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^{43}Ca AND ^{67}Zn NMR SPECTRA OF Ca^{2+}, Zn^{2+}-CONCANAVALIN A SOLUTIONS

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The half-band width of ^{43}Ca NMR of free aqueous Ca^{2+} was scarcely increased by adding more than equal molar apo-concanavalin A(apo-Con A), suggesting that slow chemical exchange, $k_{off} < 10\ s^{-1}$, occurs for the Ca^{2+} ion from Con A. In contrast with the ^{43}Ca NMR findings, the half-band width of ^{67}Zn NMR of free aqueous Zn^{2+} was markedly increased by adding apo-Con A. The ^{67}Zn NMR half-band width of Zn^{2+} in the presence of apo-Con A was decreased by adding excess Ca^{2+}, but was increased by adding excess D-mannose. These changes of the half-band width were influenced mutually by D-mannose or Ca^{2+}, respectively. The broadened half-band widths of Zn^{2+} in the presence of Con A were decreased by adding Mn^{2+}, suggesting that Mn^{2+} was substituted for Zn^{2+} at a metal binding site of Con A.

Concanavalin A(Con A), a lectin isolated from the jack bean(Canavalia ensiformis), exists as a dimer of molecular weight 55,000 composed of 2 identical monomeric units in the pH range 4.5-5.6[1-3]. Con A binds 2 metal ions per monomer unit in an order fashion near pH 5. Thus, transition metal ion, such as Mn^{2+}(native metal ion), Ni^{2+}, Co^{2+}, or Zn^{2+}, firstly binds at a site designated S1 and then Ca^{2+} can bind at a site designated S2[4-7]. The bindings of both metal ions appear to be required for saccharide binding to take place at acidic pH. However, it has been claimed that near pH 7 Ca^{2+} ion or Mn^{2+} ion alone will activate apo-Con A to give full saccharide-binding activity[6,8,9].

Our initial applications of ^{67}Zn NMR spectroscopy to biological systems have exhibited its high potential for studying Zn^{2+} bound to amino acids, peptides and macromolecules[10-13]. In this paper, we like to describe ^{43}Ca and ^{67}Zn NMR studies of Ca^{2+} and Zn^{2+} in the presence of dimeric Con A. ^{43}Ca NMR of free aqueous $^{43}Ca^{2+}$ was scarcely changed by adding apo-Con A or other effectors. The ^{43}Ca NMR findings are markedly different from those of other Ca^{2+}-binding proteins[10,14,15]. In contrast with the ^{43}Ca NMR results, ^{67}Zn NMR spectra of free aqueous $^{67}Zn^{2+}$ were markedly broadened by adding apo-Con A. Environments of Zn^{2+} in Con A solutions were sensitively changed by the presence of Ca^{2+}, D-mannose and Mn^{2+} in terms of ^{67}Zn NMR spectra.

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MATERIALS AND METHODS

Con A was purchased from Vector Laboratories(Type L-1000). It was homogeneous upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Protein concentrations were determined spectrophotometrically at pH 5.6 by using $A^{1\%}_{280,nm}{}^{1\ cm} = 12.4$[16]. To prepare apo-Con A, native Con A was demetallized by lowering the pH of the aqueous solution as described previously[16]. After stirring for 45 min at 20 °C, the solution was dialyzed against two successive 1 mM ethylenediaminetetraacetic acid solution at 4 °C. The solution was then dialyzed against three successive 0.1 M potassium acetate-0.9 M KCl buffer(pH 5.33)[16]. For ^{67}Zn NMR studies, the solution was further dialyzed against three successive 0.1 M KCl solution (pH 5.55). Reagents used were of the highest guaranteed grade and used without further purification.

