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Kinetic and Equilibrium Studies of Concanavalin A Activation by Calcium Ions†

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ABSTRACT: The kinetics of conversion of concanavalin A (Con A) to the saccharide-binding conformation by Ca^{2+} alone were studied as a function of pH by monitoring the fluorescence quenching of 4-methylumbelliferyl α-D-mannopyranoside. In the pH range 6.0-7.2, the protein conformational change associated with metal ion binding occurs upon binding of only one Ca^{2+} per P monomer. When sufficient Ca^{2+} is present to saturate the single Ca^{2+} site on P, the rate constant for the locking process, k_2, is independent of pH between 6.4 and 7.2 and furthermore is identical within experimental error with k_2 for Ca^{2+} in the presence of Mn^{2+}. Equilibrium dialysis studies demonstrate that only one Ca^{2+} is bound in the final CPL conformation with a $K_D = 23 \pm 5$ μM at 25 °C. Ca^{2+} has been completely removed from CPL by gel filtration techniques, and the resulting metal-free PL structure has no measurable affinity for saccharides. The near-UV circular dichroism spectrum of CPL is identical with that of native Con A (CMPL) while the spectrum of PL is different from those of both P and CPL. The activation energy for the Ca^{2+}-induced locking process is 14 kcal/mol at both pH 6.4 and pH 7.4 and is completely unaffected by the presence of Mn^{2+} at the higher pH. These data are consistent with a recent crystallographic report [Shoham, M., Yonath, A., Sussman, J. L., Moult, J., Traub, W., & Kalb, A. J. (1979) *J. Mol. Biol. 131*, 137-155] of minor structural differences between native and demetalized Con A. We propose that Ca^{2+} binding at S2 produces a general ordering of the ligands at this site which in turn orders those side-chain residues involved in saccharide recognition.

Concanavalin A (Con A)[1] is a metalloprotein isolated from the jack bean which has been used extensively in the study of cell surfaces. Con A is composed of identical subunits and undergoes a pH-dependent association from a primarily dimeric form below pH 6 to a tetrameric form above pH 7. The metal ions Mn^{2+} and Ca^{2+} are known to play a fundamental role in maintaining the conformational form of Con A which possesses a specific saccharide-binding site. Many of the early metal binding studies were carried out below pH 6 to avoid possible complications arising from the dimer ⇄ tetramer equilibrium (Kalb & Levitzki, 1968; Shoham et al., 1973; Sherry & Cottam, 1973). The results of this early work led

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[1] Abbreviations used: Con A, concanavalin A; EDTA, ethylenediaminetetraacetate; MUM, 4-methylumbelliferyl α-D-mannopyranoside; CD, circular dichroism; C, divalent calcium; M, divalent manganese or any other divalent first-row transition-metal ion where noted; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)-ethanesulfonic acid; αMeMann, methyl α-D-mannoside.

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to a sequential metal ion binding model in which a divalent transition metal ion (such as Mn^{2+}) must first occupy S1 before Ca^{2+} achieves an appreciable affinity for its site, S2. Both of these ions were thought to be necessary for maintaining the conformationally active form of the lectin.

Brown et al. (1977) were first to report that Con A undergoes a slow structural transition from the apo form (P) to the active, saccharide-binding form (CMPL) upon the addition of calcium (C) and manganese (M) and coined the labels "unlocked" and "locked" structural forms for P and CMPL, respectively. They also reported that Mn^{2+} occupies both S1 and S2 at pH 6.4 to initiate a similar structural transition to ultimately yield MMPL. Later studies have shown that Ca^{2+} is also capable of inducing the locking transition to the active structure without prior addition of a divalent transition metal ion (Koenig et al., 1978; Harrington & Wilkins, 1978; Christie et al., 1978; Stark & Sherry, 1979a). Although there is disagreement as to the number of binding sites Ca^{2+} occupies during this locking transition (Koenig et al., 1978; Christie et al., 1978), the resulting structural form displays the same high affinity for saccharides as the native structure, and, like the Mn^{2+} system, the Ca^{2+} ions are labile from their site(s) on PL and may be scavenged by EDTA.

We have previously measured the locking rate constants for a variety of individual metal ions and metal ion mixtures as a function of pH and found that the locking rate constant is sensitive to pH, especially for those ions, i.e., Mn^{2+} or Ca^{2+}, which singularly initiate the locking transition (Stark & Sherry, 1979a). We also erroneously reported that Zn^{2+} or Co^{2+} alone induces the locking transition but later found this was due to a Ca^{2+} contamination of our protein stock solution. Subsequent data indicated that a minor Ca^{2+} contaminant could play a catalytic role in the locking transition and hence prepare the protein to accept those metal ions (Zn^{2+} or Co^{2+}) which do not singularly initiate the locking conformational transition (Stark & Sherry, 1979b).

We report here the results of our further investigations of the Ca^{2+}–Con A system as a function of pH. Our data are consistent with the binding of only one Ca^{2+} ion per protein monomer during the rate-determining locking transition and in the final equilibrium form. Concomitant circular dichroism studies indicate that CPL is structurally very similar to CMPL but different from PL (formed upon removal of Ca^{2+} from CPL by using EDTA). The activation energies measured for the CP → CPL and CMP → CMPL conversions are identical at pH 7.2 but significantly different at pH 6.4.

