Vol. 87, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1979 Pages 598-604

METAL ION INDUCED CONFORMATIONAL CHANGES IN CONCANAVALIN A: EVIDENCE FOR SACCHARIDE BINDING TO ONE METAL FREE STRUCTURE

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Received February 23, 1979

SUMMARY

The addition of Mn^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+} or Pb^{2+} to apo-concanavalin A results in a slow conformational conversion of the protein to the active saccharide binding form. The rates of conversion are dependent upon the sample pH and identity of the ions which occupy the native transition metal and calcium ion sites yet the affinity of each metalloform for the fluorescent sugar, 4-methylumbelliferyl-α-D-mannopyranoside, is independent of these same parameters (above pH 5.6). EDTA quickly removes all metal ions from the active Mn^{2+} or Co^{2+}-concanavalin A samples leaving a metastable metal free structure which retains its high saccharide affinity for several hours at room temperature. This form of apo-concanavalin A and the metallized derivatives have equally high saccharide binding affinities in 1M NaCL but the former dramatically loses its sugar affinity as the ionic strength is lowered.

The plant lectin, concanavalin A (Con A), contains one divalent manganese and one divalent calcium ion per monomeric subunit in its native state (1). The only known function of these ions is the stabilization of a polypeptide loop (residues 11-23) near the N-terminal region of the peptide into a structural conformation which has a high affinity for carbohydrates. At low pH the metals dissociate, and the protein adopts a less ordered structure with concomitant loss of sugar binding capability (2). This apo-structure has been labeled the "unlocked" structure (3) because metal ions bind only weakly to this form and are kineticly labile. However, the readdition of a divalent transition metal ion (S1) and a calcium ion (S2) returns the protein to the thermodynamically more stable "locked" structure. This structure has a much higher affinity for metal ions, and their subsequent dissociation from this structural form occurs only slowly. Brown et al. (3) first showed that Mn^{2+} ions could bind in both S1 and S2 and induce the same protein conformational transition while more recently Harrington and Wilkins have reported that Ca^{2+} ions alone will activate

0006-291X/79/060598-07$01.00/0

the protein (4) to yield a structure which has a similar affinity for carbohydrates as the native protein at pH 7.2.

We report here the rates of the conformational locking transition upon addition of single metal ions or mixtures of metal ions to apo-"unlocked"-Con A as a function of pH and show that a metastable apo-Con A structure may be formed by EDTA removal of metal ions from protein solutions equilibrated with Mn^{2+} or Co^{2+} which retains an equally high affinity for saccharides as the native protein.

MATERIALS AND METHODS

Con A was isolated from jack bean meal (Sigma) by the Agrawal and Goldstein method (5), purified of subunit fragments (6) and demetallized by dialysis against 50 mM EDTA at pH 3. The purified apo-protein was dialyzed against 1M NaCL, concentrated to 10 mg/mℓ and stored in unbuffered water at pH 4. This apo-protein stock solution was diluted into 0.05M buffer solutions containing 1M NaCL immediately preceding each fluorescence experiment. A 4-methylumbelliferyl-α-D-mannopyranoside (Pierce Chemical Co.) stock solution was prepared in water and its concentration determined using ε = 13,600 $cm^{-1}M^{-1}$ at 318 nm (7). Con A concentrations were based on monomer molecular weight of 27,000 and $E^{1\%}_{1cm}$ = 12.4 (8). The fluorescence data were collected using a Farrand MK-1 spectrofluorimeter with the sample cell thermostated at 25.0±0.5°C.

RESULTS

The conversion rate of "unlocked" Con A (P) to "locked" Con A (PL) was measured by following the fluorescence quenching of MUM upon addition of metal ions. A representative first order quench curve resulting from the addition of Mn^{2+} to P at pH 6.4 is shown in Figure 1. The rates were independent of MUM concentration and metal ion concentrations above saturating levels (five-ten fold excess) but first order in total protein concentration. The data are consistent with a rapid pre-equilibration of added metal ions with the "unlocked" structural form followed by a slow conformational conversion of MP to MPL (2,3). MPL is the only structural form of the protein represented in these two equations which is capable

$$M + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MP \qquad (1)$$

$$MP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} MPL \qquad (2)$$

of binding the fluorescent sugar, MUM. As fluorescence quenching of MUM followed first-order kinetics over 80-90% of the curve, there is little contribution from

