Activation of Concanavalin A by Cd\(^{2+}\)

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Edward R. Pandolfoino, Douglas J. Christie, Gerhard R. Munske, Jeffry Fry, and James A. Magnuson

From the Program in Biochemistry and Biophysics, Department of Chemistry, Washington State University, Pullman, Washington 99164

Binding of Cd\(^{2+}\) to concanavalin A and the subsequent induction of saccharide-binding activity has been studied at pH 6.5. We found that Cd\(^{2+}\) bound to both metal sites, S1 and S2, and that Cd\(^{2+}\) alone would induce sugar binding in concanavalin A. Using the fluorescent sugar 4-methylumbelliferyl \(\alpha\)-D-mannopyranoside we determined that full saccharide-binding activity was obtained only when the total bound Cd\(^{2+}\) stoichiometry reached 2 ions/concanavalin A subunit. We also report evidence suggesting that the binding of Cd\(^{2+}\) to S2 is the crucial step in activation and that Cd\(^{2+}\) binding to S1 induces a form of concanavalin A similar to that induced by Zn\(^{2+}\), Ni\(^{2+}\), or Co\(^{2+}\) and different from that induced by Mn\(^{2+}\).

The jack bean lectin concanavalin A (Con A) is one of the most intensely studied and commonly used proteins. The interest in Con A stems from its ability to bind to saccharide moieties on the surfaces of cells. The selectivity of this binding and the subsequent changes in cellular activity have made this lectin a useful tool in cell biology and immunology. The biological properties of Con A have been extensively reviewed elsewhere (Bittiger and Schnebli, 1976; Chowdhury and Weiss, 1975).

Con A is a metalloprotein and its saccharide-binding activity is dependent upon the prior binding of metal ions. The interaction of Con A with metal ions and their ability to induce activity has been the subject of many papers over the last 12 years. Recently, work from several laboratories has drastically changed the previously accepted concepts about the interaction of metals with Con A.

Early work conducted near pH 5 led to the proposal of the following model (Kalb and Levitski, 1968; Shoham et al., 1973). Each Con A monomer contains one transition metal site (S1) and one Ca\(^{2+}\) site (S2). The Ca\(^{2+}\) site is not formed until the S1 site has been filled with a transition metal ion. Saccharide-binding activity requires both sites to be filled.

This model appears to be correct at low pH; however, work performed near pH 7 has shown that Con A behaves quite differently at higher pH. Ca\(^{2+}\) will bind to Con A without the prior binding of a transition metal ion at pH 6.5 (Alter et al., 1977) and three different groups have subsequently shown that Ca\(^{2+}\) alone will activate Con A (Christie et al., 1978; Harrington and Wilkins, 1978; Koenig et al., 1978). Christie et al. (1978) also showed that only one Ca\(^{2+}\) ion per Con A monomer was required to induce saccharide binding. Harrington and Wilkins (1978) and Koenig et al. (1978) also reported evidence suggesting that Mn\(^{2+}\) alone activates Con A. Brown et al. (1977), based on protein relaxation data, proposed that 2 Mn\(^{2+}\) ions must bind to Con A in order to induce sugar-binding activity. More recently, Christie et al. (1979), by repeating Koenig’s experiments near pH 7 and coupling them with direct metal-binding and sugar-binding studies, showed that it is Mn\(^{2+}\) alone that activates Con A (Christie et al., 1979; Christie et al., 1978). At pH 5 where most studies on metal requirements have been conducted, all current evidence suggests that both a transition metal ion and Ca\(^{2+}\) are essential for saccharide-binding activity. Near pH 7, therefore, Con A can exist in an active conformation with either a single Ca\(^{2+}\) ion, or a combination of a transition metal ion and a Ca\(^{2+}\) ion. In addition, Shoham et al. (1973) showed that Con A would bind methyl \(\alpha\)-D-glucopyranoside in the presence of 80 mM Ca\(^{2+}\) at pH 5.2. They further showed that Cd\(^{2+}\) would compete for either S1 or S2. These findings have also been confirmed by the observations of Sherry et al. (1975).

