The Activation of Concanavalin A by Lanthanide Ions†

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ABSTRACT: Divalent cadmium and lead and the trivalent lanthanides bind in the transition metal site (S1) of concanavalin A and induce saccharide binding to the protein in the presence of calcium. Partial activation of the protein in the presence of lanthanides alone indicates these ions bind into both transition metal (S1) and calcium sites (S2). The activity of a lanthanide–protein derivative may be increased by the addition of either calcium or a transition metal ion. The saccharide binding activity decreases in the order Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Cd^{2+} reflecting the order of binding constants for these ions in the transition metal site. Like the lanthanides, divalent cadmium substitutes for both the transition metal ion and calcium ion to partially activate the protein. Divalent lead substitutes only for the transition metal ion and partially activates the protein upon adding calcium. The data are consistent with a model in which saccharide binding activity is independent of the metal size in S1 but critically dependent upon metal size in S2.

Concanavalin A (Con A), the lectin or plant hemagglutinin obtained from *Canavalia enisformis,* contains the metals manganese and calcium in its native state. Con A exists as a dimer below pH 6.0 at sufficient ionic strength with a molecular weight of 53,000 and a tetramer above pH 7.0 or at low ionic strength (McKenzie et al., 1972). Each dimer has two transition metal sites (S1) and two calcium sites (S2) (Kalb and Levitzki, 1968). Several reports have shown that a full complement of these metals are required for optimal binding of saccharides (Agrawal and Goldstein, 1968; Uchida and Matsumoto, 1972; Karlstam, 1973) although Brewer et al. (1974) suggest that Ca^{2+} may be removed from fully metallized Con A without loss of saccharide binding activity. Earlier water proton relaxation enhancement and electron paramagnetic resonance (EPR) studies from our laboratory provided evidence of two equivalent Mn^{2+} binding sites per protein dimer with a K_D = 50 μM (Sherry and Cottam, 1973). The addition of Ca^{2+} to Mn^{2+}–Con A effects a large decrease in the water proton relaxation enhancement and a concomitant decrease in the Mn^{2+} dissociation constant. Further frequency and temperature dependent studies suggest this decrease in enhancement is primarily due to a decrease in the exchange rate of one water molecule between the primary bound coordination sphere of the Mn^{2+} and the bulk solvent (Koenig et al., 1973; Meirovitch and Kalb, 1973; Villafranca and Viola, 1974). The Mn^{2+}–Con A binding constants determined by the nuclear magnetic resonance (NMR) method is an order of magnitude larger than that determined by equilibrium dialysis at 4° (Shoham et al., 1973).

We have been interested in the use of the trivalent lanthanide ions as spectroscopic probes of calcium ion binding sites in proteins for some time. These ions have been shown to activate α-amylase (Smolka et al., 1971; Darnall and Birnbaum, 1973), enhance the conversion of trypsinogen to trypsin (Gomez et al., 1974), and substitute for calcium in thermolysin (Colman et al., 1972) and lysozyme (Dwek et al., 1971). In our previous report on Con A, both water proton relaxation rate and fluorescence titrations gave evidence of two strong and two weak lanthanide binding sites per protein dimer (Sherry and Cottam, 1973). The results suggested the lanthanide ions substitute not only for calcium ion in S2 but also bind in S1 with an association constant similar to that determined for Mn^{2+}. Based upon the ability of the metal ions, Sm^{3+}, Eu^{3+}, Tb^{3+}, Pb^{2+}, Zn^{2+}, and Ca^{2+}, to reduce the observed water proton relaxation enhancement of Gd^{3+} bound to Con A, Barber and Carver (B. H. Barber and J. P. Carver, personal communication) proposed the lanthanides bind into a third site, S3, different from S1 and S2. This third lanthanide binding site in solution was presumed identical with the Pb^{2+} binding site as determined crystallographically. We would now like to report the effect of the substitution of a variety of metal ions upon Con A's ability to bind saccharides.

Materials and Methods

Concanavalin A was isolated from jack bean meal by the affinity chromatography method (Agrawal and Goldstein, 1967) and demetallized by either dialysis against 1 *M* HCl, exhaustive dialysis at pH 3, or against sodium ethylenediaminetetraacetic acid (EDTA) at pH 5.6. The three methods were equally efficient in preparing the apoprotein although the first two are preferred to alleviate doubts concerning the complete removal of EDTA from the apoprotein in the latter method. Protein concentrations were determined from the absorbance at 280 nm using $E_{1\,cm}(1\%)$ 12.4 at pH 5.0 and reported assuming a dimer molecular weight of 53,000 at pH 5.6 (McKenzie et al., 1972). Fragment-free protein samples prepared by NH_4HCO_3 precipitation (Cunningham et al., 1972) showed the same metal binding and saccharide binding properties as the native protein and was therefore not generally used.