Materials and Methods

Con A was isolated from jack bean meal, purified of subunit fragments, demetalized by lowering the pH to 1–2, dialyzed against deionized water, concentrated, and stored in unbuffered 1 M NaCl at pH 4, 5 °C. The Con A concentrations were determined spectrophotometrically with $E^{1\%}_{1cm} = 12.4$ and are based upon a monomer molecular weight of 27 000. This apoprotein stock solution was diluted into a 0.075 M buffer solution containing 1 M NaCl to give 0.05 M buffer and 1 M NaCl at the appropriate pH immediately preceding each experiment. The Mes buffer solutions contained enough calcium (as detected by atomic absorption spectrometry) to partially activate the protein, so this contaminant was routinely removed from the buffer by using a Chelex-100 column before the addition of 1 M NaCl. The Pipes buffer solutions and 4-methylumbelliferyl α-D-mannopyranoside (MUM) stock solutions were relatively Ca^{2+} free but were routinely passed through a Chelex-100 column to remove residual metal ion contaminants. The fluorescence quenching of MUM (Stark & Sherry, 1979a; Loontiens et al., 1977) was used as a final criterion for solution purity. In our hands, a pH 7.2 buffer solution containing 100 μM Con A, 3 μM MUM, and as little as 1 μM Ca^{2+} will show a significant quenching of MUM fluorescence with time, and this was used to access the purity of our solutions. The protein and buffer solutions purified by the methods outlined above showed no fluorescence quenching with time (see curve b, Figure 1) and are thus estimated to contain ≤0.2% Ca^{2+} per protein monomer. Equilibrium constants for MUM binding were obtained from the literature (Loontiens et al., 1977; Stark & Sherry, 1979a; Christie et al., 1978; Harrington & Wilkins, 1978). Calcium-45 and [^{14}C]EDTA were purchased from Amersham Corp. The equilibrium dialysis experiments were performed in Lucite cells (0.4 mL on each side of the membrane) by mixing $^{45}Ca^{2+}$ with PL at the appropriate pH and allowing at least 5 h for the $^{45}Ca^{2+}$ to equilibrate across the dialysis membrane. No protein precipitation was evident during dialysis with this experimental procedure. The CD spectra were measured with a Cary 61 spectropolarimeter equipped with a Varian 620 L computer and are reported in terms of molar ellipticity (deg M^{-1} cm^{-1}).

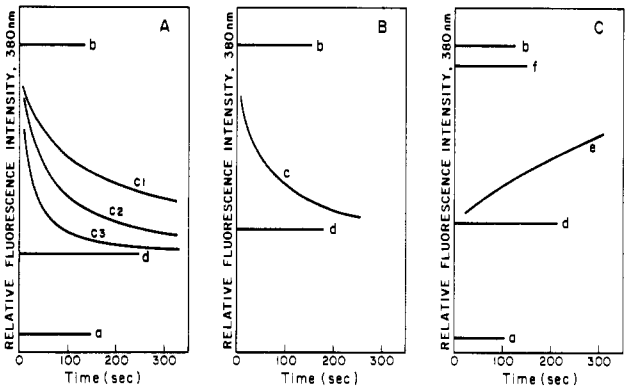

FIGURE 1: (A) Quenching of MUM fluorescence with time upon addition of Ca^{2+} to P (excess [Ca^{2+}] over [P]). (a) Con A (102 μM) in 1 M NaCl and 0.05 M Pipes, pH 6.4; (b) addition of 2.5 μM MUM to solution a; (c1–c3) addition of 1, 2, or 5 mM Ca^{2+}, respectively, to solution b; (d) solutions c1, c2, or c3 at equilibrium. (B) Quenching of MUM fluorescence with time upon addition of Ca^{2+} to P (excess [P] over [Ca^{2+}]). (b) Con A (218 μM) plus 2.5 μM MUM in 1 M NaCl and 0.025 M Pipes, pH 6.9; (c) addition of 25 μM Ca^{2+} to solution b; (d) solution c at equilibrium. (C) Increase in MUM fluorescence with time upon removal of Ca^{2+} from CPL with EDTA. (a) Con A (57 μM) in 1 M NaCl and 0.05 M Pipes, pH 6.4; (b) addition of 2.5 μM MUM to solution a; (d) equilibrium mixture after addition of 150 μM Ca^{2+} to solution b; (e) addition of 1 mM EDTA to solution d; (f) solution e at equilibrium.

Results

Kinetics of Con A Activation by Ca^{2+}. The addition of Ca^{2+} to P above pH 6 in the presence of the fluorescent sugar MUM initiates a slow quenching of MUM fluorescence (Harrington & Wilkins, 1978; Stark & Sherry, 1979a). The results of three separate experiments are illustrated in panel A of Figure 1. Curve b of Figure 1A corresponds to the fluorescence intensity of 2.5 μM MUM in the presence of P before the addition of Ca^{2+} or of 2.5 μM MUM in the absence of P. This verifies that P has no measurable affinity for MUM (Brown et al., 1977). Curves c1, c2, and c3 of Figure 1A are representative MUM fluorescence quench curves which follow the conversion of P to an active, saccharide-binding structural form (CPL) initiated by the binding of one or more Ca^{2+} ions per protein monomer (Brown et al., 1977; Koenig et al., 1978). Curve d of Figure 1A represents the MUM fluorescence remaining at equilibrium after the addition of Ca^{2+} to P. The fact that each of the three curves in Figure 1A reaches the same

fluorescence level at equilibrium indicates that the same amount of active CPL is produced in each solution. Additional Ca^{2+} or a divalent transition metal ion may be added to these equilibrated mixtures without altering the MUM fluorescence intensity further.

The apparent MUM quench rates become independent of the amount of Ca^{2+} added to initiate the process at very high Ca^{2+} concentrations. This indicates that Ca^{2+}–P binding is saturable and is consistent with the model for protein locking first proposed by Brown et al. (1977) for mixed metal ion systems. If one assumes that one or more Ca^{2+} ions are in rapid preequilibrium with P followed by the rate-determining locking transition, i.e.

$$C + P \overset{K_a^M}{\rightleftharpoons} CP \quad (1)$$

$$CP \xrightarrow{k_2} CPL \quad (2)$$

$$CPL + S \overset{K_a^S}{\rightleftharpoons} CPLS \quad (3)$$

then the first-order locking rate constant, k_2, may be extracted from the fluorescence quench data as follows.

