Interaction of Calcium and Manganese Ions with Apoconcanavalin A and Sugar Binding†

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ABSTRACT: The interaction of apoconcanavalin A (apo-Con A) with Mn^{2+} and Ca^{2+} was studied at 25 °C using fluorescence stopped flow. The reaction was monitored using 4-methylumbelliferyl α-D-mannopyranoside whose fluorescence is quenched on binding to the metalloproteins. At pH 5.0, entry of Mn^{2+} into apo is second-order (rate constant = 1.2×10^3 M^{-1} s^{-1}); at higher pH the rate constant is $>10^4$ M^{-1} s^{-1}. Reaction of excess Ca^{2+} with Mn(Con A) is pseudo-first-order with $k_{obsd} = Kk[Ca^{2+}](1 + K[Ca^{2+}])^{-1}$. This is interpreted as rapid formation of unlocked MnCa(Con A), with a formation constant $K = 3.5 \times 10^2$ M^{-1}, which transforms ($k = 0.026$ s^{-1}) to a locked form, indistinguishable from native. At pH 6.4 and 7.2, $K \geq 10^3$ M^{-1} and $k = 0.043$ and 0.050 s^{-1}, respectively. Ca(Con A) and Mn(Con A) precipitate glycogen and bind to 4-methylumbelliferyl α-D-mannopyranoside as effectively as native protein at pH 7.2. Treatment of the Ca or Mn forms with EDTA produces an apo form with a small binding capacity, which it loses slowly.

Concanavalin A (Con A)[1] is a lectin from jack bean (Chowdhury & Weiss, 1975; Bittiger & Schnebli, 1976, for reviews). The protein is able to bind specific monosaccharides and as a result can associate with cell surfaces and thus exhibit a large variety of important biological properties (Sharon & Lis, 1972). Each protomer (mol wt 27 000) of Con A contains one calcium and one manganese, both octahedrally coordinated and sharing two aspartate ligands (Edelman et al., 1972; Becker et al., 1975; Hardman & Ainsworth, 1972). A dimeric form exists at pH below 5.5, whereas the protein is mainly a tetramer at pH above 7.0 (Kalb & Lustig, 1968; McCubbin & Kay, 1971; McKenzie et al., 1972; Loontiens et al., 1977a; Huet, 1975; Huet & Claverie, 1978).

It has been believed for some time (Kalb & Levitzki, 1968; Shoham et al., 1973) that the manganese ion site (S1) had to be occupied by a transition metal ion before the second metal site (S2) could be filled by a calcium (or cadmium) ion. These strictures have been cast into doubt as the result of recent work however (Alter et al., 1977; Brown et al., 1977). Occupancy of both sites by the specific metal ions has been believed to be required for saccharide binding (Agrawal & Goldstein, 1968) and agglutination (Inbar & Sachs, 1969) but this concept may also need revising.

Concanavalin A is known to quench completely the fluorescence of 4-methylumbelliferyl α-D-mannopyranoside (MUM) (Dean & Homer, 1973; Clegg et al., 1977; Loontiens et al., 1977a,b), modify slightly the absorption spectrum of *p*-nitrophenyl α-D-mannopyranoside (PNM) (Hassing & Goldstein, 1970; Gray & Glew, 1973; Lewis et al., 1976), and precipitate glycogen at pH 7.0. We have used these characteristics (especially those toward MUM) to study at pH 5.0, 6.4, and 7.2 and 25 °C the sugar binding properties of mixtures of apo-Con A with Mn^{2+} alone and with Ca^{2+} alone. Further, we have studied the rate of interaction of Mn^{2+} and Ca^{2+} with apo-Con A at pH 5.0 using sugar binding as a monitor.

