# CHARACTERIZATION OF THE AMINO ACIDS INVOLVED IN CALCIUM BINDING IN CONCANAVALIN A

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## 1. Introduction

The saccharide-binding capacity of concanavalin A, a protein from Jack bean, is lost following demetalization of the protein by acid treatment [1]. The saccharide-binding capacity can be restored by addition of the proper metals to the demetalized protein [1–3]. The binding of a transition metal ($Co^{2+}$, $Ni^{2+}$, $Mn^{2+}$, $Zn^{2+}$) to a specific site induces the formation of a distinct site, specific for the binding of $Ca^{2+}$ ions [4]. The full saccharide-binding capacity of concanavalin A is restored when both kinds of sites are occupied by the appropriate metal ion [4]. The concanavalin A molecule, of molecular weight 55 000 daltons [5, 6], consists of two identical subunits [7, 8] and has two sites for each ligand [4].

In the course of our studies on cancanavalin A structure, the chemical nature of the metal binding sites was investigated. Two histidyl residues were shown to participate in the transition metal-binding site [9]. In this paper, similar studies aimed at the identification of the residues involved in $Ca^{2+}$ binding, are reported. The data suggest that $Ca^{2+}$ is bound to a cluster of three ligands, two carboxyls and probably one amine.

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## 2. Materials and methods

Concanavalin A, twice crystallized, in saturated NaCl, was obtained from Miles-Yeda. The protein was demetalized by acid treatment as previously described [4]. Demetalized concanavalin A was stored as an unbuffered solution in 0.2 M NaCl, at −20°. Protein concentrations were determined spectrophotometrically, using a value of $A^{1\%}_{1\,cm}$ at 280 nm = 12.4 [2].

All reagents were of the purest grade commercially available. NaCl stock solutions were made free of divalent cations by passage through a column of Chelex X 100. Twice distilled water was used throughout. The $Ni^{2+}$–concanavalin A complex was prepared as follows: the solution of demetalized protein was brought to pH 6 and the specific binding sites were saturated with $Ni^{2+}$. The resulting solution was then brought to the desired pH value. This procedure allows the transition-metal binding site to remain occupied at pH-values as low as 3 [9].

The release of protons by concanavalin A and its complexes upon addition of $Ca^{2+}$ ions was studied at constant pH, changes in pH effected by the addition of small amounts of $Ca^{2+}$ being balanced by NaOH addition. A Radiometer expanded Scale pH-meter, PHM-26c, equipped with a G-202 B electrode and K 401 calomel reference electrode was used. Carbon dioxide free helium (Matheson) was passed over the titrated solution (2.5 ml, 13–15 mg protein), ther-

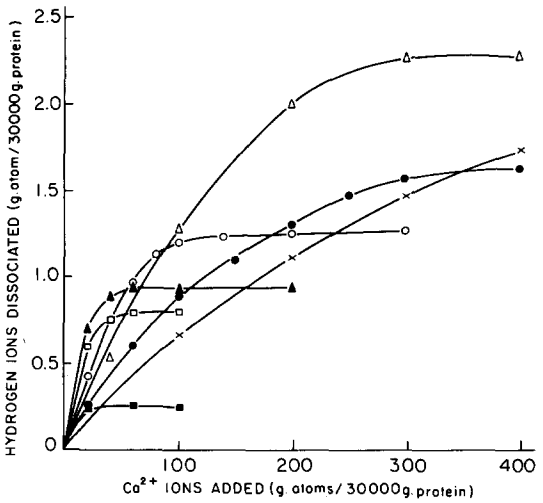


Fig. 1. Hydrogen ion dissociation isotherms for the interaction of $Ca^{2+}$ with $Ni^{2+}$–concanavalin A complex. $Ni^{2+}$–concanavalin A complex, 13–15 mg/2.5 ml, in 0.2 M NaCl. 25°. Titrants were $CaCl_2$ (0.1 to 1.0 M) and NaOH (0.05 to 0.1 N). (X—X—X): pH 3.7; (△—△—△): pH 4.12; (●—●—●): pH 4.9; (○—○—○): pH 5.41; (▲—▲—▲): pH 6.25; (□—□—□): pH 6.76; (■—■—■): pH 7.5.