The saccharide binding activity of concanavalin A was measured using affinity chromatography on Sephadex G-50. In a typical experiment, 200 μl of 0.1 m*M* metallized Con A is added to a 0.5 × 7.0 cm Sephadex G-50 column and eluted with 2 ml of pH 5.6 buffer containing 0.05 *M* sodium acetate and 0.3 *M* sodium chloride followed by 2 ml of pH 5.6 buffer containing 0.1 *M* α-D-glucose. These volumes were sufficiently large to effect separation of the active and inactive forms and to assure better than 95% recov-

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Table I: Removal of Zn^{2+} and Ca^{2+} from Metallized Concanavalin A.[a]

Dialysis Procedure	$[Zn^{2+}] \times 10^4$		$[Ca^{2+}] \times 10^4$	
	Sephadex[b] Binding Data	Atomic[c] Absorption Data	Sephadex[b] Binding Data	Atomic[c] Absorption Data
(a) Before dialysis	1.20		1.20	
(b) After 3 changes of buffer	1.20	1.28	1.20	1.22
(a) Before dialysis	1.38		1.38	
(b) After 1 change of 10^{-3} *M* EDTA and 3 changes of buffer	1.38	1.28	1.38	1.04
(a) Before dialysis	1.15		1.15	
(b) After 2 changes of 10^{-3} *M* EDTA and 3 changes of buffer	0.36	0.40	0.36	0.44
(a) Before dialysis	1.33		1.33	
(b) After 3 changes of 10^{-3} *M* EDTA and 3 changes of buffer	0.16	0.20	0.16	0.21

[a] Each initial 2-ml sample contained 87 μM Con A and the indicated amounts of bound Zn^{2+} and Ca^{2+} in 0.3 *M* sodium chloride and 0.05 *M* sodium acetate at pH 5.6. The samples were dialyzed against 18 ml of buffer or 10^{-3} *M* EDTA in buffer for 3-hr periods. [b] Only that percentage of protein which bound to a Sephadex column was assumed to contain Zn^{2+} and Ca^{2+} (2 mol of metal ion/mol of active protein). [c] The dialyzed protein samples were acid hydrolyzed and dissolved in fresh buffer before atomic absorption analysis.

ery of all protein added to the column. The saccharide binding "activity" is calculated by comparing the absorbance at 280 nm of the elutant before and after the addition of glucose. The reproducibility of the binding percentages were within ±2%. The measured activities were extremely dependent upon the degree of cross-linking in the Sephadex matrix showing increased binding percentages from Sephadex G-25 < G-50(course) < G-50(medium) < G-50(fine) ≲ G-75 = G-100. Several batches of G-50(fine) showed a large variation in binding percentages indicating nonuniformity in preparative cross-linking.

The transition metal, zinc, cadmium, lead, and calcium, solutions were prepared by dissolving reagent grade metal chlorides in distilled, deionized water. Lanthanide ion stock solutions were prepared by dissolving the oxides in acid and standardized against EDTA using Xylenol Orange as the indicator.

The longitudinal water proton relaxation rates were determined by the Carr-Purcell method with a Nuclear Magnetic Resonance Specialties PS 60-AW pulsed spectrometer operating at 24.3 MHz. A Bausch and Lomb Spectronic 505 or a Beckman Model 25 spectrophotometer was used for the absorbance measurements and a Perkin-Elmer 303 flame spectrophotometer was used to obtain the atomic absorption data. The concentration of free manganese was determined by electron spin resonance (Mildvan and Cohn, 1963) using a Varian E4 spectrometer.

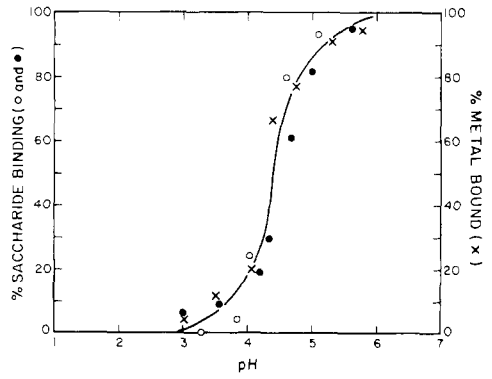

FIGURE 1: Saccharide binding and manganese binding (ESR) to concanavalin A as a function of pH. All samples contained 0.12 m*M* Con A, 0.83 m*M* calcium(II), 0.30 *M* sodium chloride, and 0.04 *M* sodium phosphate buffer at the indicated pH. In addition, the samples contained 0.83 m*M* nickel(II) (○) or manganese(II) (● and X).