^{43}Ca was obtained in 49.1 % $CaCO_3$ from Commissariat a L'Energie Atomique. ^{67}Zn was obtained in 78.63 % metal from Prochem. ^{43}Ca and ^{67}Zn NMR spectra were accumulated on a Bruker CXP-300 FT NMR spectrometer at 20.19 and 18.77 MHz, respectively, in spinning 10 mm sample tubes with external D_2O for frequency lock. An equipped transmitter provided 90° pulse widths of 80 μs for both nuclei at a peak-to-peak voltage of 300 V. Typical spectra consisted of 1,000-5,000 transients for ^{43}Ca NMR and 5,000-400,000 transients for ^{67}Zn NMR, respectively, to obtain signal/noise > 6 using 1 k-16 k data points over 2,000-5,000 spectral window in quadrature detection mode. The signal/noise ratio was improved by exponential multiplication which introduced 1-10 Hz line broadenings. The delay time was changed from 0.12 s to 4 s depending upon the line widths of samples to fit sufficient delay time. For ^{67}Zn NMR spectroscopy, a dead time(400 μs) longer than that(200 μs) for ^{43}Ca NMR spectroscopy was necessary due to accumulated ring down in the probe after more than 10^5 transients. NMR spectra were obtained after standing sample more than 1 hr at 20 °C, since bindings of metals or sugars to apo-Con A take long time[6,17,18]. We could not see any time-dependent change of NMR later than 1 hr after sample preparations.

RESULTS

^{43}Ca NMR spectra: Fig. 1 shows ^{43}Ca NMR spectra of aqueous Ca^{2+}(3 mM)(A) and Ca^{2+}(2.34 mM)-apo-Con A(4.34 mM)(B) solutions at pH 5.33 together with ^{67}Zn NMR spectra(C and D). The half-band width($\Delta\nu_{1/2}$)(0.9 Hz)(Fig. 1A) of ^{43}Ca NMR of aqueous Ca^{2+}(2.34 mM) was scarcely changed by adding apo-Con A(Fig. 2A). The $\Delta\nu_{1/2}$ of Ca^{2+}(2.34 mM)-apo-Con A(4.37 mM) solution was only 4.8 Hz(Fig. 1B). Adding up to 50 mM Zn^{2+} and/or up to 50 mM D-mannose did not essentially change the $\Delta\nu_{1/2}$ of the Ca^{2+}-Con A solution. Chemical shift of aqueous Ca^{2+} was moved nearly 4 Hz down-field by adding apo-Con A. Keeping the

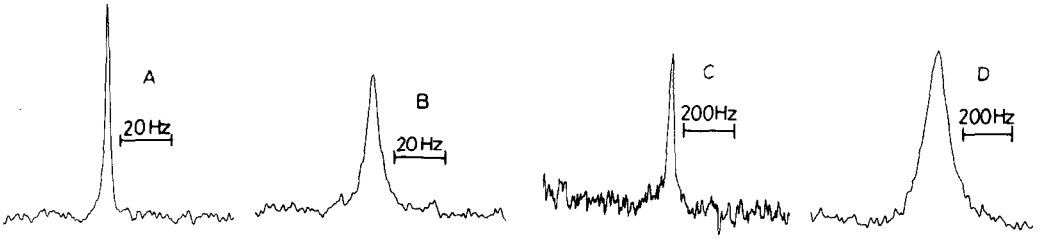

Figure 1. Typical ^{43}Ca NMR spectra of enriched aqueous $^{43}Ca^{2+}$(3 mM)(A) and enriched $^{43}Ca^{2+}$(2.34 mM)-apo-Con A(4.37 mM)(B) and ^{67}Zn NMR spectra of enriched aqueous $^{67}Zn^{2+}$(5.5 mM)(C) and enriched $^{67}Zn^{2+}$(5.5 mM)-apo-Con A(10.26 μM)(D).

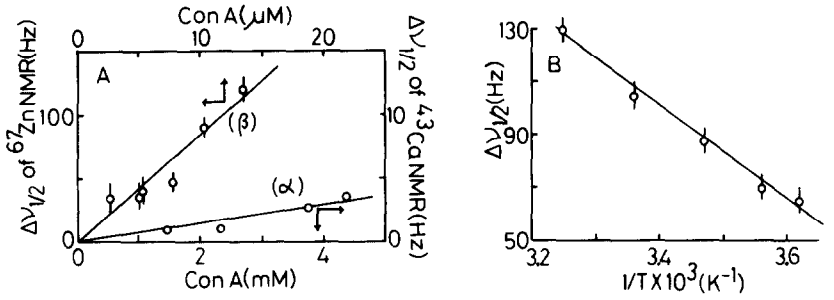

Figure 2. A: Excess relaxation rates in terms of the $\Delta\nu_{1/2}$ of ^{43}Ca and ^{67}Zn NMR spectra. (α) ^{43}Ca NMR changes of aqueous $^{43}Ca^{2+}$(3.0 mM) caused by adding apo-Con A in 0.1 M potassium acetate-0.9 M KCl(pH 5.33) buffer. (β) ^{67}Zn NMR changes of aqueous $^{67}Zn^{2+}$(5.5 mM) caused by adding apo-Con A in 0.1 M KCl(pH 5.55). B: Temperature dependence of ^{67}Zn NMR spectra of the Zn^{2+}(5.5 mM)-apo-Con A (10.26 μM) in the presence of $CaCl_2$(40 mM) and D-mannose(40 mM) in 0.1 M KCl (pH 5.55).