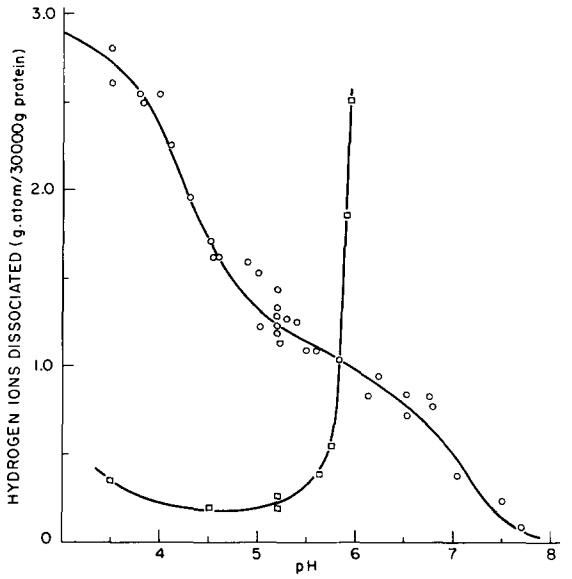


Fig. 2. pH dependence of the maximum number of protons dissociable from demetalized concanavalin A and $Ni^{2+}$–concanavalin A complex in the presence of $Ca^{2+}$ ions. (○—○—○) Data taken from experiments plotted in fig. 1 or from similar experiments. In pH range 3–5.5 the solid line is the theoretical curve computed on the basis of two ionizing groups of pK 4.25. (□—□—□) Data of hydrogen ion dissociation isotherms for the interaction of $Ca^{2+}$ ions with demetalized concanavalin A. Same conditions as in fig. 1.

mostated at 25°. Small volumes (10–20 μl) of a $CaCl_2$ solution ($10^{-2}$ to 1 M) were repeatedly added with a microsyringe. After each addition, the pH was allowed to reach a stable value and NaOH (0.01 to 0.05 N) was then added until the initial pH was restored. The amounts of $CaCl_2$ added were chosen so as to induce a pH shift no greater than 0.02 pH unit. The data were corrected for uptake of protons by the solvent.

## 3. Results

The relationship between $Ca^{2+}$binding and $H^+$ release was studied by measuring the number of protons dissociated from a $Ni^{2+}$–concanavalin A complex upon $Ca^{2+}$ addition. The number of protons released increases with $Ca^{2+}$ concentration, and tends to a maximal value at any pH in the range 3.7 to 7.5 (fig. 1). These curves can be interpreted as reflecting the progressive saturation of the $Ca^{2+}$ binding site, a certain number of protons being stoichiometrically released on binding of one $Ca^{2+}$ ion.

The release of a maximal number of protons is associated with the saturation of the $Ca^{2+}$ binding site, while the variation of this number with pH can be related to the degree of dissociation of the ionizing groups involved. These "plateau values" are plotted in fig. 2 as a function of pH. The data indicate that, on $Ca^{2+}$ binding, protons are dissociated from 3 groups of two different kinds: two ionizing groups show an apparent $pK_a$ of 4.2 (this value is based on the extrapolation of the titration curve below 3.5 since below this pH value the interaction of $Ca^{2+}$ with the protein is too weak to be directly investigated); the third group is fully protonated at pH 5.5 and dissociates at higher pH, with an apparent $pK_a$ of ~7. The sharpening of the curves as pH increases reflects the increase of the association constant for $Ca^{2+}$.

The properties of the "proton releasing-center" can be associated with that of the $Ca^{2+}$ binding site. The

pattern of release of protons on addition of $Ca^{2+}$ is observed under conditions where the $Ca^{2+}$ binding site is formed, i.e. after saturation of the transition metal binding site with $Co^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Ni^{2+}$. In the presence of $Cu^{2+}$ ions, known to prevent transition metal binding and formation of the $Ca^{2+}$ binding site [4], no protons are released on $Ca^{2+}$ addition, even in the presence of $Ni^{2+}$ ions. Moreover addition of $Ca^{2+}$ ions to demetalized concanavalin A induces only an almost negligible release of protons in the pH range 3.5–5.3. Above pH 5.3, although the release of protons remains a saturation process, the maximal number of protons released increases steeply (fig. 2). The specificities of both sites are similar: $Mg^{2+}$ ions, which do not bind to the $Ca^{2+}$ binding site [4], do not effect a release of proton from the $Ni^{2+}$–concanavalin A complex. An apparent association constant for $Ca^{2+}$ at 25°, pH 5.2, was estimated from the titration curve; its value, $\sim 2 \times 10^3$ l/mole, is in good agreement with the association constant calculated from direct binding experiments [4].

## 4. Discussion

The binding of $Ca^{2+}$ ions to concanavalin A is accompanied by the dissociation of three ionizing groups per subunit. On the basis of their apparent $pK_a$ (4.2) two of them can be unambiguously identified as carboxyls; the third group, $pK_a \sim 7$, could be assigned to an imidazole or an amino group. Studies on the chemical modifications of concanavalin A have indicated that $Ni^{2+}$ ions protect two histidines against ethoxyformylation [9], while $Ca^{2+}$ ions are not able to protect an additional histidine against derivatization. This finding makes the participation of an imidazole in $Ca^{2+}$ binding unlikely. On the other hand, modifications of 80–85% of the amines in native concanavalin A does not lead to loss of the saccharide binding activity [10] suggesting that at least some of the amines are protected when $Ca^{2+}$ is present.

Although the techniques used do not to provide clearcut evidence for the direct involvement of the three ionizing groups in $Ca^{2+}$ binding, such an interpretation is consistent with the data, as well as with the fact that carboxyls are frequently involved in $Ca^{2+}$ binding in proteins [11, 12]. Were such a structure of the calcium binding site to be confirmed, this could help to interpret the discrete changes observed by comparison of X-ray diffraction data of transition-metal concanavalin A complex and of demetalized concanavalin A [13].

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