Results

In the absence of either calcium ions or transition and metal ions, concanavalin A shows no saccharide binding activity as measured by binding to Sephadex at pH 5.6 and μ = 0.3 *M* in agreement with previous work (Kalb and Levitzki, 1968; Shoham et al., 1973; Karlstam, 1973; Uchida and Matsumoto, 1972). The addition of Ni^{2+} to 0.1 m*M* Con A in the presence of 2 m*M* Ca^{2+} results in nearly stoichiometric binding of this transition metal ion. Only that portion of Con A which contains Ni^{2+} displays saccharide binding capability. The saccharide binding activity reaches a maximum (>95%) at a Ni^{2+}/protein dimer concentration ratio of 2. A similar activity study at constant Ni^{2+} concentration and varying Ca^{2+} concentrations also suggests a 2:1 stoichiometric requirement for Ca^{2+} binding to the Con A dimer. By assuming 2 mol of calcium and transition metal ion are bound per active Con A dimer, the concentration of bound metal ions may be estimated by simply measuring the percent protein bound to Sephadex. The satisfactory agreement between this method and the atomic absorption analyses in Table I provides further evidence for a 2:1 stoichiometry requirement for both transitional metal ion and calcium ion binding. The data in Table I also prove that zinc and calcium are not easily removed from metallized Con A. Dialysis of metallized Con A against three changes of tenfold volumes of pH 5.6 buffer results in essentially no loss of either Zn^{2+} or Ca^{2+}. Further dialysis against 10^{-3} *M* EDTA slowly removes both metal ions with a subsequent loss of saccharide binding activity. Even after three changes of tenfold volumes of 10^{-3} *M* EDTA in pH 5.6 buffer, approximately 15% of the Con A remains metallized and active. Brewer et al. (1974) have reported preparing calcium deficient Mn^{2+}-Con A which retains its ability to bind methyl α-D-glucopyranoside simply by dialyzing Mn^{2+}-Ca^{2+}-Con A against large volumes of buffer. We have not been able to remove Ca^{2+} from the metallized protein even after extensive dialysis against pH 5.6 buffer. Only after dialysis for several hours against EDTA do we find a significant decrease in the Ca^{2+} concentration.

The pH dependence of Con A saccharide binding activity is shown in Figure 1. The saccharide binding in the presence of either Ni^{2+} or Mn^{2+} drops off rather dramatically as the pH is lowered below 5. The increase in free Mn^{2+}, as measured by electron spin resonance spectroscopy (Mildvan and Cohn, 1963), with decreasing pH parallels the loss of saccharide binding activity. Presumably, the similar de-

Table II: The Metal Activation of Concanavalin A.[a]

Additions (mM)	Saccharide Binding Activity ±2%
A. [Con A] = 70 μM	
None	0
Ni^{2+} (1.2)	0
Ca^{2+} (2.3)	0
Ni^{2+} (1.2) and Ca^{2+} (2.3)	90
Cd^{2+} (1.7)	13
Yb^{3+} (2.5)	3
B. [Con A] = 70 μM; [Yb^{3+}] = 1.3 mM	
Zn^{2+} (0.8)	26
Ni^{2+} (0.8)	20
Co^{2+} (0.8)	12
Mn^{2+} (0.8)	9
Cd^{2+} (0.8)	6
C. [Con A] = 63 μM; [Zn^{2+}] = 0.2 mM	
La^{3+} (0.2)	12
Nd^{3+} (0.2)	19
Eu^{3+} (0.2)	20
Ho^{3+} (0.2)	20
Tm^{3+} (0.2)	20
Lu^{3+} (0.2)	13

[a] All solutions contained 0.05 M sodium acetate and 0.3 M sodium chloride at pH 5.6. The saccharide binding activity was determined as described in the Experimental Section.