Loontiens et al. (1977) have previously shown that MUM fluorescence is completely quenched when bound to native Con A. Koenig et al. (1978), Harrington & Wilkins (1978), and Stark & Sherry (1979a) have previously shown that the equilibrium constant represented by eq 3 is identical for the CPL and CMPL (native Con A where C = Ca^{2+} and M = Mn^{2+}) forms. Therefore, at any time, t, on our quench curves in Figure 1A, the MUM fluorescence intensity, F_t, equals the product of the initial unquenched fluorescence intensity, F_i, and the mole fraction of free MUM in solution, $[S]_f/[S]_T$. It is easily shown that

$$[S]_b \equiv [CPLS]_t = \frac{F_i - F_t}{F_i}[S]_T \quad (4)$$

Substituting this equation into eq 3 gives an expression for CPL in terms of measurable fluorescence intensities at any time t along the quench curve.

$$[CPL]_t = \frac{F_i - F_t}{K_a^S F_t} \quad (5)$$

For a kinetic scheme given by eq 1–3 where the equilibria given by eq 1 and 3 are assumed fast compared to the rate-determining step in eq 2 and the Ca^{2+} concentration is in sufficient excess over that of P to ensure saturation of P, the disappearance rate of CP is given by

$$-\frac{d[CP]}{dt} = k_2[CP] \quad (6)$$

At any time after addition of excess calcium ions to P, the protein may exist in any one of three forms:

$$[P]_T = [CP]_t + [CPL]_t + [CPLS]_t \quad (7)$$

Expressing $[CPL]_t$ and $[CPLS]_t$ in terms of fluorescence, we obtain

$$[CP]_t = [P]_T - \frac{F_i - F_t}{K_a^S F_t} - \frac{F_i - F_t}{F_i}[S]_T \quad (8)$$

and a semilog plot of $[CP]_t$ vs. time gives $-k_2$ as the slope. Under conditions where $[Ca^{2+}]$ is not sufficiently high to saturate P, the fluorescence quench curve is not logarithmic since the left side of eq 8 would contain an additional term in $[Ca^{2+}]_f$ (Cardin et al., 1979). Under these conditions, an apparent pseudo-first-order rate constant, k_{obsd}, is obtained from the initial slope of the fluorescence quench curve.

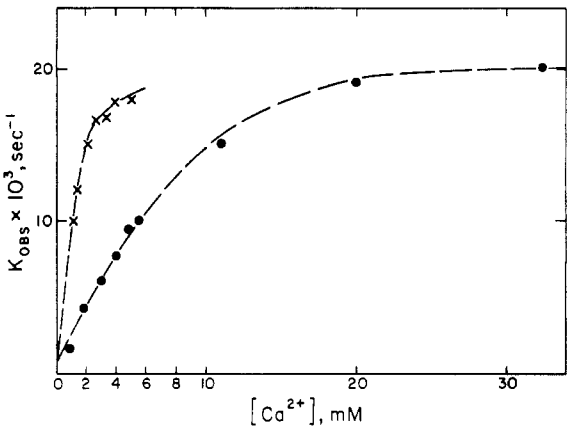

FIGURE 2: Dependence of k_{obsd} on $[Ca^{2+}]$. All samples were 1 M NaCl, 0.05 M Pipes, 102 μM Con A, and 2–3 μM MUM, 25 °C. (×) pH 7.1; (●) pH 6.4.

A plot of k_{obsd} vs. $[Ca^{2+}]$ at two different pH values is found in Figure 2. These curves are similar in form to those presented by Harrington & Wilkins (1978) for the addition of Ca^{2+} to Mn^{2+}·Con A at pH 5 and by Cardin et al. (1979) for the addition of Ca^{2+} to Co^{2+}·Con A at pH 5.3. The data reflect that the Ca^{2+} site(s) on P becomes saturated at a much lower Ca^{2+} concentration at pH 7.2 than at 6.4, and hence the value of K_a^M for Ca^{2+} binding to P must be greater at the higher pH. The curves in Figure 2 may be described by eq

$$k_{obsd} = \frac{k_2 K_a^M[Ca^{2+}]}{1 + K_a^M[Ca^{2+}]} \quad (9)$$

9 where K_a^M represents the association in eq 1 (Harrington & Wilkins, 1978). At saturating Ca^{2+} levels, the product $K_a^M[Ca^{2+}]$ becomes much greater than 1, and k_{obsd} approaches the true first-order locking rate constant, k_2. A fit of the experimental data to eq 9 gives $k_2 = (20 \pm 1) \times 10^{-3}$ s^{-1} and $K_a^M = 175$ M^{-1} at pH 6.4 and $k_2 = (17.9 \pm 1) \times 10^{-3}$ s^{-1} and $K_a^M = 1260$ M^{-1} at pH 7.2. Hence, the first-order rate constant for the conversion of P to the active, saccharide-binding form appears to be independent of pH over this range. This same conclusion was drawn for the mixed metal ion systems between pH 5.3 and 6.4 based on magnetic relaxation dispersion (Brown et al., 1977) and between pH 6.4 and 7.2 with the MUM fluorescence quenching technique (Stark & Sherry, 1979a).

The pH dependence of k_{obsd} measured for a series of solutions containing 79 μM P and 1 mM Ca^{2+} is shown in Figure 3. This concentration of Ca^{2+} will not saturate the site(s) on P at any of these pH values, so if one assumes that k_2 is independent of pH over this range this pH dependence of k_{obsd} must reflect the sensitivity of K_a^M to pH. Our failure to observe measurable rates below pH 6 simply reflects the low affinity of P for Ca^{2+} below this pH value. Other workers have shown that incubation of P with Ca^{2+} for several hours at room temperature produces the active structure even at pH 5 (Richardson & Behnke, 1976; Harrington & Wilkins, 1978).

The reasonable fit of the curves presented in Figure 2 to eq 9 lends support to the hypothesis that only one Ca^{2+} ion need be bound per P monomer to initiate the locking process. We have attempted to gain further evidence for this hypothesis by determining the kinetic order of the reaction with respect to Ca^{2+} under experimental conditions where P is in large excess. One fluorescence quence curve is presented in panel B of Figure 1 for 25 μM Ca^{2+} added to a 218 μM P solution at pH 6.9. Equations similar to those presented above for

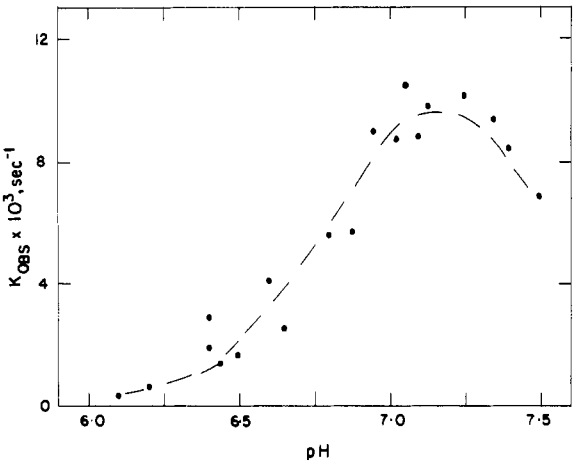

FIGURE 3: Dependence of k_{obsd} on pH. All samples were 1 M NaCl, 0.05 M Pipes, 79 μM Con A, 1 mM Ca^{2+}, and 2.6 μM MUM, 25 °C.

