Calcium-Induced Cooperativity of Manganese Binding to Concanavalin A†

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ABSTRACT: Titrations employing electron spin resonance spectroscopy and equilibrium dialysis studies have revealed that Mn^{2+} binding to concanavalin A is cooperative in the presence and noncooperative in the absence of Ca^{2+}. The degree of cooperativity increases with increasing pH. Hill coefficients range from 1.4 at pH 5.0 to 1.8 at pH 6.85. In addition to inducing cooperativity in Mn^{2+} binding, Ca^{2+} influences the pH dependence and increases the affinity of Mn^{2+} binding. In contrast to previous suggestions based mostly on work conducted near pH 5, demetallized concanavalin A does bind Ca^{2+} with an appreciable binding constant. These observations indicate that at physiological pH the role of metal ions in determining functional properties of concanavalin A is different from that suggested by metal binding studies conducted at lower pH values.

Concanavalin A[1] (Con A), a mitogenic lectin isolated from jack bean (*Canavalia ensiformis*) (Sumner and Howell, 1936), is of considerable interest because it can bind to cell surfaces and alter intracellular activities. The specificity of Con A makes it a valuable probe in the study of surface-mediated cellular responses. The association of Con A with cell surfaces is primarily due to its ability to bind particular carbohydrates. Saccharides possessing the D-arabinopyranoside configuration at positions C-3, C-4, and C-6 are bound by Con A (Goldstein et al., 1965, 1973). This sugar-binding ability is dependent upon the binding of one Ca^{2+} and one transition metal ion to each Con A subunit (Kalb and Levitzki, 1968).

Con A has been shown to exist as a dimer of identical 25 500 molecular weight subunits at pH 5.5 or below and as a tetramer of those same subunits at pH 7 or above (Kalb and Lustig, 1968; McCubbin and Kay, 1971). Work by Kalb and Levitzki (1968) which indicated that each Con A subunit binds one Ca^{2+} and one Mn^{2+} ion was verified by x-ray studies (Becker et al., 1975; Hardman and Ainsworth, 1972). These studies show that the two metal ion binding sites are very close together and, in fact, the metal ions share two ligands, Asp-10 and Asp-19.

The interaction between Ca^{2+} and Mn^{2+} ions in Con A has been the subject of considerable investigation. The experiments of Kalb and Levitzki (1968) indicate that the transition metal ion must bind first and that its binding results in the formation of a Ca^{2+} binding site. This sequential binding of metal ions is currently accepted by most workers in this field. Brewer and co-workers (1974) proposed that the role of Ca^{2+} is merely to enhance the rate of formation of the Mn^{2+}-Con A complex, which then does not require Ca^{2+} for activity. This is in conflict with the results of other studies (Kalb and Levitzki, 1968; Sherry et al., 1975) which indicate that Ca^{2+} is required for full saccharide binding activity. Numerous workers have shown that the binding of Ca^{2+} to Con A induces a change in Mn^{2+} liganding (von Goldammer and Zorn, 1974; Koening et al., 1973; Sherry and Cottam, 1973; Kalb and Pecht, 1973). An overall change in protein conformation may also occur (Richardson and Behnke, 1976; Barber and Carver, 1975; Grimaldi and Sykes, 1975).

The precise way in which one metal ion influences the binding of the other and the effect of metal ions on the protein as a whole are not yet completely understood. In an attempt to clarify the effect of Ca^{2+} on Mn^{2+} binding by Con A, we have utilized electron spin resonance (ESR) and equilibrium dialysis to study Mn^{2+} binding in the presence and absence of Ca^{2+}. Both experimental methods show that Mn^{2+} binding is strongly cooperative when Con A is preincubated with Ca^{2+} and noncooperative when no Ca^{2+} is present. The Ca^{2+}-induced cooperativity increases as the pH approaches 7. We have also investigated the binding of Ca^{2+} by demetallized Con A and found that, while Ca^{2+} binding is weak at pH 5.2, in agreement with Kalb and Levitzki (1968), there is significant Ca^{2+} binding at pH 7.0. The temperature and pH dependence of Mn^{2+} binding in the presence and absence of Ca^{2+} have also been examined. The thermodynamic parameters for the association of Mn^{2+} with Con A have been determined.