Experimental Section

Materials. Concanavalin A was prepared from Sigma jack bean meal (Agrawal & Goldstein, 1967) and also purchased from Sigma and U.S. Biochemicals. It was necessary to add calcium and manganese ions in millimolar amounts to Sigma grade IV to produce the completely metallated protein. Similar behavior was then noted for all samples of the native protein and apoprotein derived from the various sources. A sample of fragment-free Con A (Cunningham et al., 1972) gave results similar to those for untreated protein and thus the latter was used in the majority of the experiments. The Mn^{2+} and Ca^{2+} concentrations in protein samples were occasionally determined by atomic absorption analysis and corresponded well to those expected, in contrast to the experience of others (Brown et al., 1977). It has been emphasized that care should be taken in checking the metal content of native Con A (Uchida & Matsumoto, 1972; Doyle et al., 1975). Apo-Con A was prepared by acid treatment of the protein (Kalb & Levitzki, 1968; Brewer et al., 1974). The calcium and manganese contents of the apo form used in all experiments were ≤3% as shown by atomic absorption analysis as well as from the amplitude of MUM fluorescence quenching by the apo sample compared with the native form. All equipment was soaked, usually overnight, in 1% EDTA solution. Protein was stored in plastic containers. No Ca^{2+} was leached from glassware over periods of days (Sherry et al., 1975). At pH 7.2, apo-Con A precipitates significantly after a few hours, Mn(Con A) and Ca(Con A) only very slightly overnight, and native not at all for several days. Solutions were filtered prior to use. No precipitation problems were encountered at pH 6.4 or 5.0 for any of the above solutions. Molarities of protein are based on a molecular weight of 27 000 (Edelman et al., 1972) and $E^{1\%}_{1cm} = 12.4$ at 280 nm (Kalb & Lustig, 1968). All other chemicals used were reagent grade. Concentrated stock solutions of $CaCl_2$ and $MnCl_2$ were standardized by EDTA complexometric titration. Deionized water free of traces of metal ions was used throughout.

Measurements. The kinetics of binding of MUM to protein were studied by using a Gibson-Durrum stopped-flow spec-

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[1] Abbreviations used: Con A, concanavalin A; MUM, 4-methylumbelliferyl α-D-mannopyranoside; PNM, *p*-nitrophenyl α-D-mannopyranoside; MM, methyl α-D-mannopyranoside; Mn(Con A) and Ca(Con A) represent a protein or proteins arising from mixing Mn^{2+} or Ca^{2+} ions with apo-Con A. The representation is not meant to indicate necessarily only one metal ion per protomer. MnCa(Con A) represents a protein formed within a few minutes by adding a mixture of Mn^{2+} and Ca^{2+} to apo-Con A.

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trophotometer in the fluorescence mode. The excitation wavelength used was 313 nm and collection was above 360 nm. Measurements with PNM were carried out at 317 nm. Small changes in these wavelengths had no effect on the value of the rate constants measured. For examination of sugar binding, metal-protein solutions were prepared by mixing apoprotein (~0.4 mM) with metal ion (1-20 mM) and leaving at room temperature or at 4 °C until the amplitude of the fluorescence quenching of MUM by a diluted portion of it had reached a maximum. The rates of quenching of MUM (1-3 μM) by the "settled" protein (8-100 μM) were determined. Generally, excellent first-order plots were obtained. The rate constant for dissociation of MUM from the metalloprotein-MUM adduct was determined by treating a settled metal protein (10 μM) and MUM (1 μM) mixture with methyl α-D-mannopyranoside (1-10 mM) in a stopped-flow apparatus. The dissociation is preceded by a much faster step arising from a dilution relaxation of the protein-MUM mixture. In the studies of the formation of Ca or Mn protein, apo-Con A (10-20 μM) was mixed with a metal ion (0.2-5.0 mM)-MUM (1-3 μM) mixture in the stopped-flow apparatus and the rate of fluorescence quenching recorded. Decomposition of freshly formed and aged metal protein solutions (10-20 μM protein; 1-11 mM metal ion) by an EDTA (1-25 mM), MUM (1 μM) solution mixture was measured by the removal of the fluorescence quenching. The binding of MUM is followed by a slow dissociation as metal is removed. With the conditions used, the amplitude of fluorescence quenching on the oscilloscope is linearly related to the concentration of Mn(Con A) or Ca(Con A). A few experiments were carried out using PNM (20-75 μM) instead of MUM. The interaction of aged Mn(Con A) (prepared from 10 μM apo and 1-2 mM Mn^{2+}) with Ca^{2+} ions (0.1-10 mM) was studied at pH 5.0 by methods a-c and at pH 6.4 and 7.2 by a only: (a) by following fluorescence quenching or spectral change on mixing Mn(Con A) with a Ca^{2+}, MUM or PNM mixture; (b) by treating Mn(Con A) with Ca^{2+} ions in a well-mixed solution, and adding EDTA (1-10 mM) at various times [EDTA combines with Ca^{2+} or Mn^{2+} present in all species but the final "locked" MnCa(Con A) form; the concentration of the latter in the quenched solution can be assayed by determining the rate constant for its fluorescence quenching of MUM which is related to the concentration of protein (cf. Figure 1)]; (c) by mixing Mn(Con A) with Ca^{2+} ions and reacting the mixture at various times with MUM solution in a stopped flow apparatus. From the trace amplitude or half-life the amount of locked MnCa(Con A) formed could be calculated as in b. In methods c and b, blanks arise from the binding capacity of Mn(Con A) alone or after EDTA treatment, respectively, and these were allowed for in the determination.