Ca^{2+}-Con A solution at 20 °C more than 2 days did not essentially change the spectrum. These findings are quite surprising since the $\Delta\nu_{1/2}$ of ^{43}Ca NMR for aqueous Ca^{2+} has been known to be markedly broadened to more than 500 Hz by adding Ca^{2+}-binding proteins[10,14,15]. Since the association constant of Ca^{2+} to apo-Con A is 2.1-3.0 x 10^3 M^{-1}[4,17], nearly 87 % Ca^{2+} in the solution must be bound to Con A under the conditions studied here(Fig. 1B).

^{67}Zn NMR spectra: In contrast with the ^{43}Ca NMR findings, the $\Delta\nu_{1/2}$(20 Hz) of ^{67}Zn NMR of aqueous 5.5 mM Zn^{2+} was markedly increased linearly by adding apo-Con A(Fig. 1C, D and Fig. 2A). The Zn^{2+}(5.5 mM)-apo-Con A(13.25 μM) solution had a $\Delta\nu_{1/2}$ of 165 Hz. By adding more apo-Con A to the solution, the signal/noise ratio was markedly decreased, giving rise to no signal even after more than 2 x 10^5 transients under our experimental conditions. The $\Delta\nu_{1/2}$ of the Zn^{2+}-apo-Con A solution was linearly decreased by nearly 65 Hz by lowering the temperature from 308 K to 276 K. The $\Delta\nu_{1/2}$ of the Zn^{2+}-Con A solution in the presence of excess Ca^{2+} and/or D-mannose was similarly decreased by lowering the temperature(Fig. 2B). Thus, the $\Delta\nu_{1/2}$ of the Zn^{2+}-Con A solution will be dominated by the chemical exchange mechanism.

^{67}Zn NMR titrations were done for the Zn^{2+}(5.5 mM)-apo-Con A(10.26 μM) solution by adding Ca^{2+} in the presence and absence of D-mannose(Fig. 3A). The $\Delta\nu_{1/2}$ of ^{67}Zn NMR of the Zn^{2+}-Con A complex was decreased by adding excess Ca^{2+}. The decrease of the $\Delta\nu_{1/2}$ caused by adding Ca^{2+} was less marked in the presence of 40 mM D-mannose than that in the absence of 40 mM D-mannose. Apparent dissociation constant derived from theoretical curves assuming simple binding of Ca^{2+} to the Zn^{2+}-Con A solution were 3 mM and 34 mM in the absence and presence of D-mannose, respectively(Fig. 3A).

We did ^{67}Zn NMR titration studies of the Zn^{2+}(5.5 mM)-apo-Con A(10.26 μM) solution by adding D-mannose in the presence and absence of Ca^{2+}(Fig. 3B).