Materials and Methods

Con A Preparation. Con A was prepared from jack bean meal as described by Agrawal and Goldstein (1967). The preparations were characterized by a single electrophoretic band at both pH 4.5 and 7.5 (Shepard and Gurley, 1966). Further, band patterns characteristic of pure Con A (Wang et al., 1971; Abe et al., 1971) were observed upon electrophoresis of the preparations on sodium dodecyl sulfate–polyacrylamide gels (Weber and Osborn, 1969). Protein concentrations were determined spectrophotometrically and activity was measured by glycogen precipitation assays (Poretz, 1968).

Preparation of Demetallized Con A. Demetallized Con A was prepared by treatment of the protein solutions with 0.1 N HCl to bring the pH to 1.2 (Kalb and Levitzki, 1968), maintaining that pH for 30 min at room temperature. The material was then dialyzed at 4 °C against distilled water until the pH reached 5.5–6.0. The resulting Con A was shown by atomic absorption to contain less than 0.02 mol of Zn^{2+} or Mn^{2+} and less than 0.04 mol of Ca^{2+} per mol of Con A subunits.

ESR Titrations. The binding of Mn^{2+} was monitored by ESR spectroscopy by using Varian E-3 and E-9 spectrometers.

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[1] Abbreviations used: Con A, concanavalin A; ESR, electron spin resonance; Mops, morpholinopropanesulfonate; Ca^{2+}-Con A, demetallized concanavalin A preincubated with an excess of Ca^{2+}.

Sample temperature was regulated by passing compressed air, cooled in a dry ice-acetone bath, over the sample. Temperature was controlled to ±1.5 °C over a temperature range from 0 to 30 °C. Solutions at pH 5.5 and below contained 0.05 M acetate while solutions above pH 5.5 contained 0.05 M Mops. NaCl concentration was typically 0.2 M.

In solutions not containing Con A, the ESR signal was found to be directly proportional to the Mn^{2+} concentration. Binding measurements were made on various mixtures of demetallized Con A and metal ions after allowing them to incubate at 4 °C for several days to a week. To ensure that significant denaturation did not occur during this period, the concentration and activity of Con A were measured at beginning and end of each incubation period.

The concentrations of free and bound Mn^{2+} were determined from Mn^{2+} ESR spectra. The spectral intensity or observed spectral height H at any point must be a composite of contributions from molar concentrations of free Mn^{2+} given below by $[Mn]_f$ and Mn^{2+} bound to Con A, $[Mn]_b$. If H_b and H_f represent the respective heights for a 1 M solution of bound and free Mn^{2+} in a 0.25-mm ESR aqueous cell, then

$$H = [Mn]_b H_b + [Mn]_f H_f \quad (1)$$

The total Mn^{2+} concentration $[Mn]_t$ is equal to the sum of free and bound Mn^{2+}. Thus $[Mn]_b$ equals $[Mn]_t$ minus $[Mn]_f$. Upon substituting for $[Mn]_b$ in the above equation and solving for $[Mn]_f$, one obtains

$$[Mn]_f = \frac{H - [Mn]_t H_b}{H_f - H_b} \quad (2)$$

H_f is determined from the signal obtained with free Mn^{2+}; H_b is obtained from the signal when all the Mn^{2+} is bound to Con A.

Equilibrium Dialysis. Equilibrium dialysis experiments were performed by placing Con A solutions in 0.05 M Mops and 1.0 M NaCl in one compartment of Lucite dialysis cells, and the same buffer solution lacking Con A but containing Mn^{2+} and/or Ca^{2+} in the other compartment. The dialysis membrane was pretreated with sodium bicarbonate and all glassware was acid washed. The cells were incubated with mixing at 2 °C until equilibrium was reached. Routinely this required 12–14 h in the absence, and 5–7 days in the presence of Ca^{2+}.

To verify the reliability of the ESR method for determining small concentrations of free Mn^{2+}, binding of the metal to Ca^{2+}-Con A was also determined by the following procedure. Dialysis bags containing 2 mL of the Con A solutions were incubated in ten changes of 100 mL of buffer solution containing the appropriate concentrations of Mn^{2+}. Thus, at equilibrium the free Mn^{2+} concentration was equal to the Mn^{2+} concentration of the added dialysate, and measurement of the total Mn^{2+} concentration within the dialysis bags allowed the concentration of bound metal to be calculated. Mn^{2+} concentration in these experiments as well as Ca^{2+} concentrations in Ca^{2+} binding studies were monitored using a Perkin-Elmer 303 atomic absorption spectrophotometer.

