Interactions of Saccharides with Concanavalin A

RELATION BETWEEN CALCIUM IONS AND THE BINDING OF SACCHARIDES TO CONCANAVALIN A*

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SUMMARY

Previous studies (KALB, A. J., and LEVITZKI, A. (1968) Biochem. J. 109, 669; SHOHAM, M., KALB, A. J., and PECHT, I. (1973) Biochemistry 12, 1914) were interpreted as showing that transition metal and calcium ions must be bound to concanavalin A before this lectin can bind sugars. In contrast, we find that the addition of calcium ions fails to affect the following properties of manganese concanavalin A if the metalloprotein is prepared in their presence: (a) ESR spectrum of the manganese ion in the protein; (b) spin-lattice relaxation time of solvent water protons measured in the presence of the protein; and (c) spin-lattice and transverse relaxation times of the $^1$H carbons of $\alpha$-methyl-$\beta$-D-glucopyranoside (uniformly enriched with 14% $^1$H) in the presence of manganese concanavalin A. These results indicate that saccharide binding activity of the protein is independent of calcium ion binding if a transition metal ion (Mn$^{2+}$) initially binds to the protein in the presence of calcium ions. The role of calcium ion binding to concanavalin A appears to be that of accelerating the rate of formation of the final transition metal-protein complex as observed by Barber and Carver (BARBER, B. H., and CARVER, J. P. (1973) J. Biol. Chem. 248, 3353).

Concanavalin A, a phytohemagglutinin isolated from the jack bean (Canavalia ensiformis), is a metalloprotein that binds sac-

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The physical properties of Con A have been studied extensively, including determination of its structure by x-ray diffraction techniques (3, 4). Con A exists as a dimer between pH 3.5 and 5.6 with a molecular weight of 54,000; tetramers form at higher pH (5, 6). Each monomeric unit of the protein possesses a site for transition metal ions (5) and a site for calcium ions (6). Kalb and coworkers (7, 8) suggested that both sites must be occupied before saccharide binding activity can occur. Recently, Barber and Carver (9) measured the spin-lattice relaxation value ($T_1$) of solvent water protons in the presence of Mn$^{2+}$-free Con A and observed the effect of adding manganese ions and calcium ions to the solution. Their results suggest that calcium ions exert a cooperative effect in binding manganese ions to the S$_1$ site by accelerating the rate of refolding of the protein about the transition metal ion.

In this paper, we report on the relationship between calcium ions and the binding of saccharides to Con A. In contrast to previous reports (7, 8), our results indicate that saccharide binding activity of Con A is independent of calcium ion binding to the protein, provided that the S$_1$ site is fully occupied by a transition metal ion.

EXPERIMENTAL PROCEDURE

Con A containing manganese ions (Mn-Con A) was prepared according to the following procedure. Native Con A (Miles-Yeda) was demetallized by a modification of the method of Kalb and Levitzki (7). A solution of 1.0 g of native Con A in 125 ml of distilled water was adjusted to pH 1.2 by the slow addition of 1 N HCl with stirring at 25°C. After 45 min, the solution was transferred to dialysis tubing that was boiled previously in 1 ml EDTA solution, and then dialyzed against 6 liters of 1 mM EDTA at 4°C. After further dialysis against two successive 6-liter portions of distilled and deionized water, MnCl$_2$·4H$_2$O and CaCl$_2$·2H$_2$O were added, in that order, to the solution with stirring at 25°C to give 10 mM concentrations of both metal ions. The pH of the solution was adjusted to 7.4 by slow addition of 1 N NaOH with stirring, followed by the addition of an appropriate amount of Mn-Con A, concanavalin A; Mn-Con A, concanavalin A containing manganese ions; [U-14C]$\alpha$-MDG, $\alpha$-methyl-$\beta$-glucopyranoside uniformly enriched with 14% $^1$H.