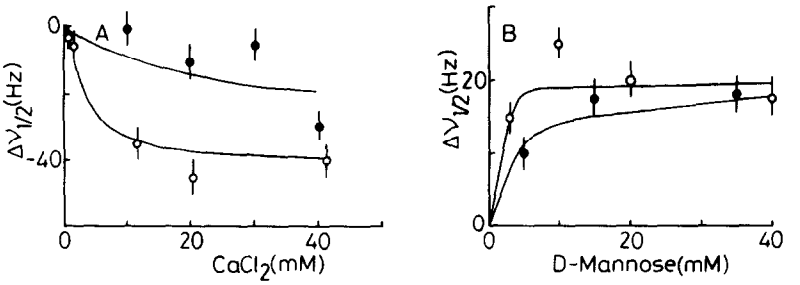

Figure 3. A: Effects of Ca^{2+} on the excess relaxation rates of ^{67}Zn NMR spectra of Zn^{2+}(5.5 mM)-apo-Con A(10.26 μM) in the absence(-O-) and presence(-●-) of 40 mM D-mannose in 0.1 M KCl(pH 5.55). The minus sign denotes the relative decrease of the excess relaxation rates caused by adding $CaCl_2$. The theoretical curves were fit to the data assuming simple binding with dissociation constants of 3 mM and 34 mM for the absence(-O-) and presence(-●-) of D-mannose, respectively. B: Effects of D-mannose on the excess relaxation rates of ^{67}Zn NMR spectra of the Zn^{2+}(5.5 mM)-apo-Con A(10.26 μM) in the absence(-O-) and presence(-●-) of 40 mM $CaCl_2$ in 0.1 M KCl(pH 5.55). The theoretical curves were fit to the data assuming simple binding with dissociation constants of 0.5 mM and 3.0 mM in the absence(-O-) and presence(-●-) of $CaCl_2$, respectively. The starting points in A and B were normalized to 0 in $\Delta\nu_{1/2}$. Thus the $\Delta\nu_{1/2}$ values described are relative values, not absolute values.

The $\Delta\nu_{1/2}$ of ^{67}Zn NMR of the Zn^{2+}-Con A solution was increased by adding excess D-mannose. The increase of $\Delta\nu_{1/2}$ caused by adding D-mannose was less marked in the presence of Ca^{2+} than that in the absence of Ca^{2+}. The apparent dissociation constant derived from theoretical curves assuming simple binding of D-mannose to the Zn^{2+}-Con A solution were nearly 3 mM and 0.5 mM in the presence and absence of 40 mM Ca^{2+}, respectively(Fig. 3B).

Mn^{2+} is a native transition metal ion and is known to be exclusively bound to the site S1[4-7]. To know whether Zn^{2+} is bound to the site S1 or to a non-specific site of Con A under the conditions studied here, we did Mn^{2+} titration studies for various Zn^{2+}-Con A solutions. The $\Delta\nu_{1/2}$ values of the Zn^{2+}-Con A solutions were reduced by adding Mn^{2+} irrespective of the presence of Ca^{2+} or D-mannose(Fig. 4). It seems likely that the decrease of the $\Delta\nu_{1/2}$ of ^{67}Zn NMR caused by adding Mn^{2+} is attributed to the increase of aqueous(uncomplexed or free) Zn^{2+} concentration as a result of Mn^{2+} substitution for Zn^{2+} bound to the site S1 of Con A.

Discussion

^{43}Ca NMR spectra: The association constant(2.1-3.0 x 10^3 M^{-1})[4,17] of Ca^{2+} to Con A is the lower limit to observe the exchange behavior of Ca^{2+} with ^{43}Ca NMR spectra. NMR of metal ion(I = 5/2 or 7/2) which is bound to macromolecule and exchanges very slowly(k_{off} < 10^3 s^{-1}) would give the $\Delta\nu_{1/2}$ essentially the same as that of free metal ion[15]. If the $\Delta\nu_{1/2}$ is totally controlled by the chemical exchange, the off-rate of Ca^{2+} from Con A may be less than 10 s^{-1}. Conformational change induced by Ca^{2+} binding at the site S2 occurs with a rate constant of 10^{-2} s^{-1} at pH 5.3 in terms of circular dichroism spectral changes