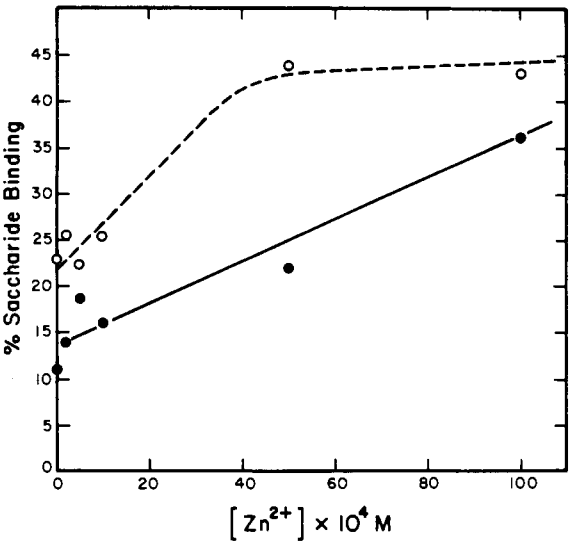

FIGURE 2: Saccharide binding activity of concanavalin A as a function of increasing zinc(II) concentration. All samples contained 63 μM Con A, 0.57 mM lanthanum(III) (●) or 0.57 mM lanthanum(III) plus 0.16 mM calcium(II) (○), 0.05 M sodium acetate, 0.30 M sodium chloride at pH 5.6, and the indicated concentrations of zinc(II).

crease in activity in the nickel derivative is due to a dissociation of Ni^{2+} from the protein. The smooth curve drawn through the binding data and the ESR data, with an apparent $pK_a \sim 4.4$, must reflect the protonation of Asp-10 and Asp-19 in the transition metal and calcium ion sites (Edelman et al., 1972).

The data in Table II outline the variation in concanavalin A saccharide binding activity as a function of added metals. In the presence of only a transition metal ion, no saccharide binding is observed. Even after 48 hr at room temperature, the transition metal–Con A solutions show only a limited increase in activity (5–8%). This small increase in saccharide binding is attributed to a leaching of small amounts of Ca^{2+} from the glassware. The substitution of the trivalent lanthanide, Yb^{3+}, for Ca^{2+} results in partial activation of concanavalian A. This is strong evidence for at least some lanthanide binding in the calcium site (S2). The addition of Yb^{3+} without a transition metal ion results in a very small but reproducibly greater activation than in the presence of calcium ion alone. This slight activity must result from partial lanthanide ion binding into the transition metal sites (S1). The decrease in saccharide binding activity as the transition metal ion is varied may simply reflect the order of transition metal ion–Con A association constants. With the exception of Zn^{2+}, the same ordering of transition metal ion association constants was found using equilibrium dialysis techniques in the absence of Ca^{2+} (Shoham et al., 1973). This same paper reported divalent cadmium binds into both transition metal and calcium sites suggesting Cd^{2+} might be capable of satisfying both metal requirements of Con A. As observed in Table II, the addition of Cd^{2+} alone results in a partial activation of Con A. It is apparent that Cd^{2+} satisfies the two site metal requirement of Con A better than does Yb^{3+}, probably due to stronger binding of the former ion into both sites (Shoham et al., 1973). The cadmium and ytterbium data would suggest that metals with considerable variation in ionic radii can bind in S1 and produce the conformational changes necessary for saccharide binding (McCubbin et al., 1971; Barber and Carver, 1975).

Considering the close approach of the transition metal and calcium ions in the active complex, the low activation of Con A induced by Yb^{3+} may reflect a repulsion between the trivalent ions in S1 and S2. The X-ray crystallographic structure of Con A indicates Mn^{2+} and Ca^{2+} are 5.3 Å apart and actually share two aspartic acid carboxyl groups as ligands (Edelman et al., 1972).

The effect of lanthanide size is also outlined in Table II. In the presence of the same concentration of Zn^{2+}, the majority of the lanthanide series show equivalent Con A activation characteristics. Only in the presence of the largest (La^{3+}) or the smallest (Lu^{3+}) lanthanide does the protein measure a lower saccharide binding activity. The decrease in lanthanide ion radii across the period has been useful in mapping the calcium ion size requirements in α-amylaze (Smolka et al., 1971) and trypsin (Gomez et al., 1974). The unusual decreases in activation of Con A at each end of the lanthanide series may reflect a sum of the size requirements for lanthanide binding in S1 and S2.

The saccharide binding activity of Con A increases with increasing Zn^{2+} concentration in the presence of La^{3+} or a mixture of La^{3+} and Ca^{2+} as shown in Figure 2. In the absence of Zn^{2+}, 63 μM Con A measures 11% activity in the presence of 0.57 mM La^{3+}. This same mixture with the addition of 0.16 mM Ca^{2+} measures 23% saccharide binding activity. The effect of metal charge is also apparent here. In the first experiment, La^{3+} must bind into both S1 and S2 to form the active protein while in the second, Ca^{2+} competes for the S2 binding site and the decreased cationic repulsion in the La^{3+}–Ca^{2+} derivative further increases the protein activity. When Zn^{2+} is added to both mixtures, the percent saccharide activity increases perceptively. The saccharide binding activity of the Zn^{2+}–La^{3+} samples increase more gradually than those which also contain Ca^{2+}. This suggests the Zn^{2+} and La^{3+} ions are competing for the transition metal site. As more Zn^{2+} is added, the increase in saccharide binding activity must reflect the formation of the Zn^{2+}(S1)–La^{3+}(S2)–Con A since Zn^{2+} is only capable of activating the protein by binding in S1. When Ca^{2+} is present, the association constant for Zn^{2+} binding into S1 is

