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Specificity of Metal Ion Interaction with Concanavalin A†

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ABSTRACT: The interaction of metal ions with concanavalin A was measured by equilibrium dialysis at 4°, pH 5.2. The transition metal binding site (S1) binds Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} ions. The values of the binding constants are in the order $K_{Mn^{2+}}$ (0.2×10^4 M^{-1}) < $K_{Co^{2+}}$ (1.6×10^4 M^{-1}) < $K_{Ni^{2+}}$ (12×10^4 M^{-1}) > $K_{Zn^{2+}}$ (1.4×10^4 M^{-1}) < $K_{Cd^{2+}}$ (6.2×10^4 M^{-1}). Ca^{2+} and Mg^{2+} ions are not bound to S1. These results suggest that S1 contains one or more nitrogenous ligands. The calcium binding site (S2) binds Ca^{2+} and Cd^{2+} equally well ($K_{Ca^{2+}} = 3.0 \times 10^3$ M^{-1}, $K_{Cd^{2+}} = 1.5 \times 10^3$ M^{-1}), Sr^{2+} very weakly, and Mg^{2+}, Ba^{2+}, Mn^{2+}, Ni^{2+}, and Sm^{3+} not at all. This suggests that S2 is a rigid site which can accommodate only divalent metal ions of radii very nearly 1 Å.

Concanavalin A, a saccharide-binding protein from Jack bean, contains metal ions in its native state (Sumner and Howell, 1936a). On removal of metal, transition metal ions can be bound at a site designated S1. On occupation of S1, a new metal binding site, S2, is formed. The saccharide binding site is created when the two metal binding sites, S1 and S2, are occupied (Kalb and Levitzki, 1968). Concanavalin A, mol wt 55,000, is composed of two identical subunits (Kalb and Lustig, 1968; Greer *et al.*, 1970). The molecule contains two of each of the metal binding sites, S1 and S2, and two saccharide binding sites (Yariv *et al.*, 1968).

In this publication we show that only divalent metal ions which have an affinity for nitrogen ligands are bound to site S1. The interaction with S2 is restricted to divalent metal ions whose radii are in a narrow range around 1 Å. In this case, it is not required that the ion should have an affinity for nitrogen.

Materials and Methods

Buffer Solutions. The solvent used in this investigation was 0.05 M sodium acetate–acetic acid buffer, pH 5.2, containing 0.2 M NaCl or, in some cases, 0.2 M $NaNO_3$, made with column-deionized, distilled water and treated with a metal-chelating resin (Chelex 100).

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Concanavalin A. The protein, prepared from Jack bean meal (Sigma Chemical Co., St Louis, Mo.) by crystallization, as described by Sumner and Howell (1936b), was made in the laboratory or purchased from Miles-Yeda, Rehovot.

Removal of Metal Ions from Concanavalin A. Demetallization was performed by acidification to pH 1.2 and dialysis as described by Kalb and Levitzki (1968). The metal ion content, as determined by atomic absorption spectroscopy, was: Mn^{2+}, 0.02 g-atom/27,000 g; Zn^{2+}, 0.02 g-atom/27,000 g. The binding parameters for Ni^{2+} ions and MeGlc[1] were as published previously (Kalb and Levitzki, 1968; Yariv *et al.*, 1968).

Reagents. $^{63}Ni^{2+}$, $^{60}Co^{2+}$, $^{45}Ca^{2+}$, $^{52}Mn^{2+}$, $^{109}Cd^{2+}$, $^{65}Zn^{2+}$, $^{203}Hg^{2+}$, and ^{14}C-labeled MeGlc were products of the Radiochemical Centre, Amersham. All other reagents were of analytical grade.

Binding Experiments. Binding of metal ions or MeGlc was measured by equilibrium dialysis (pH 5.2, 4°) according to a procedure described previously (Kalb and Levitzki, 1968). $^{63}Ni^{2+}$, $^{203}Hg^{2+}$, $^{109}Cd^{2+}$, $^{45}Ca^{2+}$, and [^{14}C]MeGlc concentrations were determined in a Packard Tri-Carb liquid scintillation spectrometer with Bray's (1960) solution as solvent. $^{52}Mn^{2+}$ and $^{60}Co^{2+}$ concentrations were determined by solid scintillation counting in a Packard γ spectrometer. Protein concentration was determined spectrophotometrically ($E_{1\,cm}^{1\%}$ at 280 nm = 12.4; Yariv *et al.*, 1968).

