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Metal-Binding Sites of Concanavalin A and their Role in the Binding of α-Methyl D-Glucopyranoside

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Binding of a transition metal ion to specific sites in concanavalin A induces the formation of specific Ca^{2+} ion-binding sites. Sites for binding α-methyl D-glucopyranoside exist only when a transition metal ion and Ca^{2+} ion are bound.

Concanavalin A, a crystalline protein from jack bean, contains bivalent metal ions that are essential for its interaction with glycogen (Sumner & Howell, 1936*a*). Removal of bivalent metal ions destroys the α-methyl D-glucopyranoside-binding sites of this protein (Yariv, Kalb & Levitzki, 1968). These observations led us to the present investigation of the binding of bivalent metal ions by concanavalin A and of the relationship of the metal ion binding sites to the binding of α-MG.*

MATERIALS AND METHODS

Buffer solution. The solvent used in this investigation was 0·05M-sodium acetate–acetic acid buffer, pH 5·2, containing 0·2M-NaCl, made with twice-distilled water and treated with a metal-chelating resin (Dowex A-1).

Concanavalin A. The protein was prepared from jack-bean meal (Sigma Chemical Company, St. Louis, Mo., U.S.A.) by crystallization, as described by Sumner & Howell (1936*b*).

Demetallized protein. Concanavalin A (8mg./ml.) was demetallized by the addition of 1M-HCl to give a pH of 1·2, measured by a glass electrode. After 30min. the acidified solution was transferred to dialysis bags (Visking 20/32) that had been treated with boiling 1mM-EDTA, and was dialysed for 3hr. against three changes of twice-distilled water at 5°. The resulting solution of P_D was stored in polyethylene bottles at −15°. The bivalent metal ion content of P_D, as determined by atomic-absorption spectroscopy (model 303; Perkin–Elmer Corp., Norwalk, Conn., U.S.A.), was: Ca^{2+}, 0·15g.atom/32000g.; Mg^{2+}, 0·16g.atom/32000g.

Reagents. $^{63}Ni^{2+}$, $^{45}Ca^{2+}$ and ^{14}C-labelled α-MG were products of The Radiochemical Centre (Amersham, Bucks.). All other reagents were of analytical grade.

Binding experiments. Binding of Ni^{2+}, of Ca^{2+} and of α-MG was measured by the method of equilibrium dialysis. Dialysis membranes were cut from Visking (20/32) dialysis tubing, treated with three changes of boiling 1mM-EDTA, stored in 0·1mM-EDTA at 5°, and washed with twice-distilled water before use. In each experiment, 1ml. of P_D solution was pipetted into one compartment and 1ml. of the buffer solution into the other compartment of the dialysis cell (model 16-E; Technilab Instruments, Los Angeles, Calif., U.S.A.). $^{63}Ni^{2+}$, $^{45}Ca^{2+}$ or [^{14}C]α-MG, and unlabelled reagents, when required, were added to either compartment. The cell was gently rotated for 16hr. at 3° and portions were then removed for assay.

$^{63}Ni^{2+}$, $^{45}Ca^{2+}$ and [^{14}C]α-MG concentrations were determined by scintillation counting in a Packard Tri-Carb liquid-scintillation spectrometer in Bray's (1960) solution. Protein concentration was determined spectrophotometrically ($E^{1\%}_{1cm.}$ at 280mμ 12·4; Yariv *et al.* 1968).

$^{63}Ni^{2+}$ and $^{45}Ca^{2+}$ were standardized by EDTA titration (Wilson & Wilson, 1960), and [^{14}C]α-MG was standardized by means of the phenol–H_2SO_4 test (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) with unlabelled α-MG (Pfanstiehl Laboratories Inc., Waukegan, Ill., U.S.A.) as standard.