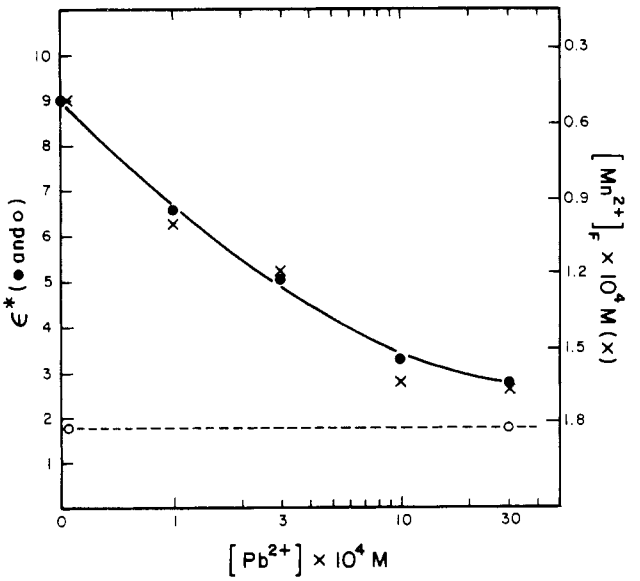

FIGURE 3: Measured water proton relaxation rate enhancement (● and ○) and free manganese(II) concentrations from EPR (x) as a function of divalent lead concentration. All samples contained 334 μM Con A, 0.3 mM total manganese(II), 0.05 M sodium acetate, 0.30 M sodium chloride at pH 5.6, and the noted concentrations of lead(II). In addition, the samples labeled ○ contained 10^{-2} M calcium(II) while those labeled ● and x are free of calcium.

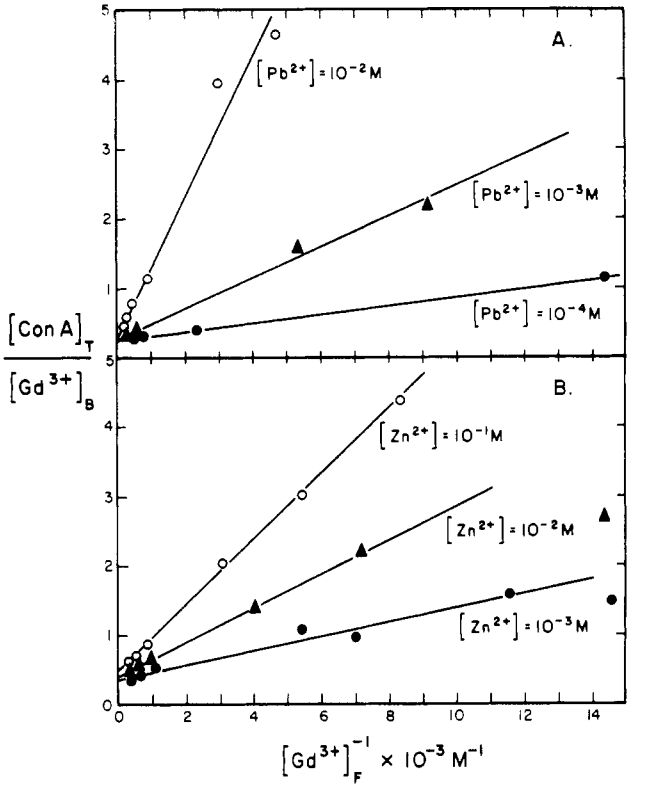

FIGURE 4: (A) A Hughes-Klotz plot of data from a water proton relaxation enhancement titration of concanavalin A with gadolinium(III) showing the effects of increasing amounts of lead(II). All samples contained 414 μM Con A, 0.05 M sodium acetate, 0.30 M sodium chloride at pH 5.6, and the noted concentrations of Pb^{2+} with Gd^{3+} varying from 0.15 to 4.5 mM. (B) Similar plots showing the effects of added zinc(II).

much larger thereby shifting the Zn^{2+}-La^{3+} competition in favor of Zn^{2+}. As a result, the saccharide binding activity reaches its maximum at a lower zinc concentration.