Labeled metal ions were standardized by EDTA titration (West, 1969) and [^{14}C]MeGlc was standardized by means of the phenol–sulfuric acid test (Dubois *et al.*, 1956) with cold MeGlc (Pfanstiehl Laboratories, Inc., Waukegan, Ill.) as standard.

TABLE I: Binding of Metal Ions to Site S1.[a]

Metal Ion	10^{-3} K_M Directly Measd (l./mol)	10^{-3} K_M Estmd from Competition with Ni^{2+} Ions (l./mol)	Equiv Binding Wt (Daltons $\times 10^{-4}$)	MeGlc Bound[b] (mol/27,000 g)
Ni^{2+}	120 ± 30		3.0 ± 0.6	0.88
Cd^{2+}	62 ± 4[c]	120[c]	2.5 ± 0.3	0.93
Co^{2+}	16 ± 4	21	2.5 ± 0.3	0.87
Zn^{2+}	14 ± 3	21	2.5 ± 0.2	0.80
Mn^{2+}	2 ± 1	2	3.0 ± 1.0	0.94
Ca^{2+}		~0		0.23
Mg^{2+}		~0		
Pb^{2+}		~0		
Sm^{3+}		~0		0.11
Hg^{2+}		~0		0.13
Ag^{+}		~0		
Tl^{+}		~0		

[a] Measured in 0.05 M CH_3CO_2Na–CH_3CO_2H, pH 5.2, 0.2 M NaCl, 4°. [b] Measured in the presence of 1 mM Ca^{2+} and calculated from single experiments on the basis of K_{MeGlc} = 4×10^3 l./mol (Yariv *et al.*, 1968). [c] Corrected for interaction with Cl^-; see text. Competing metal ion concentration was as high as 0.13 M except where solubility was limited.

Results

Binding to the Transition Metal Binding Site, S1. DIRECT BINDING EXPERIMENTS. The results of binding experiments with Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} ions were plotted according to Scatchard's equation (Scatchard, 1949)

$$r/f = -K_M r + K_M N \quad (1)$$

where r is the metal bound (gram-atoms/gram of protein), f is the molar concentration of free metal ions, K_M is the association constant for the metal M, and N is the metal bound at saturation (gram-atoms/gram of protein). The plots were rectilinear. The values of K_M from the slopes are summarized in Table I. The equivalent binding weight (N^{-1}) calculated from the x intercepts was 27,000 ± 5000. The reported value of K_{Cd} is based on a correction for the competitive formation of the $CdCl^+$ complex (Sillén and Martell, 1964).

Competitive Inhibition of Binding of Ni^{2+} Ions by Various Metal Ions. Mn^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+} ions inhibit the binding of Ni^{2+} ions competitively since the amount of Ni^{2+} bound to the protein decreases gradually to zero with increasing concentration of the competing metal ion. The data conform quantitatively to the following equation for competitive inhibition (Coleman and Vallee, 1961)

$$K_M = K_{Ni}\frac{(N - r)f}{r[M^{2+}]} \quad (2)$$

K_{Ni} ((1.2 ● 0.3) × 10^5 l./mol) and N ((3.3 ± 0.2) × 10^{-5} g of protein/g-atom) are from direct measurements with $^{63}Ni^{2+}$. $[M^{2+}]$, the free concentration of the competing metal, was taken to be equal to the total added concentration. The values of K_M calculated according to eq 2 are close to those determined from direct measurements (Table I). The relatively large discrepancy between K_{Cd} determined by the two methods may be the result of the large correction applied for the competitive formation of $CdCl^+$.

Various other metal ions were checked for their ability to compete with Ni^{2+} ions. Ca^{2+}, Hg^{2+}, Ag^+, and Tl^+ inhibit the binding of Ni^{2+} ions in a noncompetitive manner. Mg^{2+}, Pb^{2+}, and Sm^{3+} have no effect.

Formation of the Metal Binding Site, S2, and of the Saccharide Binding Site by Divalent Transition Metal Ions. Binding of MeGlc is observed when any of the ions Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, or Cd^{2+} is added to demetallized concanavalin A in the presence of Ca^{2+} ions. The binding parameters for MeGlc are identical in the presence of each of the above transition metal ions (K_{MeGlc} = (3.1 ± 1.0) × 10^3 l./mol; equivalent binding weight = (2.6 ± 0.4) × 10^4 daltons).