Results

ESR Equilibrium Measurements. The peak height of a Mn^{2+} ESR spectrum decreased upon binding to Con A. The ratio of the maximum free Mn^{2+} signal height to the maximum height of a comparable concentration of bound Mn^{2+} was found to be about 15, in good agreement with previous studies (Nicolau et al., 1969; Reed and Cohn, 1970; von Goldammer and Zorn, 1974). The binding also produces a slight shifting of the spectral maxima. The concentration of free Mn^{2+} was therefore readily determined from the amplitude of the ESR signal at a magnetic field corresponding to the low-field maximum of the aquo Mn^{2+} ESR spectrum. Here only a very small contribution from Mn^{2+} bound to Con A was observed. Titration of Con A with Mn^{2+} produced a series of spectra with non-baseline isoclinic points (Figure 1). Marriott and Griffith (1974) demonstrated that non-baseline isoclinic points may be interpreted in a manner similar to isosbestic points in absorption spectra. Although not absolute proof, this strongly suggests that the ESR spectrum is monitoring Mn^{2+} ions in two discrete environments.

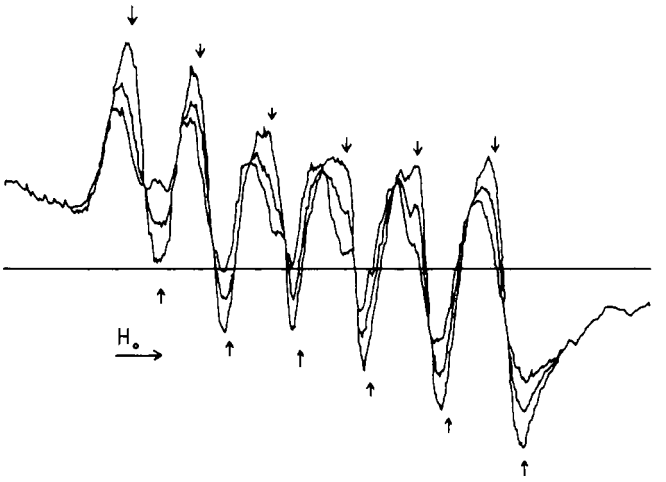

FIGURE 1: ESR spectra of Mn^{2+} bound, in differing proportions, to Con A. Mn^{2+} (1.5×10^{-4} M) and Ca^{2+} (6×10^{-4} M) were preincubated with demetallized Con A (3.2×10^{-4} M). Zn^{2+} (2×10^{-3} M) was used to displace Mn^{2+} from the protein. The series of spectra shown was obtained at various times during the course of the Mn^{2+} displacement. The spectrum with lowest intensity is the earliest and the one with the highest intensity is the latest. The arrows indicate the positions of the maxima (↓) and minima (↑) found in the spectrum obtained from a Con A solution containing a large excess of Mn^{2+}.

Effect of Ca^{2+} on Mn^{2+} Binding. When Mn^{2+} binding to demetallized Con A was analyzed using Hill plots (Van Holde, 1971), the data from ESR and equilibrium dialysis experiments yielded straight line plots with slopes approximately equal to one (Figure 2). This indicated that the Mn^{2+} binding sites on Con A subunits were acting independently. In contrast, when binding isotherms obtained in the presence of Ca^{2+} were analyzed in this way (Figure 2), slopes were consistently greater than one. Hill coefficients obtained in binding reactions at several pHs and in the presence and absence of Ca^{2+} are presented in Table I. Hill coefficients obtained from ESR titrations and equilibrium dialysis experiments were in good agreement indicating the validity of the ESR method for monitoring Mn^{2+} binding.