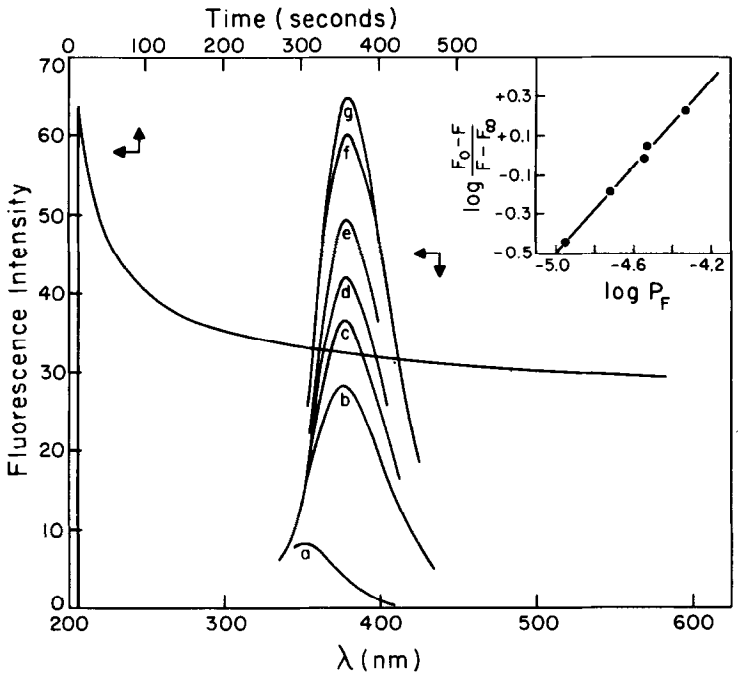

Fig. 1. Rate data for the conversion of apo-concanavalin A to the active saccharide binding form upon addition of Mn^{2+} and the resulting Mn^{2+}-Con A-MUM equilibrium constant determination. The time dependent curve represents the quenching of MUM fluorescence upon addition of 466 μM Mn^{2+} to a solution containing 49.2 μM apo-Con A, 2.4 μM MUM, 0.05M MES buffer, pH 6.4 in 1M NaCL. The wavelength scans show the increase in MUM fluorescence upon dilution of Mn^{2+}-Con A from 49.2 μM (curve b) to 2 μM (curve g). The insert shows the resulting equilibrium constant calculation.

k_{-2} to the observed rate and the equilibrium represented by equation 2 lies far to the right. The association constant for MUM binding to MPL was determined in the same sample after a sufficient incubation period to insure the complete formation of MPL by the method of Loontiens et al. (7). The conformational rate constants, k_2, and the resulting metallized protein-MUM equilibrium constants, K_a, are given for a variety of single metal ion species and mixtures of metal ions in Table I.

The most rapid locking rates at any given pH occurs upon the addition of excess divalent transition metal ions and calcium ions. These result in "locked" protein structures (MCPL) which are indistinguishable from the native form. A dramatic increase in the locking rates was always observed between pH 5.6 and 6.4 but differences between 6.4 and 7.2 were small. The locking transition is in-

Table I. Conformational Rate Constants for the MP → MPL Transition and MPL-MUM Association Constants[a]

Metal Ion(s) Added	0.05M NaOAc 1M NaCL, pH 5.6		0.05M MES 1M NaCL, pH 6.4		0.05M PIPES 1M NaCL, pH 7.2	
	$k_2 x 10^3 (sec^{-1})$	$K_a x 10^4 (M^{-1})$	$k_2 x 10^3 (sec^{-1})$	$K_a x 10^4 (M^{-1})$	$k_2 x 10^3 (sec^{-1})$	$K_a x 10^4 (M^{-1})$
$Mn^{2+} + Ca^{2+}$	15±3	3.5	23±1	3.5	24±1	3.3
$Zn^{2+} + Ca^{2+}$	15±3	3.5	23±1	3.5	24±1	3.3
$Co^{2+} + Ca^{2+}$	15±3	3.5	23±1	3.5	24±1	3.3
Mn^{2+}	2.4±0.1	1.7	13±1	3.4	12±1	3.0
Zn^{2+}	0.8±0.2	1.8	7.3±1	3.4	11±2	3.0
Co^{2+}	1.4±0.2	1.6	9.6±1	3.4	15±4	3.0
Ca^{2+}	-	-	14±1	3.6	21±1	3.4
Pb^{2+}	-	-	1.3±0.5	3.0	2±0.5	3.4
$Mn^{2+} + Pb^{2+}$	1.8±0.2	1.8	10±1	2.9	10±1	2.8
$Ca^{2+} + Pb^{2+}$	0.6±0.2	1.7	2.9±0.5	3.4	6.0±1	3.7

[a] A five to ten fold excess of the metal ion(s) was added to a solution containing 2.4 μM MUM and 40-80 μM apo-Con A in the indicated buffer solutions. The sample temperature was maintained at 25±0.2°C.

duced by single metal ion additions of Mn^{2} , Zn^{2+}, Co^{2+}, Ca^{2+} or Pb^{2+} at the two higher pH values but only measurable rates were observed with the single transition metal ions at pH 5.6.[1]