RESULTS

Binding of Ni^{2+} *ions by* P_D. The results of direct binding experiments with $^{63}Ni^{2+}$ are plotted in Fig. 1(*a*) according to eqn. 1 (Scatchard, 1949),

$$r/f = -Kr + KN \qquad (1)$$

where r is the metal bound (g.atom/g. of protein), f is the molar concentration of free metal ions, K is the intrinsic association constant and N is the metal bound (g.atom/g. of protein) at saturation. From the slope of the linear plot, K_{Ni} is $1·3 \times 10^5$l./mole. From the x-intercept, N is $2·7 \times 10^{-5}$g.atom/g. of protein. Thus the equivalent weight for the binding of nickel is $3·7 \times 10^4$g. of protein/g.atom.

Binding of other metal ions to the Ni^{2+} *ion-binding site.* Fig. 1(*a*) also contains results of experiments in which Ni^{2+} ion binding was measured in the presence of a fivefold excess of Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+} or Cu^{2+} ions. The transition metal ions tested interfere with binding of Ni^{2+} ions and undoubtedly compete with Ni^{2+} ions for the metal ion-binding site. Ca^{2+} ions, however, do not compete with Ni^{2+} ions.

* Abbreviations: α-MG, α-methyl D-glucopyranoside; P_D, demetallized protein.

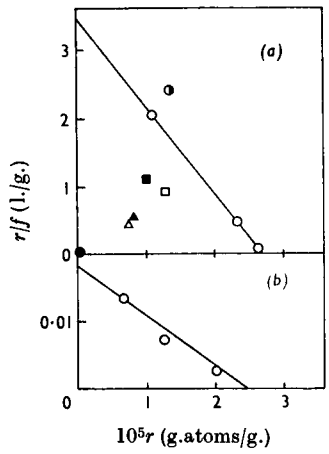

Fig. 1. (*a*) Binding of Ni^{2+} ions by concanavalin A at 3°. The P_D concentration was 6mg./ml. ○, Ni^{2+} only. The other points represent experiments in which the Ni^{2+} concentration was 0·05mM and the concentration of competing metal ion was 0·25mM: ●, Cu^{2+}; △, Co^{2+}; ▲, Zn^{2+}; ■, Fe^{2+}; □, Mn^{2+}; ◑, Ca^{2+}. (*b*) Binding of Ni^{2+} ions in the presence of 0·125mM-Cu^{2+} (○). Experimental conditions were as in (*a*). For definition of *r* and *f*, see the text.

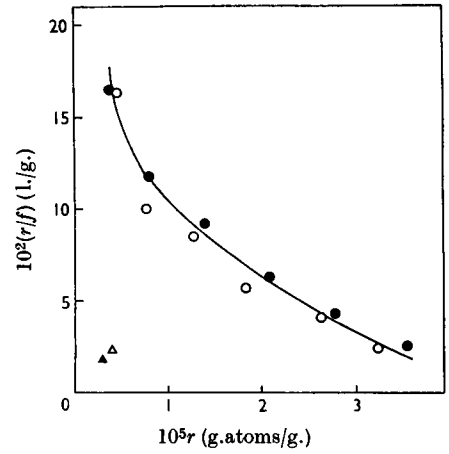

Fig. 2. Binding of Ca^{2+} by concanavalin A at 3°. The P_D concentration was 6mg./ml. ●, 0·5mM-Ni^{2+}; ○, 0·5mM-Ni^{2+} and Mg^{2+} equal to Ca^{2+} concentration; ▲, 0·2mM-Cu^{2+}; △, Ca^{2+} only. For definition of *r* and *f*, see the text.

Binding of Cu^{2+} *ions.* A more detailed experiment on the binding of Ni^{2+} ions by P_D in the presence of Cu^{2+} ions (0·125mM) is plotted in Fig. 1(*b*). Whereas the *x*-intercept, and therefore the number of Ni^{2+} ion-binding sites, is nearly the same as in the absence of Cu^{2+} ions, the slope is smaller by a factor of 200. It may therefore be concluded that Cu^{2+} and Ni^{2+} compete for the same metal ion-binding site and that Cu^{2+} is bound more strongly than Ni^{2+}. From the ratio of Ni^{2+} to Cu^{2+} concentration at half-saturation with respect to Ni^{2+}, the association constant for the Cu^{2+} ion–protein complex, K_{Cu}, is estimated to be 2×10^6l./mole.