Binding of Metal Ions to Site S2. The interaction of various metal ions with site S2 has been investigated by equilibrium dialysis in the presence of Ni^{2+} and $^{45}Ca^{2+}$ ions (Table II). Cd^{2+} ions compete effectively with Ca^{2+} ions, whereas Sr^{2+} competes only at very high concentrations. Mg^{2+}, Ba^{2+}, Ni^{2+}, or Mn^{2+} have no influence on the binding of Ca^{2+} ions. Association constants were calculated from the inhibition experiments according to eq 3. The values of K_{Ca} and N used

$$K_M = K_{Ca}\frac{(N - r)f}{r[M^{2+}]} \quad (3)$$

in eq 3 were as reported previously (Kalb and Levitzki, 1968).

[1] Abbreviation used is: MeGlc, methyl α-D-glucopyranoside.

TABLE II: Binding of Metal Ions to Site S2.[a]

Metal Ion	Assoctn Constant[b] ((l./mol) × 10^{-3})	Ionic Radius[c] (Å)	MeGlc Bound[d] (mol/27,000 g)
Mg^{2+}	~0	0.65	
Ni^{2+}	~0	0.72	0.03
Mn^{2+}	~0	0.80	
Cd^{2+}	1.5[e]	0.97	1.32[g]
Ca^{2+}	3[f]	0.99	0.98
Sr^{2+}	0.03	1.13	1.00
Ba^{2+}	~0	1.35	
Sm^{3+}	~0	1.04	0.15

[a] Measured in 0.05 M CH_3CO_2Na–CH_3CO_2H, pH 5.2, 0.2 M NaCl, 4°. [b] Calculated from competition with Ca^{2+} ions according to eq 3. [c] From Pauling (1960). [d] In the presence of 1 mM Ni^{2+} and calculated from single experiments on the basis of $K_{MeGlc} = 4 \times 10^3$ l./mol (Yariv *et al.*, 1968). [e] The solvent contains 0.2 M $NaNO_3$ instead of 0.2 M NaCl. [f] From Kalb and Levitzki (1968). [g] In the presence of 80 mM Cd^{2+} only.

The interaction of the competing ions with site S2 has been further characterized by investigating the formation of the saccharide binding site. MeGlc binds to Ni^{2+}–concanavalin A when Sr^{2+} is added. Addition of Cd^{2+} ions alone to demetallized concanavalin A is enough to induce the binding of MeGlc (Table II). However, no binding is observed in presence of Ni^{2+} alone or Ni^{2+} and Sm^{3+}.

Binding of Hg^{2+}. Hg^{2+} is bound very tightly to concanavalin A. The association constant is so high that it could be evaluated only indirectly, in the presence of Cl^-, which greatly decreases the concentration of free Hg^{2+} ions (Sillén and Martell, 1964). The association constant for Hg^{2+} ions is approximately 10^{16} l./mol and the equivalent binding weight is 1.2×10^4 daltons. Thus, two Hg^{2+} ions can be bound per subunit.

Hg^{2+} does not compete with Ni^{2+} for site S1, nor does it cause the formation of the saccharide binding site in Ni^{2+}–concanavalin A. Hg^{2+} does not prevent the binding of MeGlc in the presence of Ni^{2+} and Ca^{2+}. Thus, Hg^{2+} is bound at two sites in the subunit of concanavalin A different from S1 or S2.

Discussion

The transition metal binding site of concanavalin A can accommodate Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, or Cd^{2+} ions, but not Mg^{2+} or Ca^{2+} ions. The fact that Ca^{2+} and Mg^{2+} do not associate with nitrogen ligands, whereas Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} do (Bjerrum, 1941; Schubert, 1954; Sillén and Martell, 1964) suggests that at least one nitrogen ligand participates in metal binding in S1. The wide range of the stability constants observed here, as well as their order, are characteristic of complexes of divalent metals with simple nitrogen ligands (Vallee and Wacker, 1970). In the recently published three-dimensional structure of crystalline concanavalin A, one of the ligands in S1 is an imidazole (Edelman *et al.*, 1972; Hardman and Ainsworth, 1972). Recent chemical evidence suggests the participation of two imidazoles in S1 (Gachelin *et al.*, 1972).

The specificity of binding to S1 is governed mainly by the chemical affinity to the ligands. S1 may be a flexible site, since the larger Cd^{2+} ion has no less affinity than the smaller Zn^{2+} ion.

Binding of divalent metal ions to S2 is, in contrast, strongly dependent on the size of the ion (Table II). Ca^{2+} and Cd^{2+}, whose ionic radii are nearly 1 Å, are accommodated by S2 with nearly equal affinity. Smaller ions, *e.g.*, Mg^{2+}, Ni^{2+}, or Mn^{2+} and larger ions, *e.g.*, Sr^{2+} and Ba^{2+}, are bound weakly or not at all. Thus S2 is probably a rigid structure into which only a divalent ion of radius very close to 1 Å can fit.