The metal-binding properties of Con A and the metal requirements for activation have been shown to change dramatically between pH 5 and pH 7. It is therefore important that the binding of cadmium and its ability to induce sugar-binding activity in Con A be studied near physiological pH. Also, since we have shown that Con A can be activated by either 1 Ca\(^{2+}\) ion, presumably binding at S2, or one Mn\(^{2+}\) ion, probably binding at S1 (see “Discussion”), there is some question about the roles of the two sites in producing an active conformation. Cd\(^{2+}\) appears to be unique in its ability to bind to either site and therefore may be a very useful probe for understanding the roles of the metal sites. Because Cd\(^{2+}\) binds to both sites, however, it is necessary to define quantitatively the nature of the binding to prevent ambiguity in future interpretations. Cd\(^{2+}\) is potentially useful as an NMR probe, and a preliminary report utilizing \(^{114}\)Cd magnetic resonance to study Cd\(^{2+}\) interaction with Con A has been published by Bailey et al. (1978). One may expect to see more such work in the future. In order to accurately interpret data from \(^{114}\)Cd-NMR experiments, they must be preceded by direct metal binding and activation studies.

In light of the recent studies cited above, we have examined...
Cd²⁺ interaction with Con A at pH 6.5 in order to determine the number and location of Cd²⁺ binding sites and their role in inducing sugar binding activity in Con A.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Mops and jack bean meal were purchased from Sigma. The fluorescent sugar, 4-methylumbelliferyl α-D-mannopyranoside, was obtained from Pierce Chemical Co. and ⁸⁶⁷ Cd was obtained from New England Nuclear. Analytical reagent grade CdCl₂·2.5H₂O was from Mallinckrodt and NiSO₄·6H₂O came from J. T. Baker Chemical Co. Specpure Chemicals Limited (London) supplied the MnSO₄·5H₂O.

**Con A Preparation and Demetallization**—Con A was prepared by affinity chromatography and demetallized by dialysis against 0.1 M HCl as previously described (Alter et al., 1977). All buffers were prepared from a stock solution of 1 M NaCl which had been passed twice over a Chelex-100 column to remove divalent metal ions. Con A solutions were checked for Ca²⁺ contamination before and after all experiments and never contained more than 5% Ca²⁺ with respect to Con A monomers. Dialysis tubing was treated with EDTA before use in equilibrium dialysis experiments. All experiments were conducted in 1 M NaCl, 0.05 M Mops, pH 6.5 at 5°C and protein concentrations were determined spectrophotometrically using A₅₂₀ = 13.7 (Yariv et al., 1968).

**Cd²⁺ Binding**—Cd²⁺-binding experiments were performed by equilibrium dialysis using ⁸⁶⁶⁷Cd. Con A (1.0 mg/ml) was placed in a dialysis bag and immersed in 3.5 ml of buffer containing Cd²⁺. The experiments were conducted in polypropylene culture tubes which were gently agitated on a rotary shaker for 5 days. ⁸⁶⁷Cd was counted using a Beckman Gamma 1000.

**Con A Activation**—The activation of saccharide binding in Con A was determined by titration with MUM and observation of fluorescence quenching as previously described (Christie et al., 1978). The number of Cd²⁺ ions required for full activity was determined by dialyzing 4-ml aliquots of apo-Con A against large volumes (2 × 250 ml) of buffer containing known concentrations of Cd²⁺. After 3 days, the activity of the Con A was determined by MUM binding and the bound Cd²⁺ concentration was determined by atomic absorption using a Perkin-Elmer 300 spectrometer.

UV Difference Spectroscopy—UV difference spectra of Con A were obtained using a Cary 14 spectrophotometer with a 0 to 0.1 slide wire and temperature-controlled cuvette holders maintained at 5°C. Identical Con A concentrations were maintained in all compared samples and all spectra were run twice in triplicate. The samples were incubated for 3 days at 5°C before spectra were taken.

**RESULTS**

**Cd²⁺ Binding**—Equilibrium dialysis studies of Cd²⁺ binding to apo-Con A showed that 2 Cd²⁺ ions bound to each Con A subunit (Fig. 1A). The binding curve is complex and could result from the presence of noninteracting, nonidentical sites or from interaction between sites. Assignment of the total Cd²⁺ stoichiometry at two is supported by our observation that dialysis of apo-Con A against solutions containing a 7-fold excess of Cd²⁺ never resulted in more than 2.1 Cd²⁺ ions bound/Con A monomer. Panel B and C of Fig. 1 show that preincubation of apo-Con A with either Ca²⁺ or Ni²⁺ lowered the Cd²⁺ stoichiometry to approximately 1.0. Ca²⁺-Con A bound Cd²⁺ with a binding constant of 1.3 × 10⁵ M⁻¹ while Ni²⁺-Con A displayed a much lower affinity (2 × 10⁴ M⁻¹). It is interesting to note that the Cd²⁺ association constant for Ca²⁺-Con A is approximately that obtained from the slope of the steep portion of the apo-Con A-Cd²⁺ binding curve.