Glycogen precipitation was studied at 420 nm and 26-27 °C. The absorbance change per minute was recorded for 1 mM protein acting on 1 mg of glycogen in 1 mL of solution. The value was 1.3 for native protein at pH 7.2 and all other samples are quoted as percentages of this value.

The ESR measurements were carried out with the assistance of Dr. Alan Van Heuvelen on a Varian 4502 spectrometer. The medium used in all the studies was 0.05 M acetate and 0.3 M NaCl (pH 5.0), 0.1 M Mes and 0.9 M NaCl (pH 6.4), and 0.05 M Tris and 1.0 M NaCl (pH 7.2). A temperature of 25.0 °C was used throughout.

Results

The majority of the experiments utilized the fluorescence quenching which occurs when MUM binds with the various metalloforms of Con A. A few results were obtained using PNM, but the effects were very much smaller. The chromophoric sugars were always present in indicator concentrations. Experiments were carried out at pH 5.0 and 7.2 where Con A is predominantly dimeric and tetrameric respectively (Kalb & Lustig, 1968; McCubbin & Kay, 1971; McKenzie et al., 1972; Loontiens et al., 1977a; Huet, 1975; Huet & Claverie, 1978) and at pH 6.4 to compare with the extensive results of Brown et al. (1977). The aggregative nature of the apo, Mn, and Ca forms of the protein was, however, unknown.

Binding of MUM by Metalloforms. When either Mn^{2+} or Ca^{2+} ions (≥1 mM) were added to apo-Con A (usually 10-20 μM) at any of the pHs examined, within a second (see below) the resultant protein quenched the fluorescence of MUM. On standing at 25 °C, these solutions developed stronger quenching capacity as shown by the amplitude of the stopped-flow traces. The times involved for the development of maximum binding (quenching) decreased from hours at pH 5.0 to minutes at other pHs. Generally, Ca(Con A) "settled" quicker than Mn(Con A). For example, at pH 6.4, the approximate half-lives for settling were 2.5 min (Ca^{2+}) and 6.0 min (Mn^{2+}). These changes were examined in the absence of MUM, although concentrations of the latter were so small in all the studies described that it had no effect on the observed behavior. It was found that apo-Con A, both freshly prepared and left for one week at room temperature (Brown et al., 1977) on treatment with Mn^{2+} ion at pH 5.0 required hours for the MUM binding characteristics to become reproducible. Addition of both Mn^{2+} and Ca^{2+} in either order to apo protein gave within minutes at all pHs a constant quenching of MUM fluorescence which was indistinguishable from that of native.

The procedure used to measure k_1 and k_{-1}, the rate constants for the formation and dissociation of the metal-Con A adducts with MUM:

$$P + MUM \rightleftarrows P{\cdot}MUM \qquad k_1, k_{-1}, K \qquad (1)$$

closely followed that used for Con A itself (Clegg et al., 1977; Loontiens et al., 1977b). For the binding of MUM to the protein P, in excess, represented as a single step (Lewis et al., 1976; Clegg et al., 1977; Loontiens et al., 1977b):

$$k_{obsd} = k_1[P] + k_{-1} \qquad (2)$$

where k_{obsd} is the first-order rate constant for the approach of fluorescence quenching to its equilibrium position. Plots of k_{obsd} vs. [P] were linear (Figure 1) from which values of k_1 and k_{-1} were calculated. The value for k_{-1} was obtained, more accurately, by removing MUM from the protein with an excess of methyl α-D-mannopyranoside (MM) and observing the rate of return of MUM fluorescence.

