombin, similar to those with fragment 1, indicate a similar metal ion dependent conformational change but of smaller magnitude. As apparently only the fragment 1 portion of the molecule undergoes the transition, it would appear that the covalently linked fragment 1 is constrained from attaining the same conformation as the purified entity. This suggests that caution must be used in interpreting the results of metal ion binding studies using fragment 1 as an analogue for prothrombin.

Prothrombin fragment 1 has been shown to be the region of the prothrombin molecule responsible for phospholipid binding (Gitel et al., 1973). The 10 γ-carboxyglutamic acid residues in prothrombin all occur in fragment 1 (Fernlund et al., 1975; Howard et al., 1975), and, like prothrombin, fragment 1 binds Ca^{2+} in a highly cooperative manner (Stenflo & Ganrot, 1973; Henriksen & Jackson, 1975; Benarous et al., 1976; Bajaj et al., 1975; Mann et al., 1973). Besides Ca^{2+}, prothrombin and fragment 1 have been shown to bind other di- and trivalent cations (Prendergast & Mann, 1977; Bajaj et al., 1976; Furie et al., 1976; Nelsestuen et al., 1976).

Nelsestuen (1976) and Prendergast & Mann (1977) have shown that prothrombin fragment 1 undergoes a fluorescence transition in the presence of a variety of metal ions, which results in about a 40% quenching of the tryptophan fluorescence. In addition, Prendergast & Mann (1977) have shown that, only in the presence of metal ions (calcium) which stimulate prothrombin activation and prothrombin lipid binding, a second transition occurs which involves dimerization of prothrombin fragment 1 molecules. This second transition occurs at metal ion concentrations higher (approximately two times) than those which elicit the fluorescence change. The calcium ion concentration which is optimal for prothrombin conversion to thrombin is equivalent to that metal ion concentration which brings about the second transition.

Fluorescence quenching data alone do not allow one to decide between a minor local environmental change around a tryptophan residue or a more substantial change in the overall backbone of the protein. The technique of circular dichroism (CD) allows one to make this distinction. Circular dichroism studies available from the literature on prothrombin fragment 1 (Gabriel et al., 1975) permit the conclusion that calcium concentrations twice those required for both the aforementioned calcium-dependent fragment 1 transitions induce con-

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