**MUM Binding**—As apo-Con A is dialyzed against increasing concentrations of Cd²⁺ the MUM binding stoichiometry increases until one MUM is bound per Con A subunit (Fig. 2). Dialysis against higher concentrations of Cd²⁺ results in no significant change in either the MUM stoichiometry or binding constant.

**Determination of bound Cd²⁺ concentration in samples**

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**FIG. 1. Scatchard plots of Cd²⁺ binding to Con A.** The fraction of bound Cd²⁺ ions per total Con A subunit is represented by Y and Cd²⁺ is the free Cd²⁺ concentration. All experiments were performed in 1 M NaCl at pH 6.5 and 5°C. A, Cd²⁺ binding to apo-Con A (10.5 mg/ml); B, Cd²⁺ binding to Ca²⁺-Con A (4.6 mg/ml); C, Cd²⁺ binding to Ni²⁺-Con A (4.6 mg/ml).
analyzed for MUM-binding activity showed a relatively linear correlation between the number of Cd\(^{2+}\) ions bound and the fraction of active Con A subunits (Fig. 3). A total stoichiometry of 2 Cd\(^{2+}\) ions bound/Con A monomer was necessary to observe full MUM-binding activity.

Con A can also be activated by treatment with Ni\(^{2+}\) plus Cd\(^{2+}\) or Ca\(^{2+}\) plus Cd\(^{2+}\) and the MUM-binding properties of various metallized forms of Con A are compared in Table I.

**Conformation of Cd\(^{2+}\), Cd\(^{2+}\)-Con A**—Fig. 4 shows UV difference spectra which result from comparison of Cd\(^{2+}\), Cd\(^{2+}\)-Con A with other forms of Con A. Changes are induced upon binding of Cd\(^{2+}\) by apo-Con A and these changes are similar to those induced by other metal ions. Cd\(^{2+}\), Cd\(^{2+}\)-Con A was slightly but significantly different from Mn\(^{2+}\), Ca\(^{2+}\)-Con A as detected by UV difference spectroscopy.

**Table I**

*MUM-binding properties of different metallized forms of Con A*

<table>
<thead>
<tr>
<th>Form of Con A</th>
<th>(n)</th>
<th>(K_s \times 10^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd(^{2+}), Cd(^{2+})-Con A</td>
<td>0.99 ± 0.09</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>Ni(^{2+}), Cd(^{2+})-Con A</td>
<td>1.02 ± 0.09</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>Cd(^{2+}), Cd(^{2+})-Con A</td>
<td>1.08 ± 0.11</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Mn(^{2+}), Ca(^{2+})-Con A</td>
<td>1.10 ± 0.01</td>
<td>9.3 ± 0.1</td>
</tr>
</tbody>
</table>

*The association constant (\(K_s\)) and MUM stoichiometry with respect to Con A monomers (\(n\)) were determined from the slopes and intercepts of Scatchard plots (Scatchard, 1949).*

**DISCUSSION**

We have shown that, as previously demonstrated at pH 5.2 (Shoham et al., 1973), 2 Cd\(^{2+}\) ions bind to each Con A subunit and Cd\(^{2+}\) alone will induce sugar-binding activity at pH 6.5. Also consistent with the findings of Shoham et al. is our observation that Cd\(^{2+}\) will compete with Ca\(^{2+}\) or with a transition metal ion. Given the quite reasonable assumption that Cd\(^{2+}\) binds to S2 and transition metals bind to S1, the data from experiments with Ca\(^{2+}\)-Con A suggest that Cd\(^{2+}\) binds to S1 with a high affinity (1.3 \(\times 10^{-3}\) M\(^{-1}\)) and Ni\(^{2+}\)-Con A experiments suggest that the affinity of Cd\(^{2+}\) for S2 is much lower (2 \(\times 10^{-4}\) M\(^{-1}\)). The Scatchard plot for Cd\(^{2+}\) binding to apo-Con A is nonlinear with a steep initial slope of approximately 10\(^{-3}\) M\(^{-1}\) decreasing to near 10\(^{-4}\) M\(^{-1}\). It is not possible to determine with certainty whether this is due to the presence of two independent sites with different affinities for Cd\(^{2+}\) or the result of interaction between sites. There could be interaction between S1 and S2 within a single subunit, or between sites on different subunits or some combination of both. There is precedent in the Con A literature for either type of interaction (Kalb and Levitski, 1968; Shoham et al., 1973; Alter et al., 1977; Alter and Magnuson, 1979).