Both metal binding sites of concanavalin A are unique in their specificity as compared to metal binding sites in other proteins. Carboxypeptidase A and carbonic anhydrase bind Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} but also Hg^{2+} and Cu^{2+} (Coleman and Vallee, 1961; Lindskog and Nyman, 1964). In concanavalin A Hg^{2+} is not bound to S1. Cu^{2+}, which inhibits the binding of Ni^{2+}, is in fact not bound to S1 (Shoham, 1972). Furthermore, in carboxypeptidase and carbonic anhydrase, in contrast to S1, Zn^{2+} has a higher binding constant than Co^{2+} or Ni^{2+}.

Site S2 is much more selective than calcium sites of other proteins. Trypsin and trypsinogen bind neodymium (Darnall and Birnbaum, 1970), staphylococcal nuclease binds barium and neodymium (Arnone *et al.*, 1971; Williams, 1971), and thermolysin binds Sr^{2+}, Ba^{2+}, and rare earth metal ions at their calcium binding sites (Colman *et al.*, 1972). On the other hand, α-amylases exhibit high specificity toward Ca^{2+} and, as in concanavalin A, lanthanide ions fail to replace calcium (Levitzki and Reuben, 1973).

According to the X-ray structure of concanavalin A, the two metal binding sites are similar in the nature of their ligands and in their geometry. The only outstanding difference is that S1 contains an imidazole whereas S2 contains carboxyls only. Indeed, two carboxyls are shared by both metals (Edelman *et al.*, 1972; Hardman and Ainsworth, 1972). In view of this, the specificity of the two sites is surprisingly different. Further structural studies on the region of the two metal binding sites will be of great importance in understanding this difference.

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Interactions of Metal Ions with Biotin and Biotin Derivatives. Complexing and Hydrogen-Bond Formation of the Ureido Group†

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ABSTRACT: Earlier studies have shown that two of the three potential binding sites of *d*-biotin can coordinate with metal ions in solution; namely, the carboxylate and the thioether groups. It is shown in this paper that the third potential site, the ureido group, can also interact with metal ions. This is evidenced by line-broadening studies of the nuclear magnetic resonance spectra of biotin and some derivatives in the absence and presence of increasing amounts of the paramagnetic divalent manganese ion in deuterated dimethyl sulfoxide as solvent. That the presence of the sulfur in the biotinyl moiety enhances the interaction between Mn^{2+} and the ureido group follows from a comparison with dethiobiotin and ethyleneurea (2-imidazolidone) as ligands. The nucleophilicity of the ureido group becomes evident by the intermolecular formation of hydrogen bonds between phenol and the biotin model, ethyleneurea. Studies of the infrared spectra in chloroform revealed a significant shift of the carbonyl-stretching frequency to longer wavelengths.

The early work of Lynen and his coworkers (Lynen *et al.*, 1961; Lynen, 1967; *cf.* also Knappe, 1970) concerning carboxylation reactions in biological systems led to the conclusion that, during the enzymatic process, a nucleophilic attack occurs by the nitrogen of the ureido group of *d*-biotin at the carbon of carbon dioxide or bicarbonate. Later, Bruice and Hegarty (1970) concluded, based on experiments with model compounds, that, although the ureido group shows no nucleophilicity in *inter*molecular reactions, it is an effective nucleophile in *intra*molecular reactions with nearby electrophiles. In addition, these authors gave evidence that the oxygen of the ureido group is more nucleophilic than the nitrogen in such intramolecular reactions. They proposed a mechanism for the enzymatic carboxylation reaction with an O-carboxylated biotin as an intermediate. However, in a recent review, Moss and Lane (1971) attacked this theory. Indeed, the model used by Bruice and Hegarty in their study is so different from the biotin molecule as it occurs in the enzymatic surroundings that no unequivocal decision appears possible at this time, even though the hypothesis of Bruice and Hegarty has some appealing features.

Though it is obvious that the reactions occurring at biotin during the carboxylation process are still disputed, the following is definite. In biotin-containing enzymes, the carboxylate group of biotin is amide linked with an ϵ-amino group of lysine, which is part of the peptide chain of the enzymes and probably has no other function than attaching the coenzyme to the protein by a covalent bond. That both remaining functional groups, *i.e.*, the thioether and the ureido groups, are of importance during the enzymatic reaction has been shown independently with two different carboxylases by the carboxylation rates of free biotin as compared to different free biotin derivatives (Lynen *et al.*, 1961; Stoll *et al.*, 1968); it turned out that dethiobiotin and a biotin without the car-

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