The data were also analyzed in a manner described by Tanford (1967). This treatment expressed the free energy of association of ligands with a macromolecule in terms of an intrinsic free energy plus an excess free energy

$$\Delta G^\circ = \Delta G^\circ_{int} + RT\phi(\bar{\nu}) \quad (3)$$

or

$$K = K_{int} e^{-\phi(\bar{\nu})} \quad (4)$$

where ΔG° and ΔG°_{int} are the observed free energy and intrinsic free energy of association, respectively. R is the gas constant and T the absolute temperature. K is the observed association constant and K_{int} is the intrinsic association con-

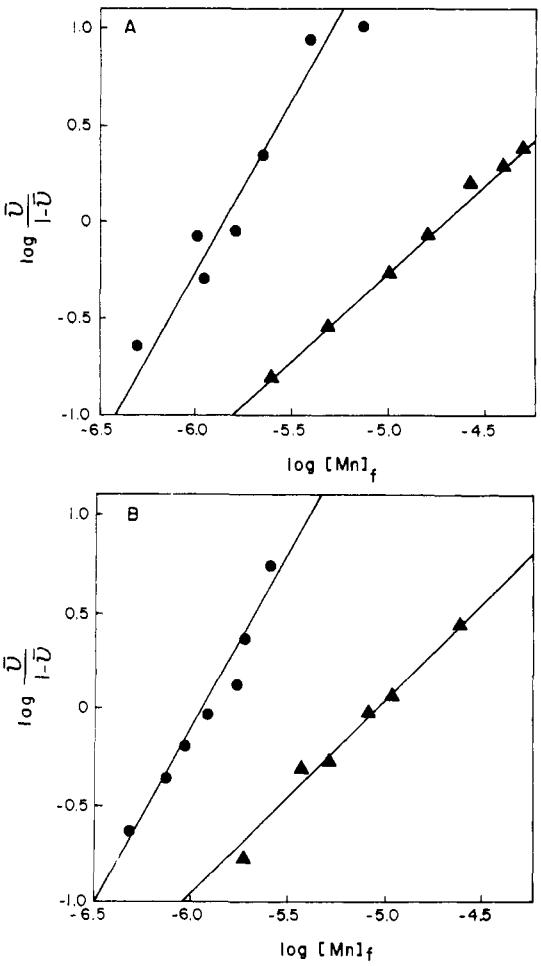

FIGURE 2: Representative Hill plots of ESR titrations (A) and equilibrium dialysis (B) experiments on the binding of Mn^{2+} to Con A. The fraction of occupied binding sites is represented by $\bar{\nu}$. The ESR experiments were carried out at pH 6.02 and 12 °C and the equilibrium dialysis experiments at pH 6.5 and 2 °C. Panel A: (▲) 2.81×10^{-4} M Con A and no Ca^{2+}; (●) 2.62×10^{-4} M Con A and 2.5×10^{-3} M Ca^{2+}. Panel B: (▲) 3.14×10^{-4} M Con A and no Ca^{2+}; (●) 2.12×10^{-4} M Con A and 2.5×10^{-3} M Ca^{2+}.

stant. The fraction of occupied binding sites is represented by $\bar{\nu}$. The interaction parameter, $\phi(\bar{\nu})$, is a completely arbitrary function which may be either an increasing function in the case of negative cooperativity or a decreasing function in the case of positive cooperativity.

The equilibrium expression may be written as

$$\frac{\bar{\nu}}{n-\bar{\nu}} = K(L) = K_{int}e^{\phi(\bar{\nu})}(L) \quad (5)$$

where n is the number of ligand binding sites per monomer and L is the concentration of the free ligand. Each monomer of Con A has a molecular weight of 25 500 and n is equal to one for the monomer. The logarithmic form of this equation is

$$\log K = \log \frac{\bar{\nu}}{1-\bar{\nu}} - \log L = \log K_{int} - 0.434\phi(\bar{\nu}) \quad (6)$$

A plot of log K vs. $\bar{\nu}$ yielded a line whose slope at any point was equal to $\phi(\bar{\nu})$ and whose y intercept is K_{int}. In the case under consideration, $\phi(\bar{\nu})$ was found to be a linear function of $\bar{\nu}$. $RT\phi(\bar{\nu})$ is the increase in free energy of association at a given site that results from the prior association of ligands to neighboring sites. K_{int}, therefore, may be viewed as the association constant for the binding of Mn^{2+} to a Con A dimer before any interacting sites are occupied. Similarly, the value

TABLE I: Hill Coefficients for the Association of Mn^{2+} to Concanavalin A.

pH	Hill coefficients	
	With Ca^{2+}	Without Ca^{2+}
6.85	1.80 ± 0.15[a]	1.05 ± 0.15
6.00	1.60 ± 0.29	0.97 ± 0.15
5.50	1.53 ± 0.26	0.88 ± 0.15
5.00	1.40 ± 0.20	0.95 ± 0.15

[a] Standard deviations obtained in fitting data with a linear least-squares analysis.