$$P{\cdot}MUM \rightleftarrows P + MUM \qquad k_{-1}, k_1 \qquad (3)$$

$$P + MM \rightarrow P{\cdot}MM \qquad k_2 \qquad (4)$$

Provided $k_2[MM] \gg k_1[MUM]$ and P·MM is fully formed, then the first-order rate constant return of fluorescence, k_{obsd}, can be equated to k_{-1}. These conditions were easily shown to apply by finding that k_{obsd} was independent of [MM] from 1 to 10 mM. MUM binding characteristics for all equilibrated forms of metalloprotein examined are shown in Table I. The results for Mn(Con A) and Ca(Con A) were independent of the concentrations of free metal ion in solution provided these exceeded 1 mM. The data for native Con A and equilibrated MnCa(Con A) are identical and in excellent agreement with those recently reported for the native (Clegg et al., 1977; Loontiens et al., 1977b). They show that dimeric and tetrameric Con A have almost identical MUM binding parameters.

TABLE I: Binding of Metalloforms of Concanavalin A to MUM.

	Mn(Con A)			Ca(Con A)			MnCa(Con A)		
pH	$10^{-5}k_1$ (M^{-1} s^{-1})	k_{-1} (s^{-1})	$10^{-4}K$ (M^{-1})	$10^{-5}k_1$ (M^{-1} s^{-1})	k_{-1} (s^{-1})	$10^{-4}K$ (M^{-1})	$10^{-5}k_1$ (M^{-1} s^{-})	k_{-1} (s^{-1})	$10^{-4}K$ (M^{-1})
5.0	2.5	9.0	2.8	0.44	3.6	1.2	1.2 (1.1)[b]	4.8 (3.4)[b]	2.5 (3.3)[b]
6.4	2.3	8.0	2.9	1.1	2.8	3.9	1.1	3.4	3.2
7.2	2.0	6.6	3.0	0.90 (0.94)[a]	2.5 (2.7)[a]	3.6 (3.5)[a]	1.0 (1.0)[c]	3.1 (3.2)[c]	3.2 (3.2)[c]

[a] Values using freshly formed material. [b] Values at 24 °C and pH 5.5 (Clegg et al., 1977). [c] Values at 24.1 °C (Loontiens et al., 1977b).

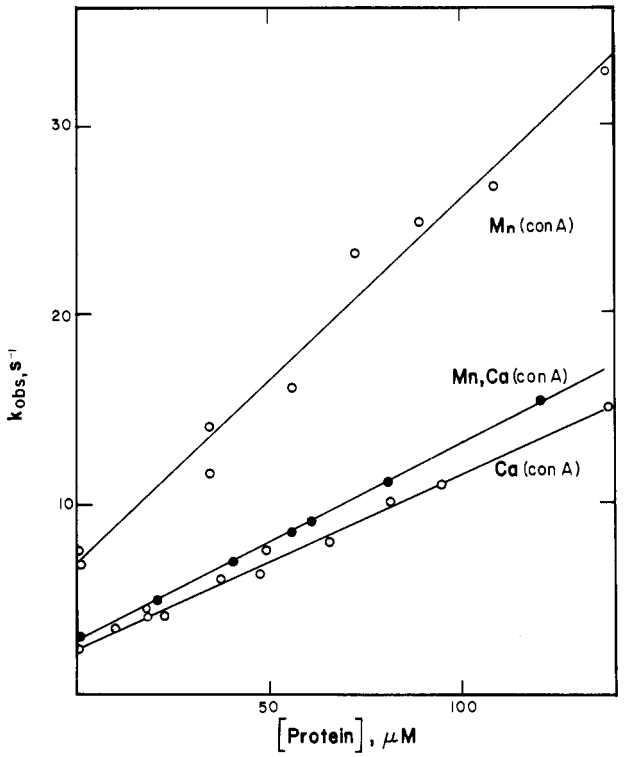

FIGURE 1: Plots of k_{obsd} (s^{-1}) vs. [protein] for binding to MUM by various metalloforms of Con A at pH 7.2 and 25 °C.

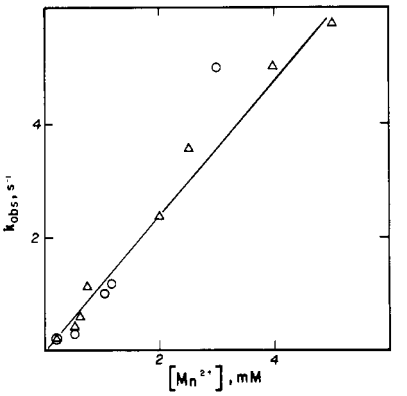

FIGURE 2: Effect of $[Mn^{2+}]$ on k_{obsd} s^{-1} for the formation of Mn(Con A) from apo-Con A at pH 5.0 and 25 °C. MUM (1-3 μM), △; PNM (20-80 μM), ○; Apo, ~20 μM.