Barber and Carver (B. H. Barber and J. P. Carver, personal communication) have recently proposed that the lanthanides bind in a third site (S3), distinct from the transition metal (S1) and calcium (S2) ion sites. They observed a large decrease in the water proton relaxation rate of a Gd^{3+}-Con A sample upon the addition of Pb^{2+}, Tb^{3+}, Eu^{3+}, or Sm^{3+} and only small changes upon the addition of Zn^{2+} and Ca^{2+}. Based upon these data, the gadolinium site was assumed identical with the crystallographically determined Sm^{3+} and Pb^{2+} site. This report prompted us to reexamine our binding data assuming a third independent lanthanide binding site.

The binding data in Table II and Figure 2 prove the lanthanides at least partially substitute for calcium in S2 to activate the protein and suggest they may also bind in the transition metal site (S1). Considering the excess metal concentrations used throughout these binding studies, the results certainly do not preclude the existence of a lanthanide specific site (S3) with a $K_D \simeq 40$ μM (Sherry and Cottam, 1973; B. H. Barber and J. P. Carver, personal communication). We have also examined the water proton relaxation rates of samples containing Gd^{3+}-Con A and Mn^{2+}-Con A and find a decrease in the enhancement ($\epsilon^* = T_{1P}^{*-1}/T_{1P}^{-1}$, where T_{1P}^{-1} is the paramagnetic contribution to the water proton relaxation rate in the presence (*) and absence of Con A) of both metal-protein complexes upon the addition of divalent Pb^{2+}. The decrease in the enhancement of each Mn^{2+}-Con A sample is paralleled by an increase in the free Mn^{2+} concentration as measured by EPR (Figure 3) and therefore is due to a displacement of Mn^{2+} from the protein and not to a change in the number of water molecules in the primary coordination sphere of the bound Mn^{2+}. The displacement of Mn^{2+} may be due to direct competition between Mn^{2+} and Pb^{2+} for S1 or to a protein conformational change induced by Pb^{2+} binding in S3 resulting in a loss of Mn^{2+} binding in S1. Calcium is known to increase the association of Mn^{2+} in S1 and, as shown in Figure 3, Pb^{2+} fails to dissociate Mn^{2+} from Mn^{2+}-Ca^{2+}-Con A. These results are consistent with measured saccharide binding activities of Mn^{2+}-Ca^{2+}-Con A in the presence of the lanthanides. When active Mn^{2+}-Ca^{2+}-Con A is dialyzed against several changes of excess lanthanide ion, the protein retains full saccharide binding activity and atomic absorption measurements reveal little decrease in either bound Mn^{2+} or Ca^{2+}. If a lanthanide is added to the protein previous to the addition of Mn^{2+} and Ca^{2+}, inhibition of saccharide binding is observed indicating the lanthanide competes with one or both of the required metals.

Further supporting evidence for lanthanide binding in S1 comes from water proton relaxation rate experiments. The enhancement observed when Gd^{3+} binds to apo-Con A is related to the mole fraction of bound gadolinium, X_b, as shown in eq 1 (Dwek, 1972). ϵ_b is the enhancement when X_b

$$\epsilon^* = X_b\epsilon_b + (1 - X_b) \qquad (1)$$

is unity. Several gadolinium titrations were performed at a constant Con A concentration in the presence of varying amounts of Zn^{2+} or Pb^{2+}. The results are presented in the form of Hughes-Klotz plots in Figure 4 (Hughes and Klotz,

$$\frac{[\text{Con A}]_T}{[Gd^{3+}]_B} = \frac{K_D}{n}\frac{1}{[Gd^{3+}]_F} + \frac{1}{n} \qquad (2)$$

1956). The intercepts of all plots reflect approximately two Gd^{3+} binding sites per Con A dimer. As expected, increas-

ing concentrations of Pb^{2+} result in increasing Gd^{3+}-Con A dissociation constants characteristic of competitive binding of these two ions (ϵ_b = 4.6 for Gd^{3+}-Con A in the presence of 10^{-2} *M* Pb^{2+}). Titrations in the presence of Zn^{2+} also reflect a competition between this ion and Gd^{3+} (ϵ_b = 6.4 for Gd^{3+}-Con A in the presence of 10^{-2} *M* Zn^{2+}). This leaves little doubt concerning Gd^{3+} binding in the transition metal site (Zn^{2+} site) but still does not prove the equivalence of this site with the Pb^{2+} site. It is apparent that Pb^{2+} is at least ten times more effective in inhibiting Gd^{3+} binding to Con A than is Zn^{2+}.