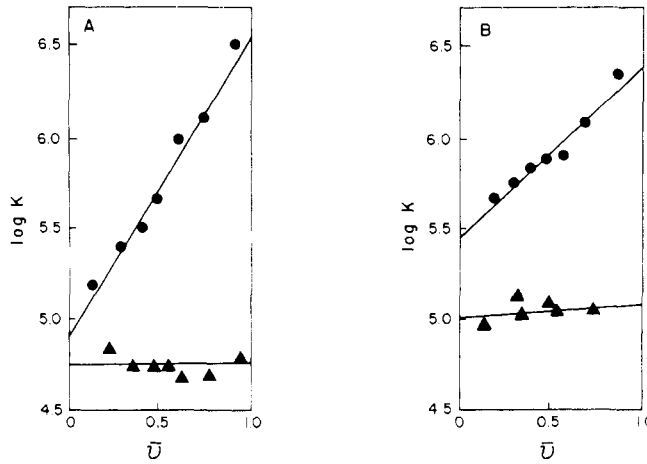

FIGURE 3: Mn^{2+} binding constant, K, as a function of fraction of occupied binding sites, $\bar{\nu}$. The analysis was carried out according to eq 6. Panel A: Results of an ESR experiment at pH 6.02 and 12 °C; (▲) 2.81×10^{-4} M Con A and no Ca^{2+}; (●) 2.62×10^{-4} M Con A and 2.5×10^{-3} M Ca^{2+}. Panel B: Results of an equilibrium dialysis experiment at pH 6.5 and 2 °C; (▲) 3.14×10^{-4} M Con A and no Ca^{2+}; (●) 2.12×10^{-4} M Con A and 2.5×10^{-3} M Ca^{2+}. The experimental conditions are the same as in Figure 2.

of K obtained at $\bar{\nu} = 1$ may be viewed as the association constant for the binding of a second Mn^{2+} ion to the dimer after the first site was occupied.

A representative plot of Mn^{2+} binding data analyzed in terms of an intrinsic association constant and an interaction parameter is displayed in Figure 3. When Mn^{2+} binding to Con A was measured in the absence of Ca^{2+} ions, the slopes of these plots were close to zero. This indicated little or no interaction between sites, as expected for a system showing no cooperativity. In the presence of excess Ca^{2+}, however, positive slopes demonstrating cooperativity were obtained.

The affinity of Mn^{2+} for Con A in the presence or absence of Ca^{2+} varied with pH, increasing as the pH approached neutrality (Figure 4). In the presence of Ca^{2+} the affinity of Con A for Mn^{2+} increased, the sites became cooperative, and metal binding showed a strong pH dependence. Using the treatment of cooperative binding of Mn^{2+} as described above, the intrinsic binding constant was found to change slightly over the pH range 5–7. The affinity of the second Mn^{2+} for Ca^{2+}-Con A and affinity of Mn^{2+} for demetallized Con A, however, were quite pH dependent. It may be important to note that Con A is undergoing a transition from dimer to tetramer through this pH range (Kalb and Lustig, 1968; McCubbin and Kay, 1971).

The effect of temperature on the association of Mn^{2+} with Con A in the absence of Ca^{2+} is shown in Figure 5. A detailed measurement of the temperature dependence of binding constants in the presence of Ca^{2+} was not carried out. Preliminary

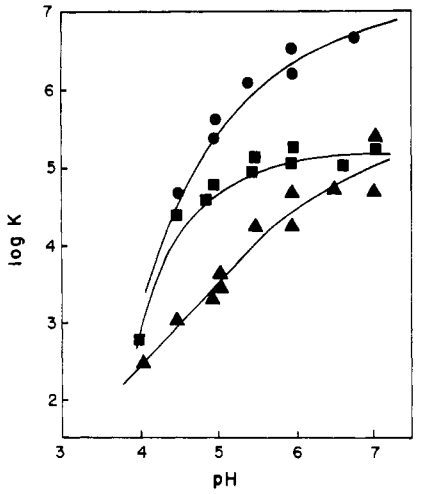

FIGURE 4: The pH dependence of the association constants for the binding of Mn^{2+} to Con A. (▲) Binding of Mn^{2+} to demetallized Con A; (■) binding of the first Mn^{2+} to Con A in a solution containing a tenfold excess of Ca^{2+} with respect to Con A monomers; (●) binding of the second Mn^{2+} to Con A in a solution containing the same Ca^{2+} excess described above. The association constants for the binding of the first and second Mn^{2+} ions to Con A were obtained as described in the text. All solutions were buffered as described in Materials and Methods and maintained between 12 and 16 °C.