Our data for PNM binding by Con A (not shown) also agree with literature values (Lewis et al., 1976). PNM was used mainly to check trends observed with MUM (see Figures 2 and 3).

Glycogen precipitation did not occur in the presence of any metalloprotein, including native, at pH 5.0 but was rapid at pH 7.2. For Mn(Con A) the rate was 75% and for Ca(Con A) it was 52% of that of the native.

Interaction of Ca^{2+} and Mn^{2+} Ions with Apo-Con A. We were unable to determine directly the changes in situ of the concentration of free metal ion or protein-bound metal ion. Attempts using metal indicators (murexide and metalphthalein), calcium-ion selective electrodes, direct UV spectral changes (Doyle et al., 1975) and pH changes (Gachelin et al., 1972) were unsuccessful. The changes were either too small in magnitude or the response of the electrodes too sluggish to function effectively. Indicators unfortunately interacted with the protein. It was necessary therefore to use sugar binding differences of apo- and metallated protein to monitor metal binding, although there were some ambiguities in their use. When Ca^{2+} was reacted with an apo-Con A/MUM mixture at pH 6.4 and 7.2, the stopped-flow fluorescence quenching traces were identical irrespective of metal ion concentration (≥1.0 mM). A similar result was obtained with a series of Mn^{2+} concentrations (0.1-5.0 mM). The fluorescence quenching of MUM is therefore limited by the rate of its binding to protein, and not by the formation of the metal-protein bond. From these results, it could be estimated that the second-order rate constant for reaction of these metal ions at pHs 6.4 and 7.2 with apo-Con A was $\geq 5 \times 10^4$ M^{-1} s^{-1}. However, the binding of Mn^{2+} ions (0.2-10 mM) to apo protein at pH 5.0 was slower than MUM binding to freshly prepared Mn(Con A) and thus the fluorescence quenching of MUM could be used to monitor the formation of Mn(Con A).

$$Mn^{2+} + \text{apo-Con A} \rightleftarrows Mn(\text{Con A}) \qquad k_2, k_{-2} \quad (5)$$

$$Mn(\text{Con A}) + \text{MUM} \rightleftarrows Mn(\text{Con A})\cdot\text{MUM} \qquad \text{rapid} \quad (6)$$

$$k_{obsd} = k_2[Mn^{2+}] + k_{-2} \quad (7)$$

The linear relationship between k_{obsd} (pseudo-first-order rate constant for MUM quenching) and $[Mn^{2+}]$, used in excess over apo-Con A, is shown in Figure 2 which also includes some data using PNM as monitor. The value of k_2 is 1.2×10^3 M^{-1} s^{-1} and $k_{-2} \leq 0.5$ s^{-1}. The small fluorescence quenching observed when Ca^{2+} ions were added to apo-Con A at pH 5.0 precluded accurate measurements of a similar kind to that of Mn^{2+}. However, results indicated that calcium ion binding was faster than sugar binding at $[Ca^{2+}] = 10$ mM but not at $[Ca^{2+}] = 1$ mM, so that it appears that Ca^{2+} too reacts more slowly at the lower pH.

Interaction of Ca^{2+} Ions with Mn(Con A). A complete study was possible at pH 5.0. Mn(Con A) which had been allowed to equilibrate was treated with Ca^{2+} ions in the presence of MUM. A fast fluorescence quenching, due to production of the Mn(Con A)·MUM adduct (previously examined), was followed by a much slower fluorescence quenching leading finally to a protein form indistinguishable from that of native. This slow change was nicely first-order. The plot of the first-order rate constant, k_{obsd}, vs. $[Ca^{2+}]$ is shown in Figure 3. Changing the $[Mn^{2+}]$ from 1 to 10 mM or replacing MUM by PNM had no effect on the rate constants. The reaction was