Binding of Ca^{2+} *ions.* Fig. 2 summarizes the results of direct binding experiments with $^{45}Ca^{2+}$. In the absence of added metal ions, as well as in the presence of Cu^{2+}, very little Ca^{2+} is bound by the protein. In the presence of Ni^{2+}, however, Ca^{2+} ion-binding is greatly increased. Mg^{2+} ions do not compete for the Ca^{2+} ion-binding site. The upward curvature of the plot indicates heterogeneity of affinity constants. Since saturation is not attained at practicable concentrations of Ca^{2+} ions, it is possible only to set a lower limit of $3{\cdot}5 \times 10^{-5}$g.-atom/g. of protein at saturation. Hence, the equivalent weight of a Ca^{2+} ion-binding site is no greater than $2{\cdot}9 \times 10^4$. The concentration of free Ca^{2+} ions in the vicinity of half-saturation is 0·3mM, and thus $\bar{K}_{Ca} = 3 \times 10^3$l./mole may be regarded as the 'average association constant' for Ca^{2+}.

Table 1. *Binding of α-methyl* D-*glucopyranoside by concanavalin A at 3° with 6 mg. of demetallized protein/ml.*

Transition metal ion	$10^4 \times$ Concn. (M) of transition metal ion	Non-transition metal ion	$10^4 \times$ Concn. (M) of non-transition metal ion	α-MG bound* (mole/32000g.)
None	—	None	—	0·09
None	—	Ca^{2+}	16·0	0·24
Ni^{2+}	5·0	None	—	0·13
Ni^{2+}	5·0	Mg^{2+}	24·0	0·16
Cu^{2+}	2·0	Ca^{2+}	16·0	0·25
Ni^{2+}	5·0	Ca^{2+}	16·0	0·91
Mn^{2+}	12·5	Ca^{2+}	10·0	0·96

* Maximal coverage, calculated from single experiments on the basis of $K_{\alpha\text{-MG}} = 4 \times 10^3$l./mole.

Binding of α-MG. The results of measurements of α-MG binding by P_D are summarized in Table 1. Very little α-MG is bound when no bivalent metal ions are added. Further, no single added metal ion enables the protein to bind α-MG. Binding of α-MG approaches 1 mole/32000g. of protein only when two metal ions are present: Ca^{2+} and Ni^{2+} or Mn^{2+}. Mg^{2+} ions cannot play the role of Ca^{2+}. Cu^{2+} ions, even in the presence of Ca^{2+} ions, do not enable P_D to bind α-MG.

In Fig. 3, the results of measurements of α-MG binding in the presence of Ni^{2+} (0·5mM) at two

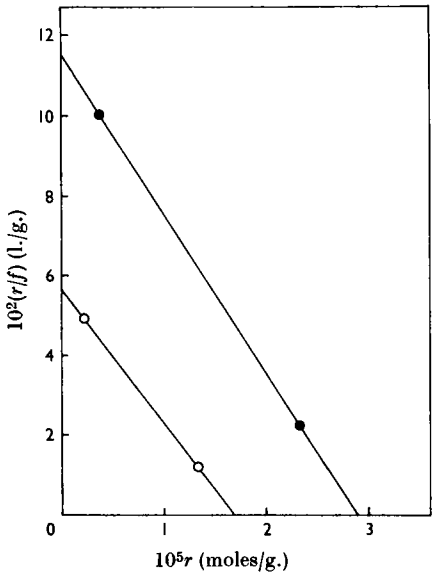

Fig. 3. Binding of α-MG by concanavalin A at 3°. The P_D concentration was 6mg./ml. and the Ni^{2+} concentration 0·5mM. Ca^{2+} concentration: 0·2mM (○); 1·6mM (●). For definition of r and f, see the text.

different concentrations of Ca^{2+} are plotted according to eqn. 1. Straight lines drawn through pairs of points corresponding to a single Ca^{2+} concentration are nearly parallel to each other. The x-intercepts, however, increase with Ca^{2+} ion concentration. At the lower Ca^{2+} concentration, which corresponds to $1{\cdot}40 \times 10^{-5}$g.atom of calcium bound/g. of protein, the x-intercept is $1{\cdot}65 \times 10^{-5}$ mole of α-MG bound/g. of protein. At the higher Ca^{2+} concentration ($3{\cdot}53 \times 10^{-5}$g.atom of calcium bound/g. of protein), the x-intercept is $2{\cdot}89 \times 10^{-5}$ mole of α-MG bound/g. of protein. The latter value of N corresponds to an equivalent binding weight of $3{\cdot}5 \times 10^4$g. of protein/mole of α-MG. From the average of both slopes, $K_{\alpha\text{-MG}}$ is calculated to be $3{\cdot}8 \times 10^3$l./mole.