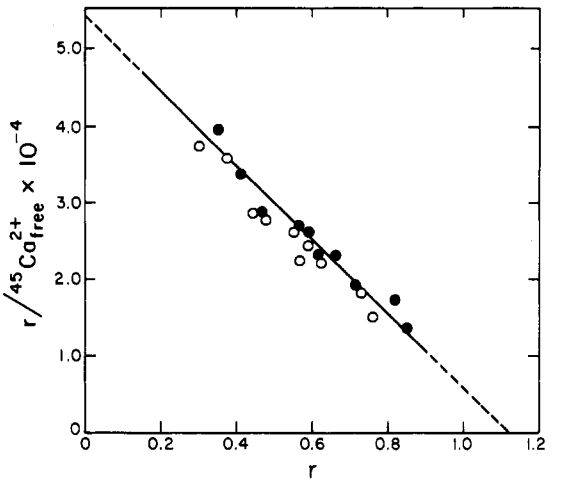

FIGURE 4: Scatchard plot of $^{45}Ca^{2+}$ binding to Con A in the PL structural form. Experiments were conducted in 1 M NaCl and 0.05 M Mes at pH 6.4 and in 0.05 M Pipes at pH 7.2, both at 25 °C.

conditions where the Ca^{2+} concentration is rate limiting clearly show that $-d[CP]/(dt)$ is first order in $[Ca^{2+}]$ over the initial 70% of the fluorescence quench curve. The observed rate constant determined from the data shown is 7.4×10^{-3} s^{-1}. Attempts to increase P to concentrations which would ensure the complete binding of Ca^{2+} have led to slight solution turbity and hence light scattering problems.

Preparation and Characterization of PL. Following the lead of other investigators (Koenig et al., 1978; Harrington & Wilkins, 1978), we have examined the properties of PL prepared by removal of Ca^{2+} from CPL by using EDTA. The addition of 6.5-fold molar excess of EDTA to an equilibrated CPL–MUM mixture results in a near complete reversal of the MUM fluorescence to its original value (Figure 1C, curve e). This corresponds to removal of Ca^{2+} from CPL with a rate constant $k_{off} = 5.8 \times 10^{-3}$ s^{-1}. The final steady-state MUM fluorescence level demonstrated considerable variation from one sample to the next, never reaching the same level of MUM fluorescence as originally observed with P before the addition of Ca^{2+} (compare curves f and b, Figure 1C). This suggested to us, as had been reported earlier (Koenig et al., 1978; Harrington & Wilkins, 1978), that PL retains a small affinity for the saccharide. Several unsuccessful attempts at measuring the PL–MUM binding constant led us to conclude that the residual MUM fluorescence remaining after the addition of EDTA to CPL results from an incomplete removal of metal ions by the chelate. Separation of [^{45}Ca]EDTA from PL by gel filtration under identical experimental conditions, i.e., 1 M NaCl and pH 6.4, as in the fluoresence experiment revealed that 2–5% of the PL retained a bound $^{45}Ca^{2+}$, and this quantity is enough to account for the residual fluorescence shown in Figure 1C, curve f. Metal-free PL could only be prepared by gel filtration of the [^{45}Ca]EDTA–PL mixture through a Bio-Gel P6 column equilibrated and eluted with 1 mM EDTA in 1 M NaCl. PL prepared by this method had no detectable affinity for MUM (an upper limit for the sugar affinity is estimated at 150 M^{-1}) and yet retains the locked structure capable of rapidly binding sugars upon readdition of metal ions (see below). Metal-free PL slowly reverts to the P structure with a half-life of several days at 5 °C (Koenig et al., 1978; Harrington & Wilkins, 1978; Stark & Sherry, 1979a).

The excess EDTA, doped with [^{14}C]EDTA, could be completely removed from metal-free PL in a second gel filtration step by passing the mixture through a Bio-Gel P6 column which had been previously washed with EDTA followed by several washings of ultrapure 1 M NaCl. The use of ultrapure NaCl assured against metal ion recontamination of the protein. The resulting metal-free, EDTA-free PL sample was stored in a plastic container at 4 °C. Equilibrium dialysis experiments were performed by adding $^{45}Ca^{2+}$ to metal-free PL and allowing the excess free $^{45}Ca^{2+}$ to equilibrate across a dialysis membrane. The combined results of two experiments at pH 6.4 and 7.2 are presented in the form of a Scatchard plot in Figure 4. The data indicate that PL has one high-affinity Ca^{2+} site per protein monomer with $K_D = 23 \pm 5$ μM. This value of K_D together with $k_{off} = 5.8 \times 10^{-3}$ s^{-1} calculated from the data presented in Figure 1C allowed us to estimate a second-order rate constant of 252 M^{-1} s^{-1} for Ca^{2+} binding to PL.

The addition of excess Ca^{2+} to PL results in an immediate (within the time of mixing) fluorescence quenching of MUM to the same maximum value as observed for the slow, time-dependent conversion of P in the presence of Ca^{2+} or Mn^{2+} plus Ca^{2+}. Excess Mn^{2+} produces the same rapid quenching of MUM when added to PL, but MnPL does not quench MUM to the same extent as CPL. These observations indicate that metal-free PL prepared by the above procedures retains the locked structure capable of binding with MUM but does so only after metal ions are reintroduced into the PL structure. Mn^{2+} or Ca^{2+} may be incrementally titrated into a PL–MUM sample, and in both cases maximum MUM fluorescence quenching is achieved after the addition of 1 equiv of metal ion per PL monomer. This verifies that indeed only 1 equiv of Mn^{2+} (Christie et al., 1979) or 1 equiv of Ca^{2+} (Christie et al., 1978) is required to yield a fully active, saccharide-binding Con A structure. The protein–MUM equilibrium constants measured with the fluorescence techniques previously outlined (Loontiens et al., 1977; Stark & Sherry, 1979a) for these derivatives are the following: for CPL prepared by adding Ca^{2+} to PL, $(3.2 \pm 0.2) \times 10^4$ M^{-1}; for MnPL prepared by adding Mn^{2+} to PL, $(2.9 \pm 0.2) \times 10^4$ M^{-1}. Other individual metal ions which do not normally initiate the locking transition, i.e., Tb^{3+}, Zn^{2+}, or Co^{2+}, also enhance the affinity of PL for MUM, but this will be the subject of a future paper.