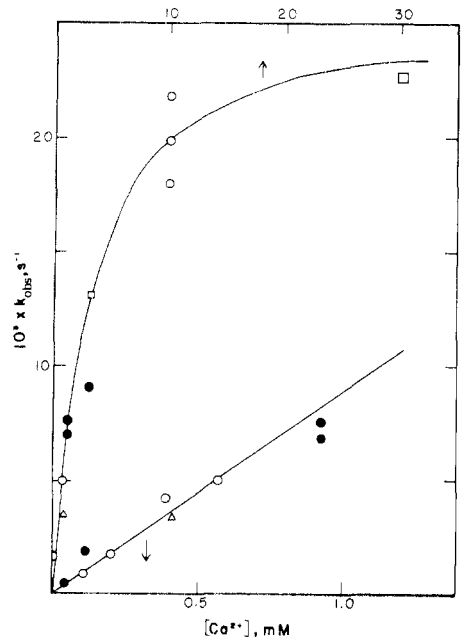

FIGURE 3: Effect of $[Ca^{2+}]$ on k_{obsd} (s^{-1}) for the formation of CaMn(Con A) from Mn(Con A) at pH 5.0 and 25 °C. Mixture of Mn^{2+} (1–10 mM) and apo-Con A (10 μM) left overnight, treated with various concentrations of Ca^{2+} and sugar binding monitored. MUM: (●) method a; (○) method b; (△) method c. PNM: □ method a. The solid curves corresponds to eq 10; see text. The lower curve refers to lower $[Ca^{2+}]$, the upper curve to the whole range of $[Ca^{2+}]$ used.

also studied by methods (see Experimental Section) in which MUM was not present in the reaction mixture, but was used to monitor the formation of the final MnCa(Con A) form. All methods gave consistent results (see Figure 3).

The behavior observed is consistent with a scheme:

$$\text{Mn(Con A)} + Ca^{2+} \rightleftharpoons \underset{\text{intermediate form}}{\text{MnCa(Con A)}} \qquad K \qquad (8)$$

$$\text{MnCa(Con A)} \rightarrow \underset{\text{final form}}{\text{MnCa(Con A)}} \qquad k \qquad (9)$$

if the intermediate form binds sugar comparable to that of Mn(Con A) but significantly less than does the final form (identified with native). The second step is irreversible since formation of the final form is complete at all Ca^{2+} concentrations used down to 0.1 mM. For this scheme:

$$\frac{d \ln [\text{final form}]}{dt} = k_{obsd} = \frac{Kk[Ca^{2+}]}{1 + K[Ca^{2+}]} \qquad (10)$$

The solid line in Figure 3 describes eq 10, with $K = 3.5 \times 10^2$ M^{-1} and $k = 0.026$ s^{-1}. At high $[Ca^{2+}]$, the intermediate form is rapidly produced and the transformation of this to the final species is then observed ($t_{1/2} = 26$ s). Some idea of the relative labilities of the intermediate and final forms is shown by an experiment in which 0.2 M EDTA was added 20 s after mixing Mn(Con A) (20 μM apo; 2 mM Mn^{2+}) and Ca^{2+} ions (20 mM). The solution was analyzed (vs. MUM) and 35–40% of the final native concentration was found. This indicated that all the forms but the final one are decomposed by EDTA.

At pH 6.4 and 7.2, the values of K were larger than 10^3 M^{-1} since the limiting region had been reached even at as low concentrations of Ca^{2+} as 1 mM. The corresponding values of k are 0.043 and 0.050 s^{-1} respectively.

Reaction of Metalloforms with EDTA and Properties of the Residual Protein. Addition of EDTA to Mn(Con A) and Ca(Con A) at all pHs[2] quickly reduced MUM binding to the proteins and at pH 7.2 removed their ability to precipitate glycogen. The loss of MUM binding (as shown by the return of fluorescence) on adding EDTA to equilibrated metal–Con A samples was first-order with rate constants, s^{-1}: (for Mn) 0.1 (pH 5.0), 0.069 (pH 6.4), and 0.046 (pH 7.2); and (for Ca) 0.03 (pH 5.0), 0.01 (pH 6.4) and 0.007 (pH 7.2). These values were independent of [EDTA] from 1 to 25 mM. PNM gave similar results. ESR experiments showed that 90 ± 10% of Mn(Con A) was converted into $Mn(EDTA)^{2-}$ within 3 min of adding EDTA at pH 5.0 and 7.2.[3] Addition of EDTA to Ca(Con A) and more especially Mn(Con A) did not destroy all the MUM binding tendency. Typically, some 10–30% of the original would remain (judging from amplitudes) and over a period of some time only was all binding lost. The protein remaining after treatment of Mn(Con A) with EDTA lost its sugar binding with a half-life of about 2 h at pH 5.0 and faster at higher pHs. Addition of EDTA to either the equilibrated CaMn(Con A) or the native protein at pH 5.0 or 7.2 had no effect, for at least several hours, on its ability to bind MUM.

