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LOCATION OF Mn^{2+} In CONCANAVALIN A CONTAINING ONLY A Mn^{2+} ION[1]

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SUMMARY

The metal-sugar distances in two metallized forms of concanavalin A have been compared by ^{19}F magnetic resonance techniques. Using relaxation times measured at two different frequencies we have shown that the distance between the Mn^{2+} ion and the bound sugar in concanavalin A containing only Mn^{2+} is essentially identical to that found in concanavalin A containing both Mn^{2+} and Ca^{2+}. Our results rule out the possibility that Mn^{2+} activates concanavalin A by binding at the Ca^{2+} site (S2) and would suggest that Mn^{2+} alone can induce an active saccharide binding conformation by binding at the transition metal site (S1).

In spite of having received a great deal of attention in the past several years, the role of metal ions in inducing an active (sugar binding) conformation in concanavalin A (Con A)[3] remains a source of much controversy. While most workers concur with the model proposed for activation at pH 5, there is much disagreement about the role of metal ions in the more physiologically relevant pH range of 6.5 - 7.0.

At pH 5 Mn^{2+} binds to a site designated S1 which induces the formation of a second site, S2, which binds Ca^{2+}. Sugar binding activation requires

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[3]Abbreviations used are: Con A, concanavalin A; apo-Con A, demetallized Con A; NTFAGlcn, N-triflouroacetyl-D-glucosamine; MUM, 4-methylumbelliferyl α-D-mannopyranoside. Con A containing only a Ca^{2+} ion is designated as Ca-Con A; Con A containing only a Mn^{2+} ion is designated as Mn-Con A; and Con A containing a transition metal ion, such as Mn^{2+}, and a Ca^{2+} ion is designated Mn-Ca-Con A.

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the presence of both metal ions (1,2). At pH 6.5 or above, this model does not hold. In this pH range Ca^{2+} can bind to demetallized Con A (apo-Con A) in the absence of Mn^{2+} (3) and this form of Con A (Ca^{2+}-Con A) has full saccharide binding activity (4,5,6). In addition, it has been found that Mn^{2+} alone will also activate Con A above pH 6.5 (5,6,7). Koenig and co-workers (5,8) have proposed that Mn^{2+} binds to both S1 and S2 and suggested that the form containing two Mn^{2+} ions is the stable sugar-binding conformation. Their findings are in direct conflict with those of Christie et al. (7) who showed by repetition of the experiments of Brown et al. (8) coupled with direct binding studies that only one Mn^{2+} binds per Con A subunit and that only one Mn^{2+} is required to activate the protein.

The crucial information which is needed and which may help to end this controversy is the location of the Mn^{2+} ion or ions in Mn-Con A. In this communication, we provide this information and show that activation of Con A does not require the occupation of S2 by Mn^{2+}.

MATERIALS AND METHODS

Con A was prepared as previously described (3). All solutions were at 25°C, pH 6.6, and contained 1.0 M NaCl prepared by two passages over Chelex-100 (Bio-Rad), and 0.02 - 0.05 M Mops (Sigma). Apo-Con A was prepared by treatment in 0.1 M HCl as previously described (4). All glassware was thoroughly acid-washed and plastic containers were used whenever possible. Ca^{2+} contamination was monitored by atomic absorption spectroscopy at the beginning and end of every experiment and never exceeded 3% with respect to Con A monomers. N-trifluoroacetyl-D-glucosamine (NTFAGlcn) was synthesized by the method of Wolfrom and Conigliaro (9). Spin-lattice relaxation times (T_1) were determined from inversion recovery experiments using a Bruker HXS-270 NMR spectrometer operating at 254 MHz. Spin-spin relaxation times (T_2) were determined from the linewidths of the ^{19}F resonances of NTFAGlcn using a Bruker WH-90 operating at 84.7 MHz. Relaxation times for the bound sugar (T_b) were calculated assuming fast exchange conditions (see reference 10 for justification) using the following equation

$$\frac{1}{T_{obs}} = x_f \frac{1}{T_f} + x_b \frac{1}{T_b}$$

where T_{obs} is the experimentally observed relaxation time, T_f and T_b are the relaxation times for the free and bound sugar, and x_f and x_b are the mole fractions of free and bound sugar. T_f and T_{obs} were determined by either of two methods. For T_1 determinations, aliquots of apo-Con A were

dialyzed against three changes of tenfold volumes of buffer containing 1.0 mM concentrations of metals and 5 mM NTFAGlcn. The T_1 of the dialysate was used as T_f and the T_1 of the Con A solution as T_{obs}. Measurements of T_2 were performed as described by Alter and Magnuson (10) by adding sugar directly to Con A pretreated with 1.5 equivalents of the appropriate metals. In these experiments T_{obs} was the T_2 measured and T_f was the T_2 obtained after addition of a large excess of methyl α-D-mannopyranoside. Mole fractions were calculated using binding constants determined by titration of Con A with NTFAGlcn as previously described (10). Bound relaxation times were assumed to be dominated by the proximity of the Mn^{2+} ion. This assumption was justified by the observation of Alter and Magnuson (10) that diamagnetic Zn-Ca-Con A bound NTFAGlcn but caused no ^{19}F line broadening and by our observation that binding to Ca-Con A also had no effect on relaxation times.