Near-UV Circular Dichroism Spectra. We have examined the near-UV CD spectra of CPL, PL, and other metalized derivatives in an effort to learn more about the conformational differences of the various derivatives. Cardin et al. (1979) have shown that the near-UV spectrum of Con A shows qualitatively different sensitivities to metal ions which bind at S1 vs. those which bind at S2. Several near-UV CD spectra of different forms of the protein at pH 6.4 are shown in Figure 5. Those forms of Con A which have a high affinity for

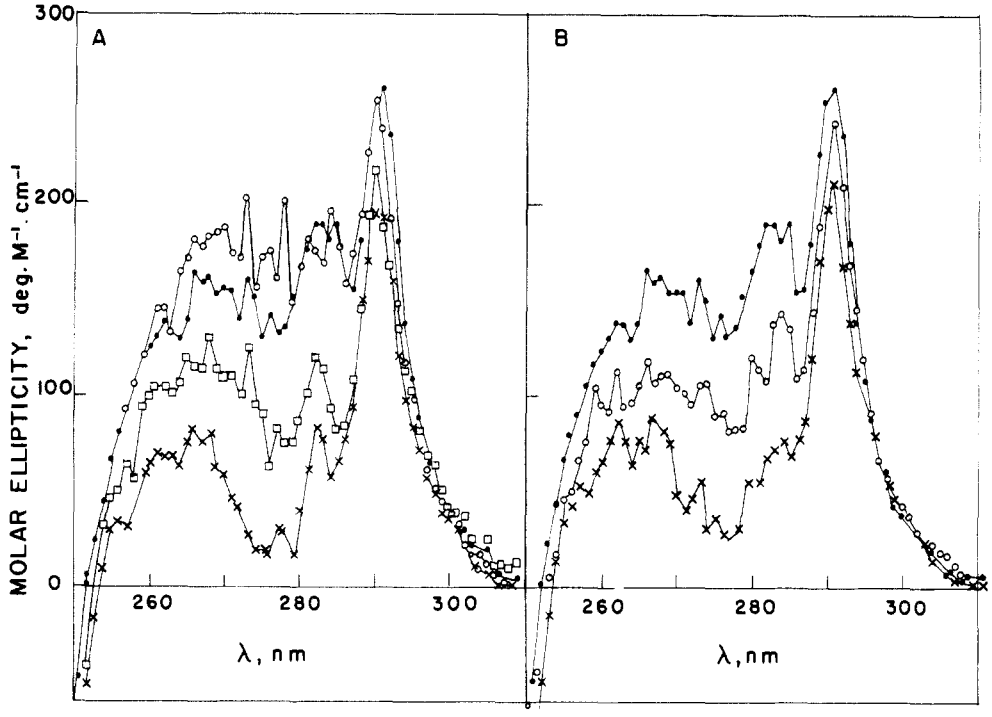

FIGURE 5: Near-UV CD spectra of various forms of Con A. (A) (●) 36.4 μM P; (×) 36.4 μM P and 0.66 mM Ca^{2+} (CPL) or 0.66 mM Mn^{2+} (MnPL); (□) 36.4 μM P, 0.66 mM Ca^{2+}, and 16 mM αMeMann (CPLS, S = αMeMann); (○) 36.4 μM P and 0.66 mM Co^{2+} (CoP). (B) (●) 36.4 μM P; (×) 36.4 μM P and 0.66 mM Ca^{2+} (CPL); (○) 36.4 μM PL. All spectra were recorded 16–20 h after the addition of the appropriate metal ions to Con A, and all samples contained 1 M NaCl and 0.05 M Pipes at pH 6.4, 25 °C.

saccharides (CPL, MnPL, and CMPL, where M = Mn^{2+} or Co^{2+}) have identical CD spectra, quite different from that of CoP, which has no appreciable saccharide affinity (Cardin et al., 1979). The addition of αMeMann to CPL (Figure 5A) results in a general increase in intensity between 260 and 280 nm, very similar to the effect of αMeMann on the CD spectrum of CMPL, where M = Co^{2+} (Cardin et al., 1979). The removal of Ca^{2+} from CPL by EDTA to form PL results in a CD spectrum (Figure 5B) very similar to that shown by CPL + αMeMann. Parallel MUM fluorescence assays carried out on this same mixture showed immediate MUM binding upon readdition of Ca^{2+} to PL. This verifies that protein which generated the middle CD spectrum in Figure 5B was indeed PL and not simply a mixture of P and CPL. The addition of αMeMann to this PL sample resulted in very minor increases in intensity between 265 and 271 nm. The near-UV CD spectra of P, CPL, and PL were also measured at 10 °C (data now shown). All three species showed uniform intensity increases of about 20% throughout the spectral range 250–310 nm, with no significant changes in peak positions.

The difference CD spectra generated from the CD spectra of PL, CPL, CPLS, and P (Figure 6) serve to identify small conformational differences between these species. When Ca^{2+} is present in only one species, the difference CD spectra show a relatively narrow maximum at 292 nm as well as a broader intensity difference below 289 nm (Figure 6A,B). In the difference spectrum for P and PL, there is only a small shoulder at 292 nm (Figure 6C). Thus, the CD spectrum above 290 nm appears to be quite sensitive to the presence of Ca^{2+} and only slightly affected by the conformational differences between P and PL. The spectral effects of αMeMann binding to CPL appear largely below 290 nm (Figure 6D).