Discussion

Metalloforms of Con A and Sugar Binding. It is well established that manganese ions (and other transition metal ions) bind to apo-Con A even in the absence of Ca^{2+} ions and the binding constants have been measured by a variety of methods (Gachelin et al., 1972; Shoham et al., 1973; Sherry & Cottam, 1973; Alter et al., 1977; Brown et al., 1977). It is also clear that Mn^{2+} binds stronger at higher pH (Nicolau et al., 1969; Sherry et al., 1975; Alter et al., 1977; Brown et al., 1977). If the first-order loss of MUM binding by Mn(Con A) on adding EDTA represents dissociation into Mn^{2+}, as the ESR experiments suggest, then the value of 0.1 s^{-1} for k_{-2} (eq 5) taken in conjunction with k_2 (1.2×10^3 M^{-1} s^{-1}) leads to a binding constant of 1.2×10^4 M^{-1} at pH 5.0. The value of k_2/k_{-2} is $>10^4$ at pH 6.4 and 7.2. These values are certainly of the correct order of magnitude (e.g., 6×10^3 (pH 5.0); 4×10^5 (pH 7.0), Alter et al., 1977). They indicate that all the protein is manganese bound in our MUM binding experiments and the independence of the binding rate constants k_1 and k_{-1} on concentration of Mn^{2+} (≥ 1 mM) strongly suggests that we are examining *one* species. This is probably a singly bound Mn^{2+} at pH 5.0 and a doubly bound Mn^{2+} (at S1 and S2) at pH 6.4 and 7.2 from the work of Brown et al. (1977).

The few reports on the interaction of Mn^{2+}–Con A protein with sugars are somewhat contradictory. Mixtures of Mn^{2+} or other transition metal ions with apo-Con A are reported to give a limited increase in saccharide activity (Kalb & Levitzki, 1968; Shoham et al., 1973; Sherry et al., 1975; Grimaldi & Sykes, 1975). It has been suggested that this may be caused by incomplete isomerization of the protein to the final conformation (Brewer et al., 1974) or to the fact that most reported work is at pH 5.2 (and higher pH favors sugar binding; Alter et al., 1977). Mn^{2+} ion added to apo is a fully active protein (towards mannan) if left a few hours (Richardson & Behnke, 1976) and toward agglutination at pH 7.0 (Agrawal & Goldstein, 1968a,b).

Our findings are that Mn(Con A) has a substantial ability

[2] The stability constants and the formation rate constants of EDTA complexes of Ca^{2+} and Mn^{2+} are sufficiently large for EDTA to be an effective scavenger of released metal ions from the protein forms.

[3] The relative signal amplitudes of solutions of 0.4 mM Mn^{2+}, native Con A, and Mn(Con A) are 13:2:1 in good agreement with recent findings (Alter et al., 1977).

to quench the fluorescence of MUM (Table I) and, since MM can remove this effect, both sugars probably bind at a common site. This is about 13 Å from the manganese site (Brewer et al., 1973; Hardman & Ainsworth, 1976; Becker et al., 1976). At pH 7.2, Mn(Con A) has the ability to precipitate glycogen, comparable in rate to that of the native.

Only recently has it been reported that Ca^{2+} binds to apo-Con A, weakly at ph 5.0 and quite strongly and in a complex manner at pH 7.0 (Alter et al., 1977). Very slowly, calcium ion added to apo-Con A induces mannan precipitation (Richardson & Behnke, 1976). Our results show that a mixture of Ca^{2+} and apo-Con A left for some time develops strong MUM binding characteristics at all pHs (Table I).[4]

The Binding of MUM to Ca(Con A) at pH 6.4 and 7.2 Is Particularly Striking Showing Quite Similar Characteristics to Those of Native. Indeed the addition of Mn^{2+} to Ca(Con A) does not change the fluorescence quenching flow trace even though native protein is formed (Figure 1).[4] The difference is in the effect of EDTA which removes the capacity for MUM binding from Ca(Con A) but leaves native protein unaffected. Once again, our data indicate we are dealing with one protein species and, at pH 6.4 and 7.2, this is likely to have Ca^{2+} at both sites.[4] The Ca^{2+}-apo-Con A mixture agglutinates glycogen quite effectively at pH 7.2.