The abbreviations used are: Con A, concanavalin A; Mn-Con A, concanavalin A containing manganese ions; [U-14C]$\alpha$-MDG, $\alpha$-methyl-$\beta$-glucopyranoside uniformly enriched with 14% $^1$H.
of NaCl to give an ionic strength of 1.0. The resulting mixture was stirred at 25° for 30 min, then centrifuged at 10,000 rpm in an SS-34 Sorvall rotors for 15 min. The supernatant was dialyzed against 6 liters of 1 M NaCl at 4°, and chromatographed on Sephadex according to the procedure of Agrawal and Goldstein (10). The pooled fractions containing protein were dialyzed against three successive 6-liter portions of 1 M NaCl at 4°, followed by dialysis against four successive 6-liter portions of distilled and deionized water at 4°, and then lyophilized to give 600 mg of Mn-Con A. The protein gave one band on polyacrylamide disc gel electrophoresis at pH 4.3, corresponding to the band obtained for native Con A. Mn-Con A was as active as native Con A in gel electrophoresis at pH 4.3, corresponding to the band obtained for Con A. Mn-Con A in the absence of added calcium was also active as native Con A in agglutinating sheep erythrocytes at 25°. Solutions of the protein were prepared in 0.1 N sodium acetate buffer, pH 5.60, with the ionic strength adjusted to 1.0 by addition of NaCl. Atomic absorption analysis of solutions of the protein (1 mg per ml) indicates the presence of 2 eq of manganese ions per dimer and a total concentration of calcium ions equal to 0.26 eq per protein dimer (0.18 eq per monomer). (This preparation of Mn-Con A will hereafter be referred to as calcium-deficient Mn-Con A.) No significant amounts of manganese or calcium ions were present in the buffer as determined by atomic absorption analysis.

ESR measurements were performed using a Varian E-9 ESR spectrometer operating at 9.5 GHz. The concentration of protein used in these experiments was 20 mg per ml. Description of the carbon magnetic resonance (11) and the T1 water proton measurements (12) have been reported previously.

RESULTS AND DISCUSSION

The observed T1 values of the carbons of [(U-13C)α-MDG in the presence of calcium-deficient Mn-Con A in pH 5.60 0.1 N acetate buffer (µ = 1.0) at 25° are given in Table I. These values agree with those obtained previously when pH 5.60 0.1 M phosphate (µ = 1.0) was used as buffer (11, 13). Acetate was chosen as a buffer for the present study in preference to phosphate since the latter forms a precipitate with calcium ions. The transverse relaxation values (T2) for the carbons of [13C]-α-MDG under the same conditions in the two buffers are also the same (13). The observed T1 and T2 values of the sugar are dependent on the affinity constant and kinetics of exchange of [(U-13C)α-MDG with Con A as well as the orientation of the bound sugar relative to the manganese ion in the protein (11, 13). Addition of calcium chloride (100 mM) to the solution failed to change the observed T1 (Table I) or T2 values of the sugar.

The magnetic field dependence of T1 of solvent water protons

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<td>Effects of calcium ions on spin-lattice relaxation (T1) values of [(U-13C)α-methyl-α-D-glucopyranoside in presence of calcium-deficient manganese-containing concanavalin A (48 mg per ml)</td>
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* T1 values were determined accurately to ±0.03 s.

The protein was calculated to have approximately 0.15 Eq of calcium ions bound per protein monomer, assuming that the association constant for calcium ion binding is 3 X 103 M⁻¹ (7), and taking into account the concentration of protein. Total concentration of calcium ions was calculated to be 0.32 mM.

The ratio of protein bound to unbound sugar was 0.071. Total sugar concentration was 25 mM.

in the presence of calcium-deficient Mn-Con A in pH 5.60 0.1 N acetate buffer (µ = 1.0) is shown in Fig. 1. These values agree with previous values obtained in pH 5.60 0.1 M phosphate buffer (µ = 1.0) (12). Addition of calcium chloride (100 mM) to the solution failed to change the T1 values of the solvent water protons (Fig. 1).

The ESR spectrum of calcium-deficient Mn-Con A in pH 5.60 0.1 M acetate buffer (µ = 1.0) (Fig. 2A) is the same as that reported for Con A in the presence of manganese ions and calcium ions (14). In the presence of calcium chloride (10 mM) the ESR spectrum of the protein is unchanged (Fig. 2B).