It is clear that the rates of conversion of apo-Con A to its active sugar binding conformation are extremely sensitive to the identity of the metal ions which occupy S1 and S2. Yet, the sugar binding characteristics of the various active metalloforms show little discrimination as to the identity of the ions which occupy the metal sites. The MUM association constants with each metal-protein derivative at pH 6.4 and 7.2 fall within the range $3.3 \pm 0.4 x 10^4 M^{-1}$. Sim-

[1] An omission in the Table does not necessarily mean a given metal ion fails to induce the locked protein conformation but merely that little locking is not apparent during the first 20-30 minutes upon mixing these ions with apo-Con A. For example, Harrington and Wilkins, Biochemistry, 17, 4245-4250 (1978) have shown that Ca^{2+} alone does promote the formation of an active, sugar binding structure at pH 5 after incubation for several hours.

ilar affinities are noted for the Mn^{2+}-Ca^{2+}, Zn^{2+}-Ca^{2+}, and Co^{2+}-Ca^{2+} derivatives at pH 5.6 but all other metalloforms at this pH have a lower affinity for MUM. The association constants reported in Table I are in excellent agreement with those reported by Loontiens, et al. (7) and Harrington and Wilkins (4) for the native protein.

The metal ion lability of each metalloform was examined by adding excess EDTA (2x metal concentration) to the equilibrated metal-protein derivative in the presence of MUM. EDTA does not remove metals from the Mn^{2+}-Ca^{2+}, Zn^{2+}-Ca^{2+}, or Co^{2+}-Ca^{2+} derivatives quickly (2,3) and consequently these metalloforms have been labeled "locked" to indicate that the metal ions are not in rapid equilibrium with free ions in solution. The addition of EDTA then produces no detectable change in MUM binding over a period of several hours at room temperature. A similar response to MUM fluorescence upon addition of EDTA is noted in the Mn^{2+}-Con A, Zn^{2+}-Con A and Co^{2+}-Con A samples yet water proton relaxation experiments on the Mn^{2+}-Con A derivative and visible spectrophotometric examinations of the Co^{2+}-Con A derivative show quite conclusively that these metal ions dissociate entirely and rapidly ($t_{\frac{1}{2}} \approx 20$ sec) upon addition of excess EDTA. Thus, an active apo-Con A structure may be generated in 1M NaCL at pH 5.6, 6.4 or 7.2 which maintains an equally high affinity for MUM. This metastable structure slowly unfolds to the unlocked apo structure with a $t_{\frac{1}{2}} \approx 2.5$ hours at pH 6.4 and $t_{\frac{1}{2}} \approx 1.8$ hours at pH 5.6. This may be contrasted with similar experiments in 0.3M NaCL which indicated that the apo-protein produced upon addition of EDTA to Mn^{2+}-Con A did indeed remain in the active conformational structure but its affinity for sugars is considerably lower (2). EDTA also rapidly removes metal ions from the Ca^{2+}-Con A, Pb^{2+}-Con A, and Ca^{2+}-Pb^{2+}-Con A samples but these apoproteins show a decreased affinity for MUM.

DISCUSSION

Our results show that a variety of individual metal ions or mixtures of ions added to demetallized Con A converts the protein to the conformational

form which has a high affinity for saccharides. The rates of conversion are dependent upon the identity of the ions added and the sample pH. Since Mn^{2+}(3), Ca^{2+} (9), or Cd^{2+} (10) are known to bind in both S1 and S2 sites, we conclude that these sites must be occupied for the protein to adopt the conformation which binds MUM. This does not mean that both S1 and S2 remain filled after MPL has formed. Christie, et al., have shown that only one equivalent of Ca^{2+} is required per Con A monomer to yield a full complement of MUM binding at equilibrium (11) but this certainly does not preclude Ca^{2+} occupancy of both S1 and S2 during the MP → MPL conformational change. Thus, we tentatively conclude that Zn^{2+}, Co^{2+}, and Pb^{2+} are also able to fill these sites and convert Con A to its active structure with the rates indicated in Table I.

The affinity of Con A for MUM is independent of S1 and S2 site occupancy in 1M NaCL solutions. Indeed, an apo-protein may be prepared which retains its high affinity for MUM for several hours at room temperature. The protein structures formed after single additions of Mn^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+} or Pb^{2+} are not identical; the divalent transition metal ions induce a structure which retains a high MUM affinity after removal of the ions while the Ca^{2+} and Pb^{2+} induced structures have a lower affinity for MUM after these ions are removed with EDTA. This latter structure is similar to that formed in 0.3M NaCL with the single addition of Mn^{2+} to apo-Con A (2).

Acknowledgements.

This work was supported, in part, by The National Institute of Arthritis, Metabolism, and Digestive Diseases, Department of Health, Education and Welfare (AM 16947), The Robert A. Welch Foundation (AT-584), and a Biomedical Research Support Grant (RR-07133) from the General Research Support Branch, Division of Research Resources, NIH.

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