Aliquots of the same samples used for distance determinations were tested for saccharaide binding activity by fluorescence quenching titrations using 4-methylumbelliferyl α-D-mannopyranoside (MUM) (4). In all cases the Con A was greater than 95% active. Distances between the ^{19}F-sugar and the bound Mn^{2+} ion were calculated using the Solomon-Bloembergen equations (11). A correlation time of 7×10^{-9} sec, determined from the ratio of relaxation times at two frequencies, was used.

RESULTS AND DISCUSSION

Table 1 shows the relaxation times for NTFAGlcn bound to Mn-Ca-Con A and Mn-Con A and the Mn^{2+}-sugar distances calculated from them. There is clearly no significant difference between the two forms of Con A with respect to the distance separating the Mn^{2+} ion and the sugar. Our earlier studies showed that one Mn^{2+} ion is bound per Con A subunit under these conditions (7) and the Mn-Con A used in these experiments was shown to be fully active and free of Ca^{2+} contamination. Therefore, the most reasonable conclusion is that the metal ion in Mn-Con A is binding at the S1 site.

TABLE 1

	Sugar Anomer	Ka(M^{-1})	T_1(sec)[a]	T_2(msec)[a]	Distance(Å)
Mn-Ca-Con A[b]	α	800	0.21	2.4	14.4 ± 0.2[c]
Mn-Ca-Con A	β	800	0.46	4.5	16.4 ± 0.3
Mn-Con A	α	1000	0.19	2.1	14.2 ± 0.2
Mn-Con A	β	1000	0.38	3.8	15.9 ± 0.3

a Bound relaxation times calculated as described in the text.
b Con A concentrations (with respect to monomers) was 7.8×10^{-4} M for T_1 experiments and 2.4×10^{-4}M for T_2 experiments.
c Error estimated on the basis of the least accurate measurement (linewidth determinations at 90 MHz).

Metal-sugar distances for Mn-Ca-Con A have been determined by several laboratories using both NMR (10,12,13) and X-ray crystallographic (14,15) data. The distances we have measured for Mn-Ca-Con A are in excellent agreement with those reported values. The X-ray studies have also shown the Ca^{2+} site (S2) to be approximately 5 Å closer to the sugar than the Mn^{2+} site (S1). Because of the sixth power dependence of relaxation time on distance, Mn^{2+} occupation of S2 in Mn-Con A would be easily detected in our experiments. This is clearly not the case.

The small increase in Mn^{2+}-sugar distance we observed when Ca^{2+} was added to Mn-Con A, while not statistically significant, was consistently observed in all our experiments. We know from earlier studies that the addition of Ca^{2+} induces changes in the Mn^{2+} (3,16) and MUM (7) binding properties of Con A. It is possible that this distance change is a reflection of that same alteration.

It is interesting to note that while Mn^{2+} can activate Con A by binding at S1, other transition metals cannot (7). Zn^{2+}, Co^{2+} and Ni^{2+}, which are assumed to bind at S1, do not activate Con A until S2 is filled with Ca^{2+}. We have also recently found that Cd^{2+}, which binds to both metal sites, probably must occupy S2 to induce a saccharide binding conformation in Con A (17). These findings suggest that Mn^{2+} may not be binding to exactly the same site as these other metals. X-ray studies on Con A containing other transition metal ions could prove very interesting.

It should be noted that the distances we report for Mn-Ca-Con A differ slightly from those previously published by our laboratory (10). The earlier measurements were based on T_2 values at a single frequency and an estimated correlation time. Also, these earlier experiments were conducted in less than stoichiometric amounts of Mn^{2+} in order to reduce the linewidth of the free sugar. We feel that the present data represent the best determination for this system.

It is now possible to conclude that, near physiological pH, Con A can be activated by either a single Ca^{2+} ion (4), a combination of Mn^{2+} at S1 and Ca^{2+} at S2, or a single Mn^{2+} ion (7) binding at S1.

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