The observed properties of calcium-deficient Mn-Con A indicate that the saccharide binding activity of the protein is independent of calcium ion binding to the protein. However, previous studies by Kalb and Levitaki (7) and Shoham et al. (8)
have suggested that saccharide binding activity of Con A requires that transition metal and calcium ions be bound to the protein. Their conclusions were based on equilibrium dialysis studies at 4°C of radioactive transition metal ions binding to demetallized Con A.

Using ESR techniques and $T_1$ measurements of solvent water protons, Barber and Carver (9) have shown that addition of manganese ions to Mn$^{2+}$-free Con A at 24°C results in a slow decrease in the $T_1$ values of the solvent water protons and that the rate is pH-dependent. The decrease in $T_1$ of solvent water protons indicates a change in the interaction of water molecules with the manganese ion in the protein (cf. Ref. 15). Addition of excess calcium ions at any time during the process results in an accelerated decrease in the $T_1$ of solvent water protons to a final value which is identical with the $T_1$ value eventually reached for water protons in the presence of manganese ions and Mn$^{2+}$-free Con A. The authors suggest that calcium ions serve to accelerate refolding of the protein about the manganese ion to form the final transition metal ion-protein complex. Addition of $\alpha$-methyl-d-glucopyranoside to this final complex in the presence of calcium ions was shown to reduce the observed $T_1$ of water protons by approximately 15%. This effect has been observed previously upon addition of this same glycoside to calcium-deficient Mn-Con A that was shown to possess full saccharide binding activity as judged from hemagglutination studies of the protein (12).

In this study, we have presented evidence that suggests removal of the calcium ions after formation of the transition metal ion-Con A complex in the presence of calcium ions does not affect saccharide binding activity of the protein. Thus, it appears that the role of calcium ion binding to Con A is that of accelerating the rate of formation of the final transition metal ion-protein complex as observed by Barber and Carver (9). This effect appears to be novel among other known interactions between calcium ions and proteins.

The results of our study and those of Barber and Carver (9) suggest that the conclusions reached by Kalb and co-workers (7, 8) concerning metal ion binding to Con A be re-examined. The association constants for the binding of manganese ions and other transition metal ions to Con A, as determined by equilibrium dialysis in the absence of calcium ions, most likely represent values associated with the incompletely formed transition metal site. Mn-Con A can be subjected to extensive dialysis against distilled water followed by gel chromatography on Sephadex without detectable loss in the manganese ion content of the protein (determined by atomic absorption analysis). This result indicates tight binding of the metal ion to the protein. The high affinity of manganese ions for Con A is consistent with the suggestion by Barber and Carver that final formation of the transition metal ion site, which can be accelerated by the presence of calcium ions, involves binding of another protein ligand to the transition metal ion, possibly aspartic acid 19 associated with the refolding of residues 12 to 22 about the S$_2$ site. The inability of Kalb and co-workers to detect significant saccharide binding activity to Con A in the presence of only transition metal ions before addition of calcium ions is probably caused by incomplete isomerization of the protein to the final transition metal ion-protein complex under the conditions of their experiment. The recent experiments by Kalb and Pecht (16) on the visible absorption and circular dichroism of the cobalt complexes of Con A as well as the work by Meirovitch and Kalb (17) on the magnetic resonance relaxation of $^1$H and $^{17}$O in aqueous solutions of Con A also appear to have been performed on the partially refolded transition metal ion complexes of Con A.

The association constant for calcium ion binding to Con A determined by equilibrium dialysis is $3 \times 10^9$ M$^{-1}$ (7). It is of interest that nearly all of the saccharide binding studies reported by Goldstein and co-workers (cf. Ref. 18) were performed without addition of calcium ions to their sample solutions. Under the conditions of their experiments, we calculate that only fractional amounts of calcium ions were bound to Con A, yet the saccharide binding activity of Con A was reproducible. This provides further evidence that calcium ions are not required for Con A to bind saccharides.

In summary, we have provided evidence that saccharide binding to Con A is independent of the binding of calcium ions to the protein once the transition metal ion site in Con A is formed in the presence of calcium ions.

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