Activation Energies. We measured the activation energy for the Ca^{2+}-initiated locking process limited by k_2 under conditions which ensure saturation of the Ca^{2+} site at each temperature, i.e., $K_a^M[Ca^{2+}] > 1$ in eq 9. For purposes of comparison, we also measured the activation energy for the

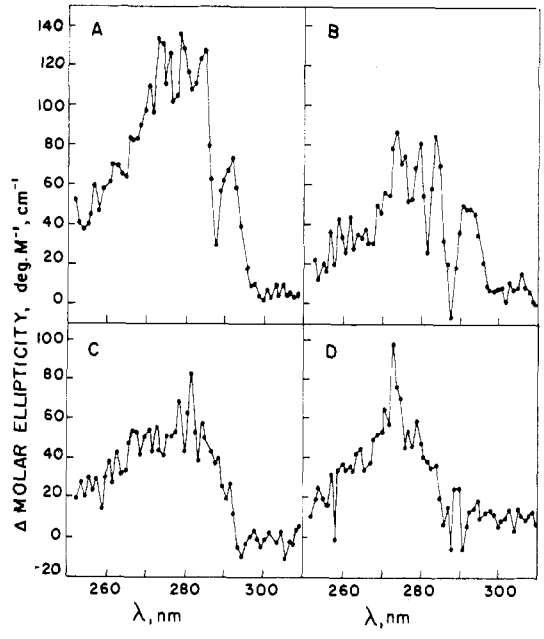

FIGURE 6: Difference near-UV CD spectra generated from the spectra in Figure 5: (A) P – CPL; (B) PL – CPL; (C) P – PL; (D) CPLS – CPL.

locking process initiated with Ca^{2+} plus Mn^{2+}. Arrhenius plots of the kinetic data are shown in Figure 7, and a summary of the activation energies is found in Table I.

At pH 7.2, the presence of Mn^{2+} in addition to Ca^{2+} appears to have no significant effect upon the activation energy for the locking process. Not only is the same concentration of Ca^{2+} required to saturate P in the presence and absence of Mn^{2+} but identical values of k_2 and E_a are obtained for the two samples. At pH 6.4, the same value of k_2 (at 25 °C) is obtained for the Ca^{2+} vs. Ca^{2+} plus Mn^{2+} samples. However, a much greater concentration of Ca^{2+} is required to saturate P in the absence of Mn^{2+} than in the presence of Mn^{2+}, and

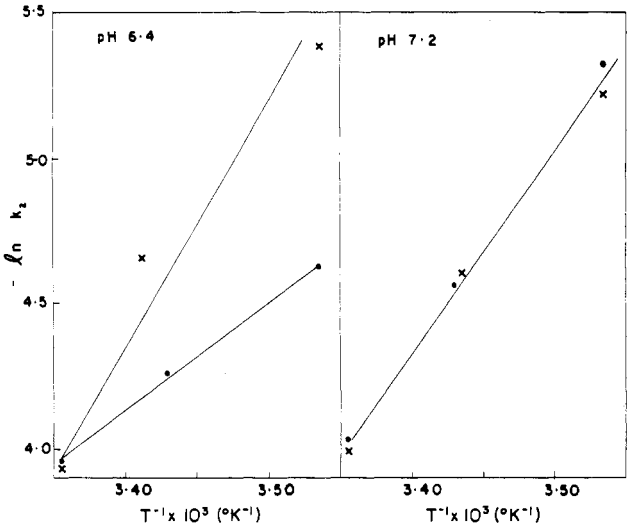

FIGURE 7: Arrhenius plots of the locking rate data. All rate constants were determined in 1 M NaCl, 0.05 M Pipes, 80–100 μM Con A, and 2–3 μM MUM. (A) pH 6.4: (×) 22 mM Ca^{2+}, E_a = 16 kcal/mol; (●) 3 mM each Ca^{2+} and Mn^{2+}, E_a = 7 kcal/mol. (B) pH 7.2: (×) 4 mM Ca^{2+}, E_a = 14 kcal/mol; (●) 3 mM each Ca^{2+} and Mn^{2+}, E_a = 14 kcal/mol.

Table I: pH and Metal Ion Dependence of the Activation Parameters for Conversion of Con A to Its Saccharide-Binding Conformation

pH	metal ion(s) added	metal ion[a] concn (mM) required to saturate P	$k_2 \times 10^3$ (s^{-1}) at 25 °C	E_a (kcal/mol)
6.4	Ca^{2+}	22	20 ± 2	16 ± 2
	Ca^{2+} plus Mn^{2+}	3	20 ± 2	7 ± 2
7.2	Ca^{2+}	4	18 ± 2	14 ± 2
	Ca^{2+} plus Mn^{2+}	3	17 ± 2	14 ± 2

[a] The total concentration of each metal ion species added. In addition, all samples contained 100 μM Con A, 3 μM MUM, 1 M NaCl, and 0.05 M Pipes.

the activation energy appears to be significantly lower for the Mn^{2+} plus Ca^{2+} sample.

Discussion

The data presented above verify earlier reports (Koenig et al., 1978; Harrington & Wilkins, 1978; Christie et al., 1978) that Ca^{2+} alone will convert Con A from the inactive conformation (P) to an active, saccharide-binding conformation (PL). The half-life for this conversion at 25 °C is extremely pH dependent, varying from several hours at pH 5 (Harrington & Wilkins, 1978) to ~35 s at pH 7.2 (this work). This variation is interpreted as reflecting the difference in affinity of P for Ca^{2+} over this pH range. Association constants for Ca^{2+} binding with P are estimated as 175 M^{-1} for pH 6.4 and 1260 M^{-1} for pH 7.2. There are no data in the literature to directly compare these values to, but we can compare them with reported association constants of 313 M^{-1} for Ca^{2+} binding to Co^{2+}·P at pH 5.3 (Cardin et al., 1979), 350 M^{-1} for Ca^{2+} binding to Mn^{2+}·P at pH 5 (Harrington & Wilkins, 1978), and ~1500 M^{-1} for Ca^{2+} binding to Mn^{2+}·P at pH 6.4 (Brown et al., 1977). Thus, the affinity of P for Ca^{2+} increases approximately 6–8-fold when S1 is occupied by a transition metal ion at pH 6.4.