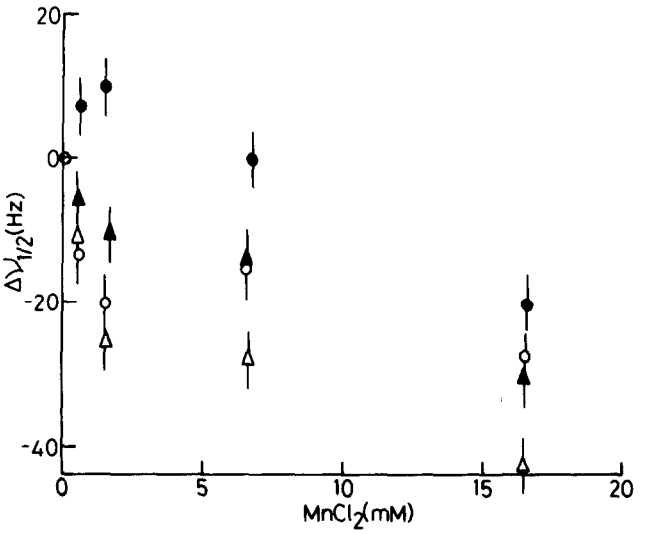

Figure 4. Effects of $MnCl_2$ on the excess relaxation rates of ^{67}Zn NMR spectra of Zn^{2+}(5.5 mM)-apo-Con A(10.26 μM)(-Δ-), Zn^{2+}(5.5 mM)-apo-Con A(10.26 μM)-D-mannose(40 mM)(-▲-), Zn^{2+}(5.5 mM)-apo-Con A(10.26 μM)-$CaCl_2$(40 mM)(-●-) and Zn^{2+}(5.5 mM)-apo-Con A(10.26 μM)-$CaCl_2$(40 mM)-D-mannose(40 mM)(-○-) solutions in 0.1 M KCl(pH 5.55). The starting points are normalized to 0 in $\Delta\nu_{1/2}$. Thus the $\Delta\nu_{1/2}$ values described are relative values, not absolute values.

of Con A[17,19]. Therefore, it seems likely that the rate of the conformational change of apo-Con A caused by Ca^{2+} is much slower than that of the Ca^{2+}-binding to apo-Con A.

^{67}Zn NMR spectra: The decrease of the $\Delta\nu_{1/2}$ of the ^{67}Zn NMR for the Zn^{2+}-Con A solution caused by adding Ca^{2+} may be attributed to the decrease of chemcial exchange rate of Zn^{2+}. The increase of symmetry around Zn^{2+} may also contribute to the ^{67}Zn NMR change caused by adding Ca^{2+}. The apparent dissociation constants, 10^{-3}-10^{-2} M, of Ca^{2+} from Con A determined from ^{67}Zn NMR are much larger than that, 10^{-4} M, determined from other method[4]. A conformational change[17-19] around the site S1 indirectly caused by adding Ca^{2+} must have the apparent dissociation constants of 10^{-3}-10^{-2} M for Ca^{2+}. The $\Delta\nu_{1/2}$ change of ^{67}Zn NMR of the Zn^{2+}-Con A solution caused by Ca^{2+} was attenuated in the presence of D-mannose(Fig. 3A). Thus, the carbohydrate binding can change the structure around the site S1 and/or site S2 in terms of ^{67}Zn NMR spectra.

The $\Delta\nu_{1/2}$ decrease of ^{67}Zn NMR for the Zn^{2+}-Con A solution caused by adding D-mannose may be attributed to the increase of the exchange rate of Zn^{2+} and/or to the decrease of symmetry around Zn^{2+} on Con A[10,11]. Conformational changes of Con A caused by Ca^{2+}[17,18] may change the binding behavior of the carbohydrate to Con A. The apparent dissociation constant, 10^{-3}-10^{-4} M, of D-mannose from Con A determined from ^{67}Zn NMR may not directly reflect the

D-mannose binding to Con A since the dissociation constant of D-mannose from Con A is 10^{-5}-10^{-6} M[20,21].

It seems unlikely that Mn^{2+} bound at a site different from the site S1 in the Zn^{2+}-Con A solutions and caused the ^{67}Zn NMR changes in an indirect manner. The decrease of the $\Delta\nu_{1/2}$ of the Zn^{2+}-Con A solutions by adding Mn^{2+} will be mainly attributed to the dissociation of Zn^{2+} from Con A. Thus, the ^{67}Zn NMR broadenings observed by adding apo-Con A will come from the Zn^{2+} binding to the specific site, the site S1, of Con A. ^{67}Zn NMR spectra of the Zn^{2+}-Con A solutions will directly reflect the environment of the Zn^{2+} binding site, the site S1, of Con A. It is interesting to note that the binding of Ca^{2+} or D-mannose influences the environment of the site S1 indirectly. Relatively high dissociation constants of Ca^{2+} and D-mannose from Con A determined from ^{67}Zn NMR may reflect the change of whole protein structure caused by those effectors. The cooperative effects of Ca^{2+} and the carbohydrate on the structure of the Zn^{2+} binding site are also to be noted. Further studies on the allosteric effect are on the way by us.

In concluding remarks, it was found that Ca^{2+} exchanges very slowly from Con A in terms of ^{43}Ca NMR spectra and that Ca^{2+} and D-mannose cooperatively affect the environment of Zn^{2+} at the site S1 of Con A in terms of ^{67}Zn NMR spectra. It should be emphasized here that ^{67}Zn NMR can offer quite valuable information on the binding site of the metal ion in macromolecule.

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