Interaction of Ca^{2+} and Mn^{2+} Ions with Apoprotein. The decrease in the value for the rate constant for the formation of Mn(Con A) at pH 5.0 compared with pH 6.4 and 7.2 is a behavior noted with metal ion interaction with apocarbonic anhydrase and apocarboxypeptidase (Billo et al., 1978) and with simpler ligands, in general, and reflects the reduced reactivity associated with the protonated form. The reaction between Mn^{2+} and apo-Con A remains second-order up to 10 mM [Mn^{2+}] (Figure 2) and this indicates that the entry into site S1 is being measured. The reaction between "aged" Mn(Con A) and Ca^{2+} ions is particularly interesting. From the change with time of the T_1^{-1} of water protons under the influence of the manganese, after apo-Con A was mixed with Mn^{2+} and Ca^{2+}, the scheme in eq 8 and 9 was proposed (Grimaldi & Sykes, 1975; Brown et al., 1977). The intermediate was termed the "unlocked" and the final form, identical with native, was referred to as the "locked" protein (Brown et al., 1977). At pH 5.3 and 23 °C, the values of K and k were estimated as $\sim 10^3$ M^{-1} and 1×10^{-2} s^{-1}, respectively. Our values, using sugar binding as a monitor of events at the metal site, are in quite satisfactory agreement with these and indicate that the alterations at the manganese site responsible for solvent proton relaxation (Brown et al., 1977) trigger the changes at the sugar site which lead to the correct conformation for sugar binding. At pH 6.4 and 7.2, the limiting region only is observed even at [Ca^{2+}] as low as 1 mM. The calcium ion must rapidly and substantially produce the unlocked form, but marked MUM binding has to await the conformational change in eq 9. The rate constant for this process is almost pH independent (see also Brown et al., 1977). The increase in MUM binding by solutions of Mn(Con A) or Ca(Con A) on standing presumably also reflects an unlocked to locked transformation, although our results would indicate that the unlocked form can bind to a limited extent. The locking speeds for the metalloprotein forms at all pHs are in the order CaMn(Con A) > Ca(Con A) > Mn(Con A). The slow time course for Mn(Con A) has been ascribed to weak Mn^{2+} binding at S2 and a higher energy barrier for the transition (Brown et al., 1977). Previous investigators have stressed the need to leave Mn^{2+} in contact with apo-Con A for considerable times before reproducible behavior is observed (Alter et al., 1977; Brown et al., 1977). The speeding up to the final MUM form when a Ca^{2+}/Mn^{2+} mixture, rather than either ion alone, is added to apo is undoubtedly related to the observation that Ca^{2+} increases the binding of the manganese (Sherry & Cottam, 1973; Alter et al., 1977; Brown et al., 1977), alters the ESR of the manganese (Nicolau et al., 1969) and accentuates UV differences (Doyle et al., 1975). In addition there is an attendant overall change in protein conformation (Barber and Carver, 1975; Grimaldi and Sykes, 1975; Richardson and Behnke, 1976; Alter et al., 1977; Brown et al., 1977). The protein remaining after EDTA treatment of the Mn or Ca forms and, which has residual MUM binding, may correspond to a recently reported metastable apo-Con A which binds to MM with an affinity approximately 10% of the double Ca^{2+} locked form (Koenig et al., 1978).

[4] Koenig et al. (1978) report that from the effects of added α-MM on the rate of Mn^{2+} displacement of Ca^{2+} from the S1 site of locked (our aged) Ca(Con A) that the latter binds saccharide as strongly as native ($K \sim 10^4$ at pH 6.4). At high [Ca^{2+}], the metal occupies both sites and can induce the unlocked to locked conformational change.

Note Added in Proof

After this manuscript was submitted, Sherry et al. (1978) reported first-order conformational rate constants (corresponding to k) using PNM and polarography. Binding ability to PNM of the apoprotein formed by adding EDTA to metal forms of Con A was also demonstrated. D. J. Christie, G. M. Alter, and J. A. Magnuson (manuscript in preparation) have shown similar binding constants of MUM to Ca(Con A) as to native at pH 7.0.

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