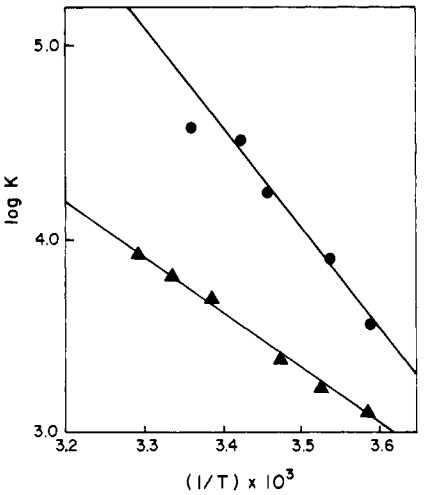

FIGURE 5: Temperature dependence of the association constants for the binding of Mn^{2+} to Con A. (▲) Binding constants obtained at pH 5.1 in the absence of Ca^{2+}; (●) binding constants obtained at pH 5.1 in the presence of a tenfold excess of Ca^{2+} with respect to Con A monomers.

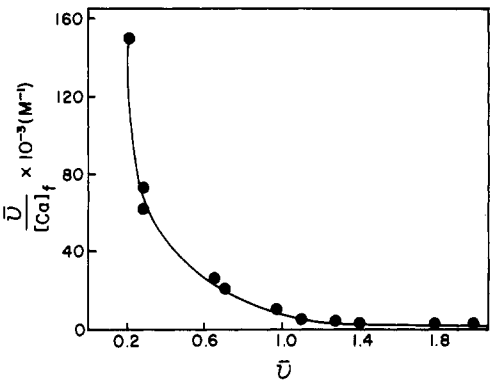

FIGURE 6: Scatchard plot of Ca^{2+} binding to demetallized Con A at pH 7.0. Solution contained Con A (7.2×10^{-4} M), Mops (0.05 M), and NaCl (0.15 M) and was maintained at 2 °C.

TABLE II: Thermodynamic Parameters for Mn^{2+} Binding to Concanavalin A.

	pH	ΔG^a (kcal/mol)	ΔH^b (kcal/mol)	ΔS (cal deg^{-1} mol^{-1})
With Ca^{2+}	5.1	5.62 ± 1.28[c]	25.2 ± 2.1[d]	105.2 ± 12.1[e]
Without Ca^{2+}	5.1	4.38 ± 0.88	13.68 ± 0.51	62.7 ± 4.8

[a] Measured at 15 °C and near saturation with respect to Mn^{2+} and expressed in terms of moles of Mn^{2+} bound. [b] Van't Hoff enthalpies. [c] Listed errors are standard deviations. [d] Listed errors are standard deviations of the slopes of fitted straight lines. [e] Listed errors are estimates derived from uncertainties in ΔG and ΔS.

results, however, were obtained at pH 5.1. Since there is substantial cooperativity in the presence of Ca^{2+} at this pH the results are difficult to interpret unambiguously. Approximate thermodynamic parameters were calculated from these experiments (Table II).

Ca^{2+} Binding by Demetallized Con A. In agreement with previous work (Kalb and Levitzki, 1968; Shoham et al., 1973), we found that Ca^{2+} binding by demetallized Con A was essentially undetectable by atomic absorption techniques at pH 5.2. Equilibrium dialysis experiments conducted at pH 7, however, gave the Scatchard plot (Scatchard, 1949) shown in Figure 6. The Ca^{2+} binding is obviously complex and difficult to interpret; nevertheless, it is clear that Ca^{2+} is bound by demetallized Con A at pH 7.