In our activity studies with the lanthanides, we observed an increase in the saccharide binding activity when Pb^{2+} was added to Zn^{2+}-Ln^{3+}-Con A and a slight decrease in activity when Pb^{2+} was added to Zn^{2+}-Ca^{2+}-Con A. Divalent lead interferes with the binding of the required metal ions either through direct competition or through a induced protein conformation change. A comparison of the activation of Con A in the presence of a tenfold excess of Ca^{2+} upon the addition of Mn^{2+}, Zn^{2+}, or Pb^{2+} is found in Figure 5. No measurable activity is found with Mn^{2+}, Zn^{2+}, or Pb^{2+} in the absence of Ca^{2+}. If we assume as before that both the transition metal site (S1) and the calcium site (S2) of each Con A monomer must be filled to induce saccharide binding, then Pb^{2+} must be acting as a transition metal substitute. However, unlike the Mn^{2+} and Zn^{2+} derivatives which reach a maximal activity near a 2:1 ratio of metal to Con A dimer, the Pb^{2+} derivative continues to increase in activity above a 8:1 ratio. The reduced Sephadex binding activity of the Pb^{2+}-Ca^{2+}-Con A must simply reflect the weaker binding of Pb^{2+} into S1. This implies the strong Pb^{2+} site ($K_D \leq 40\ \mu M$) is not the transition metal site.

Discussion

The results show that a variety of metal ions may fill the normal transition metal (S1) and calcium (S2) sites in concanavalin A to form an active saccharide binding protein. The extent of protein activation appears to parallel the relative affinities of S1 and S2 for the various metals. Con A is partially activated in the presence of lanthanides alone indicating these ions bind weakly into S1 and S2. Addition of transition metal ions or calcium ions to the lanthanide-Con A complex results in a further increase in saccharide binding activity. Thus, the addition of Ni^{2+} must increase the binding constant of the lanthanide in S2 while the addition of Ca^{2+} increases the lanthanide binding constant in S1. Shoham et al. (1973) have also reported partial binding of methyl α-D-glucopyranoside to Sm^{3+}-Ca^{2+}-Con A and to Ni^{2+}-Sm^{3+}-Con A even though the Sm^{3+} binding constants in S1 and S2 were too low to measure under the conditions of their experiments.

We previously reported two strong Gd^{3+} (K_D = 40 ± 4 μM) binding sites and two weak Gd^{3+} (K_D = 200 ± 50 μM) binding sites per Con A dimer and tentatively assigned these to S1 and S2, respectively (Sherry and Cottam, 1973). Barber and Carver (B. H. Barber and J. P. Carver, personal communication) find two strong Gd^{3+} binding constants (K_D = 40 μM) and several nonspecific weaker sites per protein dimer. The water proton relaxation results presented in Figure 5 indicate there are approximately two sites per protein dimer in which Pb^{2+} competes with Gd^{3+} and approximately two sites per protein dimer in which Zn^{2+} and Gd^{3+} compete. Proving the nonequivalency of these sites is complicated by the fact that Pb^{2+} displaces Mn^{2+} from S1 (Figure 3) and apparently does so by direct

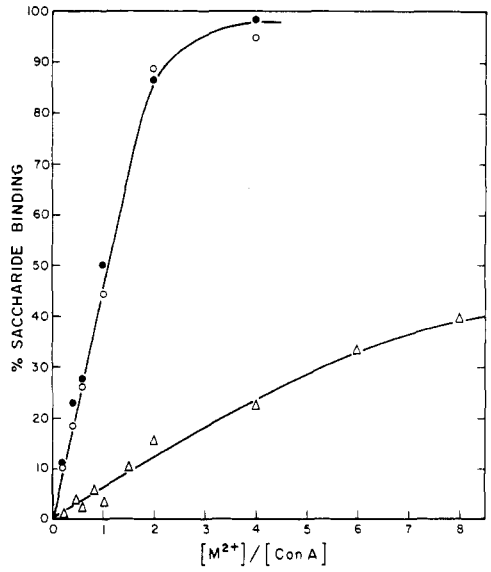

FIGURE 5: The activation of concanavalin A upon the addition of manganese(II), zinc(II), or lead(II). All samples contained 67 μM Con A, 0.67 m*M* calcium(II), 0.30 *M* sodium chloride, 0.05 *M* sodium acetate at pH 5.6, and the noted concentrations of manganese(II) (●), zinc(II) (○), or lead(II) (△).