Harrington & Wilkins (1978) and Koenig et al. (1978) first reported activation of Con A by Ca^{2+} in the absence of a transition metal ion. These authors assumed that Ca^{2+} occupies both S1 and S2 during the locking process, although a footnote added to the latter article indicated that they had experimentally verified this assumption. Christie et al. (1978) later challenged this assumption and showed that only one Ca^{2+} ion per P monomer is required to achieve a CPL structure which has one MUM binding site per protein monomer. The kinetic and equilibrium results presented here strongly suggest that only *one* Ca^{2+} is bound during the locking process, and *one* is bound to the final PL structure. It is possible that a second Ca^{2+} ion may bind with CP at very high Ca^{2+} concentrations, but the observation that a stoichiometric quantity of Ca^{2+} with P will completely form the CPL structure above pH 6 (Christie et al., 1978) would argue against the hypothesis that Ca^{2+} must occupy both S1 and S2 for the locking transition to occur.

An interesting feature of the PL structure created by removal of Ca^{2+} from its one site is that a number of individual ions can now occupy a site on PL, presumably the same site formerly occupied by Ca^{2+}, to enhance the affinity of the protein for monosaccharides. We find that PL has little or no affinity for saccharides in the *complete* absence of metal ions. This structural form has a very high affinity for Mn^{2+} (Christie et al., 1979) and for Ca^{2+} (this work), so even minor metal ion contaminations will give PL the appearance of having a weak saccharide affinity. The readdition of 1 equiv of Mn^{2+} *and* Ca^{2+} to metal-free PL rapidly produces CMPL with properties identical with the CMPL structure formed directly from P. Ca^{2+} may be titrated directly into one site on PL to regenerate CPL which displays a similar affinity for MUM as the CMPL structure. The addition of one Mn^{2+} to PL also produces MnPL, but this seems to display a slightly lower affinity for MUM than CPL. This same conclusion was reached by Christie et al. (1979) for MnPL (formed directly by the addition of Mn^{2+} to P at pH 6.4) binding with MUM at 5 °C.

The near-UV circular dichroism spectra of PL show distinct differences from both P and CPL between 260 and 300 nm. Model peptide studies have shown that either one or several aromatic residues could be responsible for the observed differences (Strickland, 1974). The PL – CPL difference spectrum could be due either to localized conformational changes near the metal ion binding region or to a more general loosening of the overall protein conformation. However, the fluorescence assays show that upon addition of metal ion PL attains the sugar-binding conformation much more rapidly than does P, and this argues for relatively small localized differences in conformation between PL and CPL. Since the sugar binding affinity and CD spectra of CPL and CMPL are identical, it appears that these two structures have identical conformations.

A comparison of sugar binding affinities and CD spectra of these various forms as a function of pH leads to an interesting hypothesis. Cardin et al. (1979), working at pH 5.3, found a unique CD spectrum for MP, where M = Mn^{2+}, Ni^{2+}, or Co^{2+}. None of these MP forms have appreciable sugar affinity. The corresponding CMPL species have full sugar affinity and identical CD spectra markedly different from that of MP. Our studies at pH 6.4 show the same correlation between sugar binding affinity and CD spectra with the important difference that the MP group of pH 5.3 is changed. Now MnPL has a CD spectrum identical with those of CPL and CMPL, and all three derivatives display a high sugar affinity. CoP, however, has the same CD spectra at pH 5.3 and 6.4 and does not bind sugars at either pH. One could

hypothesize that, unlike Co^{2+}, Mn^{2+} has an appreciable affinity for S2 above pH 6 and that occupancy of this site is mandatory in initiating the locking process. Pandolfino et al. (1980) have measured the distance between Mn^{2+} and a sugar bound to MnPL at pH 6.6 and concluded that Mn^{2+} binds at S1 in the final equilibrium structure. The observation that Mn^{2+} initiates the locking process at a considerably slower rate than does Ca^{2+} (Harrington & Wilkins, 1978; Koenig et al., 1978; Christie et al., 1980) would be consistent with a weaker affinity of S2 for Mn^{2+}. CPL and MnPL CD spectra at pH 6.4 plus their common ability to bind sugars suggest that CPL and MnPL have identical protein conformations at this pH even though the Ca^{2+} and Mn^{2+} ions may be bound at different sites in the two structures. The slightly greater affinity of CPL over MnPL for MUM may parallel Ca^{2+} binding at S2 in CPL vs. Mn^{2+} binding at S1 in MnPL.

Shoham et al. (1979) have recently reported that the crystal structure of P is only slightly different from the native structure in the metal ion binding regions. One predominant feature in the crystal at pH 5 is a strong charge–charge interaction between His-24 and Asp-19 in P which removes Asp-19 from its normal Ca^{2+}-ligating position in the native structure. This would explain the very low affinity of P for Ca^{2+} below pH 6. As His-24 is titrated above pH 6, the salt bridge to Asp-19 is destroyed, allowing movement of this residue toward the normal Ca^{2+}-ligating position and hence increasing the affinity of P for Ca^{2+} in the absence of Mn^{2+}. The fact that our measured k_{obsd} vs. pH curve (Figure 3) occurs over too narrow a pH range to parallel the deprotonation of a single residue may be due to a combination of effects: (a) the locking rate is too slow to measure accurately below pH 6 with the techniques outlined in this paper, and, therefore, we have not measured the entire pH profile; (b) the protein dimer → tetramer association which takes place over this pH range may influence the measured k_{obsd} values; (c) the higher pH region may be partly obscured by protein denaturation, which is a consistent problem above pH 7. Nevertheless, the change in k_{obsd} and the concomitant change in K_a^M for Ca^{2+} binding to P over this pH range are consistent with the observations of Shoham et al. (1979).

The activation energies determined in this work are significantly smaller than those reported previously for the locking transition at pH 5.3 (Brown et al., 1977; Cardin et al., 1979), at pH 5.6 (Sherry et al., 1978), and at pH 6.4 (Christie et al., 1980). We feel that our earlier reported value may be high because P was not saturated with metal ions under the conditions of those experiments, and hence the temperature dependence of the equilibrium constant represented by eq 1 could contribute to an error in the measured E_a for the locking process. The techniques used by Cardin et al. (1979), Brown et al. (1977), and Christie et al. (1980) to determine the activation energy are more direct than the MUM fluorescence assay employed here in that they do not involve the subsequent sugar-binding step by the locked form. Although we have made provision for the temperature dependence of the sugar affinity, the very presence of the sugar may provide for an alternate mechanism of locking (Koenig et al., 1978). With this qualification in mind that the locking process may be sensitive to the presence of sugar, some interesting molecular interpretations can still be made based on the pH and metal ion dependence of the activation energies measured in this work (Table I).