Discussion

The results presented here indicate a need to reevaluate the biological relevancy of the current theories of metal ion interaction with Con A. Virtually all of the metal binding studies so far reported have been conducted between pH 5 and 6, while many interesting biological properties of Con A, including interaction with cell surfaces, are studied at or near neutrality. Here we report basic differences in the functional properties of Con A between pH 5 and 7. The primary differences are related to the role of Ca^{2+}. The currently accepted model requires that a transition metal ion bind before a Ca^{2+} binding site is available on Con A. The work which led to this model (Kalb and Levitzki, 1968) was conducted at pH 5.2. Our results indicated that the association of Ca^{2+} to Con A is very weak at this pH; however, as the pH approaches 7, Ca^{2+} does bind to demetallized Con A with an appreciable affinity. An average association constant of approximately 6×10^4 M^{-1} at half-saturation can be estimated from our data. It is of particular importance to note that transition metal ion contamination was monitored throughout these experiments and, on a molar basis, was consistently less than 2% with respect to Con A monomers. Scatchard analysis of the Ca^{2+} binding indicates that it is not simple, and, in light of recent results (Richardson and Behnke, 1976), the possibility that there is more than one Ca^{2+} binding site cannot be ruled out. Although our results do not permit a more detailed analysis of the binding, we have shown that Ca^{2+} binds to demetallized Con A in the absence of transition metals.

Preincubation of demetallized Con A with Ca^{2+} causes the nature of Mn^{2+} binding to change. While Mn^{2+} binds to noninteracting sites in the absence of Ca^{2+}, in the presence of this metal the Mn^{2+} binding becomes cooperative. This cooperativity, although present at pH 5, is most pronounced as the pH approaches 7. Since Con A subunits are present in solution as tightly linked dimers, the observation that the Hill coefficients approach two seems quite reasonable. The increasing ability of Con A to bind Ca^{2+} from pH 5 to 7 is probably re-

sponsible for an increasing cooperativity between subunits of the dimer. At present, however, we cannot rule out the possibility that the self-association of Con A dimers which is occurring throughout this pH range may be contributing in some way to the increase in the Hill coefficient. Indeed, the self-association itself may be a result of Ca^{2+} binding.

Preincubation with Ca^{2+}, however, markedly reduces the pH dependence of the binding of the first Mn^{2+} to Con A between pH 4.5 and 7.0. The presence of Ca^{2+}, therefore, favors the proper ionization state of the critical residues linked to Mn^{2+} binding at this first site. This could be due to the presence of Ca^{2+} at the metal binding site causing deprotonation of a residue in the Mn^{2+} site prior to the association of Mn^{2+}, though again we cannot entirely rule out a less direct interaction.

The determination of thermodynamic parameters of Mn^{2+} binding provides some interesting observations. The entropy changes associated with metal binding are particularly noteworthy (Table II). Entropies of ionization and salt bridge disruption are negative and on the order of -5 to -15 cal deg^{-1} mol^{-1} (Kauzmann, 1959). The large positive entropy values observed cannot arise from ionization processes but probably result from rearrangements in protein structure or from dehydration of the protein and the Mn^{2+} ion or from a combination of both possibilities. Crystal structure analysis (Becker et al., 1975) suggests that four waters are released by Mn^{2+} in the binding reaction. Assuming that each water released contributes approximately 8 cal deg^{-1} mol^{-1} (Kauzmann, 1959) to the observed entropy, this cannot account for the entropy change observed with or without Ca^{2+}. The large positive values for the entropy are consistent with a protein rearrangement following Mn^{2+} binding with dehydration of a portion of the protein. The larger ΔS value observed in the presence of Ca^{2+} is consistent with a larger portion of the protein being involved under these conditions.

Our results indicate the need to reexamine the prevalent assumptions about metal binding to Con A. These assumptions are based on studies conducted well below the pH at which biological experiments are performed. Work by Sawyer and co-workers (Sawyer et al., 1974) indicates that Con A undergoes significant conformational changes between pH 5 and 7. Our observations indicate functional consequences of such changes. Results reported here are particularly relevant to the biological activity of Con A. For instance, the strong cooperativity of Mn^{2+} binding to Ca^{2+}-Con A may be very important to the saccharide binding characteristics of the lectin near neutrality. We suggest that the metal binding differences observed between pH 5 and 7 may be quite significant in terms of the ability of Con A to interact with cells at physiological conditions.

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