binding in S1 (Figure 5). However, three pieces of evidence lead us to conclude that the strong Gd^{3+} binding site (K_D = 40 μM) is not S1 or S2 but rather equivalent to the strong Pb^{2+} site (S3). First, Barber and Carver (B. H. Barber and J. P. Carver, personal communication) found similar ϵ_b values for Gd^{3+} binding to Zn^{2+}-Ca^{2+}-Con A and to apo-Con A and our atomic absorption data indicate that a lanthanide will not displace Mn^{2+} or Ca^{2+} from the active metallized protein. Second, we observe significantly different ϵ_b values for Gd^{3+}-Con A in the presence of 10^{-2} *M* Pb^{2+} (ϵ_b = 4.6) than in the presence of 10^{-2} *M* Zn^{2+} (ϵ_b = 6.4) and the latter value is equivalent to that previously reported for Gd^{3+} binding to apo-Con A (Sherry and Cottam, 1973). This suggests the ϵ_b = 6.4 is characteristic of Gd^{3+} binding in S3 (the normal Pb^{2+} site) while the ϵ_b = 4.6 most probably reflects Gd^{3+} binding into the many nonspecific sites. Finally, we observe nearly equal Tb^{3+} fluorescence quantum yields when this ion is added to apo-Con A or to Mn^{2+}-Ca^{2+}-Con A. The efficiency of energy transfer from an aromatic amino acid (at or near S3) to Tb^{3+} is not changed when the protein undergoes the known metal induced conformation changes required for saccharide binding (Barber and Carver, 1975).

Our pH studies show the removal of Con A from Sephadex parallels the dissociation of the required metals from the protein. The pH dependency illustrated in Figure 1 is similar to that reported for the Con A-dextran precipitation reaction (So and Goldstein, 1967) suggesting the Sephadex binding experiments reported here may parallel the agglutination process over this pH range. However, the agglutination reaction at pH 7 is induced upon the addition of only one divalent cation (Agrawal and Goldstein, 1968) suggesting the tetramer has an altered metal ion requirement. Other physical techniques have been used to help elucidate the amino acids involved in the binding of a saccharide to the protein. The difference pH titration curve of Con A in the presence and absence of methyl α-D-mannopyranoside reflects an alteration of the p*K* of approximately two carboxyl groups (p*K* ≈ 3.8) per mannoside binding site (Hassing et al., 1971). The change in fluorescence quantum yield

of 4-methylumbelliferyl α-D-mannopyranoside upon binding to Con A allowed a measurement of this binding constant as a function of pH, again revealing a binding dependence upon a group with a p$K \approx 3.5$ (Dean and Homer, 1973). It is interesting to note that Con A retains its capacity to bind this fluorescent derivative, *p*-nitrophenyl α-D-mannopyranoside, methyl α-D-mannopyranoside, and yeast mannan at pH 6.5 and pH 2.4 even though the protein fails to agglutinate the latter polysaccharide at the lower pH (Hassing and Goldstein, 1970). The reported binding constants of the monosaccharides were 5–10-fold lower at pH 2.4 than at pH 6.5. The addition of the required metals above pH 5 may simply increase the binding constant of saccharides to the protein and thereby induce the agglutination process.

It was recently suggested that divalent calcium is not required for the binding of methyl α-D-glucopyranoside to Con A but its role is merely to accelerate the rate of formation of the transition metal ion–protein complex in the proper conformational form (Brewer et al., 1974). Our observations are in complete disagreement with this hypothesis. We find only a small activation of Mn^{2+}–Con A as measured by Sephadex binding even if the protein is kept at room temperature for several days. Furthermore, we find no loss in Mn^{2+} or Ca^{2+} from Con A as measured by atomic absorption techniques and no decrease in Sephadex binding activity after extensive dialysis (3–4 days) against pH 5.6 acetate buffer or against this same buffer containing 1 m*M* Tb^{3+}. In view of the reported binding of methyl α-D-mannopyranoside to Con A even at pH 2 (Hassing and Goldstein, 1970), it is likely that Ca^{2+} is not required for the binding of this monosaccharide to Con A at pH 5.6. However, we must conclude that transition metal ions and calcium ions (or appropriate substitutes) must be present at pH 5.6 to induce the agglutination reaction and that a loss of either ion results in a reversal of this activation process.

In summary, at least six specific metal sites exist in each concanavalin A dimer at pH 5.6: two transition metal sites (S1), two divalent calcium sites (S2), and approximately two sites specific for divalent lead and the trivalent lanthanides (S3). Only metals in sites S1 and S2 control the activation of the protein. As a variety of metal ions bind in S1 (the divalent transition metal ions, Zn^{2+} and Cd^{2+}, and the trivalent lanthanides) to partially activate the protein, metal size must be of little importance in this site. The S2 requirements seem more stringent. Only those ions with ionic radii near 1 Å (Ca^{2+}, Cd^{2+}, and several of the trivalent lanthanides) bind in S2 and trigger the conformational changes necessary for agglutination.

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