The observed difference in E_a for the CP → CPL vs. CMP → CMPL processes at pH 6.4 may reflect changes in protonation at or near the S1 binding site that are important at pH 6.4 and below. In the pH range 5–7, the most likely candidate is His-24. With a normal pK_a for His-24, below pH 6 this residue will be protonated and therefore interacting in a charge–charge interaction with the ionized carboxyl of Asp-19 (Shoham et al., 1979). This interaction must be disrupted to free the side chains to serve as ligands to the metal ions. The energy considerations appear to be such that binding of Mn^{2+} results in the disruption of the charge–charge interaction, freeing the Asp-19 side chain to ligand with Ca^{2+}. This would explain the low affinity of P for Ca^{2+} in the absence of Mn^{2+} below pH 6. As the pH is increased and approaches the pK_a of His-24, the charge–charge interaction will weaken, and the affinity of the protein for Ca^{2+} in the absence of Mn^{2+} will increase, allowing Ca^{2+} alone to produce a locked protein conformation. However, the observed difference in E_a for the CP → CPL vs. CMP → CMPL processes at pH 6.4 indicates that Mn^{2+} binding is still a favorable event. As the pH exceeds the pK of His-24, the charge–charge interaction is completely disrupted, and the role of Mn^{2+} in the locking process becomes incidental as shown by its lack of effect on the activation energy at pH 7.2.

A comment is in order about the proposed trans → cis isomerization of a peptide bond in the rate-determining step of the CP → CPL locking process (Brown et al., 1977; Koenig et al., 1978). This proposal was based upon early crystallographic results which suggested that a cis peptide bond might occur between Ala-207 and Asp-208 (Hardman & Ainsworth, 1972) and the similarities between their estimated activation energy for the locking process (~22 kcal/mol) and known cis–trans isomerization activation energies for X–proline peptide bonds (Lin & Brandts, 1978; Roques et al., 1977; Cheng & Bovey, 1977). Reeke et al. (1978) found that the trans configuration between Ala-207 and Asp-208 gave a better fit to the electron-density map of demetalized Con A crystals, and this then would be consistent with a trans → cis isomerization accompanying the locking process. Shoham et al. (1979), however, reported that a cis configuration between these same residues fits their electron-density maps of demetalized Con A very well, and so the question of a correlation between the locking process and a peptide isomerization remains open. As outlined above, the activation energies reported in this work are significantly lower than the values measured previously at lower pH, and this suggests that a trans → cis isomerization process may not be rate limiting in the locking process. However, the activation energies reported here are also subject to possible complications arising from dimer → tetramer protein equilibria which occur in this pH range, and so conclusions regarding the mechanism of locking must await further data. The present data are consistent with the very minor structural differences between the native and demetalized crystal structures recently reported by Shoham et al. (1979). The binding of metal ions in S1 and S2 causes a general ordering or compacting of side-chain ligands around these ions, which in turn orders those residues such as Tyr-12, Tyr-100, Asp-16, Asp-208 and Arg-228 involved in saccharide recognition. Metal ion occupancy of S2 must be critical to this ordering process as Ca^{2+} is capable of initiating this process with S1 unoccupied.

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Kinetics of Binding of Methyl α- and β-D-Galactopyranoside to Peanut Agglutinin: A Carbon-13 Nuclear Magnetic Resonance Study†

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ABSTRACT: The binding kinetics of methyl α- and methyl β-D-galactopyranoside to the anti-T lectin from peanuts were studied by ^{13}C NMR, employing methyl galactopyranosides specifically enriched in ^{13}C at C-1. Association and dissociation rate constants, as well as their activation parameters, are reported. The association rate constants, 4.6×10^4 M^{-1} s^{-1} for the α-galactopyranoside and 3.6×10^4 M^{-1} s^{-1} for the β-galactopyranoside, are several orders of magnitude below those expected for a diffusion-controlled process. For both anomers, the association rate constant was temperature independent, implying that the association process occurs without a significant activation enthalpy. However, a considerable association activation entropy was found for both ligands. The dissociation rate constants were in the range of 9–46 s^{-1} within a temperature range of 5–35 °C for the α-galactopyranoside, and in the range of 9–39 s^{-1} within a temperature range of 5–25 °C for the β-galactopyranoside. A considerable dissociation activation enthalpy of ca. 10 kcal mol^{-1} was found for both anomers. A two-step binding model, consistent with the present NMR data and with previous UV and CD spectroscopic data, is presented.

Considerable information regarding the specificities and dimensions of lectin–carbohydrate binding sites has been obtained from simple inhibition studies (Goldstein & Hayes, 1978; Pereira & Kabat, 1979). From the physicochemical point of view, however, the recognition sites of most of these proteins are still incompletely characterized, and more detailed information from different techniques is needed for an understanding of the mechanism of carbohydrate binding.

The dynamics of lectin–saccharide interactions have been the subject of only a few investigations. NMR has been used to study the binding kinetics of glucosides and mannosides to concanavalin A (Brewer et al., 1972; Grimaldi & Sykes, 1975) and to probe the nature of *N*-acetylglucosamine bound to wheat germ agglutinin (Neurohr et al., 1980a). In addition, stopped-flow measurements on the binding of 4-methylumbelliferyl and *p*-nitrophenyl derivatives of mannopyranosides and glucopyranosides to concanavalin A have been reported, most recently by Van Landschoot et al. (1980a) and Farina & Wilkins (1980), and also a T-jump relaxation study on the binding of *p*-nitrophenyl β-D-galactopyranoside to the lectin from *Ricinus communis* (RCA_I) (Podder et al., 1978).

We recently reported the determination of association constants and thermodynamic parameters for the binding of sugars to peanut agglutinin (PNA)[1] by UV difference spectroscopy (Neurohr et al., 1980b). This protein is a tetramer, of 27 500 protomer weight (Lotan et al., 1975); it has four binding sites and contains one atom each of Ca and Mg per subunit (Neurohr et al., 1980b). Peanut agglutinin is specific

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[1] Abbreviations used: PNA, peanut agglutinin; PBS, phosphate-buffered saline (0.01 M phosphate and 0.15 M NaCl, pH 7.2).

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