The ambiguity about the precise nature of the Cd\(^{2+}\) binding, plus the fact that Cd\(^{2+}\) binds to both metal sites, prevents us from being able to firmly establish the minimum requirements for Cd\(^{2+}\) activation of Con A. Although we can conclude that full MUM-binding activity is not obtained until the total Cd\(^{2+}\) stoichiometry is 2 ions/subunit, this does not necessarily mean that both ions are required. It is possible that only the Cd\(^{2+}\) in S2 is necessary to induce a sugar-binding conformation. Since S2 appears to have a lower Cd\(^{2+}\) affinity than S1, S2 would be filled after S1 was already saturated.

Although no firm answer is possible, this question can be addressed in the following manner. If one makes the simplifying assumption that the Cd\(^{2+}\) binding sites are independent, then the binding curve in Fig. 1A can be approximated by assuming that S1 binds Cd\(^{2+}\) with an association constant of 10\(^2\) M\(^{-1}\) and S2 binds Cd\(^{2+}\) with 2 \(\times 10^4\) M\(^{-1}\) as the binding
constant. Although other studies have shown interaction between S1 and S2 the validity of this simplifying assumption is supported by our observation that the binding curves for Cd" association with Ni"-Con A and Ca"-Con A will combine to give a reasonable fit to the apo-Con A Cd" binding curve (Fig. 1). It is now possible to generate a curve of the type in Fig. 3 in which the total Cd" bound is plotted against the fraction of activated Con A. Curves were obtained based on each of the following assumptions: 1) Cd" in S1 alone activates; 2) Cd" must occupy both S1 and S2; 3) Cd" in S2 alone, regardless of S1 occupation, activates. The first two assumptions yielded very poor correlation with the experimental data. When 0.2 Cd" ion is bound/subunit, this assumption predicts that 4% of the Con A should be active while the experimentally determined line shows 8% activation. With 1.0 Cd'" bound, 31% should theoretically be active and 45% is actually binding MUM. The fit improves at higher Cd" levels with 1.5 Cd" ions bound predicting 61% activity compared with 66% observed and 1.8 Cd" ions bound yielding 93% theoretical activity compared with 90% based on the experimental line. This assumption is also consistent with the previous results of Christie et al. (1978) which showed that only 1 Ca" ion (presumably binding at S2) was necessary to fully activate Con A.

It is also important to recall that only 1 Mn" ion is required to induce MUM binding in Con A (Christie et al., 1979). Preliminary measurements of the metal-sugar distance (similar to those of Alter and Magnuson 1974) using 31P NMR have indicated that the Mn" ion in Mn"-Con A is binding at a site identical with or very close to S1.2 This suggests that Mn" in S1 will activate Con A. Therefore, the preceding analysis suggests that Cd" is not a good substitute for Mn" in S1 since Cd" in S1 alone did not activate. This conclusion is supported further by comparison of the MUM-binding constants for Mn", Ca"-Con A with Cd", Cd"- or Cd"-Co"-Con A. Mn"-Con A displays a MUM association constant of 9.3 x 10^4 M^{-1} while either of the Cd"-containing forms show an affinity below 1 x 10^5 M^{-1}. The UV difference spectrum comparing Cd", Cd"-Con A with Mn"-, Ca"-Con A also suggests that Cd" induces a slightly different conformation than do Mn" and Ca".

Other metal ions which bind to S1 including Zn", Co", and Ni", will not activate Con A without the addition of a 2nd ion

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1 E. R. Pandolfino and J. A. Magnuson, unpublished observations.

2 D. J. Christie and J. A. Magnuson, unpublished observations.