DISCUSSION

Concanavalin A binds bivalent metal ions at two different binding sites. One kind of site, S1, binds transition metal ions but not Ca^{2+} or Mg^{2+} ions. The equivalent weight of S1 was found in the present study to be $3{\cdot}7 \times 10^4$. Since the molecular weight of concanavalin A is $5{\cdot}5 \times 10^4$ (Kalb & Lustig, 1968) it may be inferred that each molecule has two S1 sites. The high value found here for the equivalent weight of S1 is attributed to partial denaturation of the protein. Indeed, binding studies on a different preparation of P_D led to an equivalent weight of $2{\cdot}9 \times 10^4$, which is much closer to one-half of the molecular weight.

The equivalent weight of an α-MG site is $3{\cdot}2 \times 10^4$ Yariv *et al.* 1968), which is nearly the same as that of S1. It is therefore possible that the concanavalin A molecule consists of two sub-units, each of which has one site for a transition metal ion and one site for α-MG.

The Ca^{2+} ion-binding site, S2, does not exist in P_D. However, when S1 is occupied by Ni^{2+} or by Mn^{2+}, S2 is formed. Cu^{2+} ions, which have the greatest affinity for S1, do not induce formation of S2. The exceptional behaviour of Cu^{2+} may be related to its unique stereochemistry among bivalent transition metal ions. S2 is highly selective for Ca^{2+}. The affinity of Ca^{2+} for S2 is, however, rather low ($\bar{K}_{Ca}$ 3×10^3). Comparison of the upper limit of the equivalent weight of S2 ($2{\cdot}9 \times 10^4$) with the molecular weight of concanavalin A ($5{\cdot}5 \times 10^4$) indicates that there are at least two such sites per molecule. The upward concavity of the Scatchard plot for Ca^{2+} binding (Fig. 2) may be the result of electrostatic repulsion between a Ca^{2+} ion bound to the first site and an entering Ca^{2+} ion. However, there may also be an intrinsic difference between the two Ca^{2+} ion-binding sites. Alternatively, one may postulate heterogeneity of the entire S2 population. With the limited range and accuracy of our results, it is not possible to decide among these possibilities.

Concanavalin A binds α-MG only when S1 is occupied by a transition metal ion that can create a site for Ca^{2+} and when this site, too, is occupied by Ca^{2+}. When S1 is empty or when it is occupied by Cu^{2+}, S2 is not formed and Ca^{2+} cannot be bound. Consequently, no site for α-MG exists. That an α-MG-binding site exists only when S2 contains Ca^{2+} is most strikingly demonstrated by the close correspondence between Ca^{2+} coverage and maximal α-MG coverage (Fig. 3).

We have demonstrated that the existence of a saccharide-binding site in concanavalin A depends on the occupation by Ca^{2+} of a site that itself is formed only when a different metal ion-binding site is occupied by a suitable transition metal ion. A mechanism for site induction of this sort is hitherto unknown. One might guess, however, that it is not a rare mechanism, since its basic components, metal ion-binding ligands and configurational flexibility, are not uncommon in proteins.

The fact that S1 may be occupied by any of several paramagnetic metal ions presents the possibility of investigating the structure of S1 with the aid of the magnetic properties of these metal ions. However, the ease with which concanavalin A can be crystallized, as well as the possibility that the molecule is composed of sub-units of molecular weight as low as $2{\cdot}8 \times 10^4$, may make X-ray

crystallography the most promising technique for investigating the structural and operational details of the sites of concanavalin A.

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