The Proton Relaxation Enhancement Properties of Concanavalin A*

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SUMMARY

The measurement of the proton relaxation rate (1/T₁) enhancement (ε₂*) for the concanavalin A (Con A)-Mn²⁺ complex as a function of time following the addition of Mn²⁺ indicates a pH-dependent rate of structural rearrangement at the bound Mn²⁺ site. The addition of excess Ca²⁺ interrupts this kinetic process and results in a reduced time-independent value of ε₂*. The further addition of α-methyl-D-glucoside to the Con A-Mn²⁺-Ca²⁺ complex also alters the observable ε₂*. Using the recently published 2 A resolution crystal structure of Con A, the results are interpreted as arising from the gradual folding of the loop of residues 12 to 22 over the initial Mn²⁺ binding site (formed by residues 8, 10, and 24) to close the Mn²⁺ off from free access by water molecules.

Concanavalin A, a protein isolated from the jack bean (1, 2), is one of a number of plant lectins shown to bind to and agglutinate transformed cells (3, 4). The specific action of Con A is thought to involve the binding of the lectin to a particular carbohydrate moiety exposed at the transformed cell surface (5, 6). Nontransformed cell cultures exhibit a range of abilities to bind Con A, but are not agglutinable unless previously subjected to mild proteolytic action at the cell surface (6). A more detailed understanding of the structural features of Con A, and the molecular basis of its interaction with the carbohydrate-containing receptor, would be of value in assessing proposed models and interpreting the cell surface changes accompanying transformation. The subunit composition of purified Con A has been described by Wang et al. (7). The structure was found to have an intact subunit molecular weight of 27,000. Sedimentation data indicate that in the pH range 3.5 to 5.8 Con A exists as a dimer of molecular weight approximately 55,000. At pH 6.0 and above, higher molecular weight forms are seen (8, 9). Agglutination of transformed cells by Con A can be competitively reversed by certain carbohydrates. The saccharide binding ability, and the agglutinability of transformed cells in culture, is dependent upon divalent metal ions (3, 10). Kalb and Levitzki (11), on the basis of equilibrium dialysis experiments, have assessed the metal ion requirements of Con A. They concluded that both S1 and S2 sites must be occupied in order to obtain significant binding of α-methyl-D-glucopyranoside.

As part of the effort in our laboratory to develop useful NMR probes of cell surface structure, we have investigated the solvent proton relaxation enhancement (12) properties of the Mn²⁺-Con A complex. In this technique the fact is utilized that Mn²⁺, a paramagnetic ion, considerably shortens the spin-lattice relaxation time, 1/T₁, of the protons of H₂O molecules in the hydration sphere of the ion, and does so differentially when the ion is bound to a macromolecule or free in solution. Since the proton spin-lattice relaxation rate, 1/T₁, is usually increased in the presence of a specific Mn²⁺ binding macromolecule, a spin-lattice relaxation enhancement parameter, ε₂*, can be defined (12). ε₂* represents the ratio of the paramagnetic ion contribution to the proton relaxation rate in the presence and absence of the macromolecule for a specified paramagnetic ion concentration. A consideration of the contributions to the water relaxation time enables one to write ε₂* in terms of three parameters ε₁*, ε₂, and X₅.

ε₂* = X₅ ε₁* + (1 - X₅) ε₂

where X₅ represents that mole fraction of the total Mn²⁺ that is bound to the macromolecular site and ε₂ has the value 1 assuming no differential viscosity effects between the Mn²⁺ hydrate in the macromolecular solution and the buffer alone. ε₁* is a number which is characteristic of the complex which the Mn²⁺ forms with the macromolecule, in this case Con A. In a completely analogous manner, the enhancement parameter ε₀ can be defined for the spin-spin relaxation rate, 1/T₂.

EXPERIMENTAL PROCEDURE

The buffers used in this study, 0.05 mM sodium acetate in 0.2 mM NaCl, were prepared in glass distilled H₂O using BDH Analar grade NaCl, sodium acetate, and acetic acid. CaCl₂ was provided in the form of CaCl₂. 2 H₂O, sodium acetate in 0.2 M H₂O (BDH Analar) and MnCl₂ as MnCl₂. 4 H₂O (Fisher Certified), and α-methyl-D-(+)-glucoside was obtained from BDH Biochemical. The Mn²⁺ stock concentration was 1.25 mM in distilled H₂O. Con A was obtained from Sigma Chemical Company as a lyophilized powder (Grade IV, salt-free, essentially free of carbohydrates, Lot 101C-5390). Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the Sigma product showed the same four bands, with similar relative intensities, reported by Wang et al. (7). Con A concentrations were determined using the optical density at 280 nm and the extinction coefficient, E₅₀₀ of 11.4 reported by Agrawal and Goldstein (13). Stock solutions of Con A prepared with no prior treatment were found to have longer H₂O solvent T₁ relaxation times (~800 ms for 16 mg per ml) than solutions of similar Con A concentration subjected to the demetallization procedure of Kalb and Levitzki (11). Comparison of the ESR spectrum of the Mn²⁺ bound to Con A in the stock solution (14) with a spectrum of a known amount of Con A-bound Mn²⁺, indicated...
that fewer than 1 in 18 Mn\(^{2+}\) binding sites were occupied by Mn\(^{2+}\) in the stock solution. Consequently, for the results reported, Con A was used as obtained from Sigma. All NMR measurements reported were made at 32 MHz on a Bruker variable frequency pulsed spectrometer (B-KR322s) at the ambient probe temperature of 24\(^\circ\)C.\(T_1\) values were determined from a semilogarithmic plot of the signal amplitude following a 180\(^\circ\)-90\(^\circ\) pulse series as a function of the pulse separation \(\tau\). Samples were examined in glass tubes (7.5 mm diameter, Wilmad 513B-1PP). The samples used were 60 \(\mu\)l of stock Con A solution with 5-\(\mu\)l additions of the appropriate metal ion or sugar stock. The length of time required to obtain the \(T_1\) value for each point in the kinetic experiments was 1 to 2 min. ESR spectra were obtained at room temperature (24\(^\circ\)C) on a Varian E-6 spectrometer operating at 9.1 GHz (X-band). Each sample (~50 \(\mu\)l) was taken up in a glass disposable microsampling pipette (Corning Glass Works), which was then inserted into the microwave cavity in a reproducible position.

**RESULTS**

When Mn\(^{2+}\) is added to a buffered stock solution of Con A the relaxation time \(T_1\) is found to change appreciably with time. The enhancements obtained at three different pH values are given in Fig. 1 as a function of time following the Mn\(^{2+}\) addition. A pH dependence of the rate of change of \(\epsilon_1^*\) is clearly evident. Mn\(^{2+}\) ESR spectra were obtained for a Con A solution of 10.9 mg per ml (404 \(\mu\)M Mn\(^{2+}\) sites assuming one site per 27,000 mol wt) at a MnCl\(_2\) concentration of 96 \(\mu\)M from 15 to 80 min after the addition of the Mn\(^{2+}\). No free Mn\(^{2+}\) signal was observed. The estimated lower limit of detection was 8 \(\mu\)M. In view of this result, and the fact that all Con A concentrations used in the NMR experiments were considerably greater than 11.0 mg per ml and that Mn\(^{2+}\) concentrations were less than 100 \(\mu\)M, \(\epsilon_1^*\) values reported can be regarded directly as \(\epsilon_M\) values (i.e. \(X_5 = 1\)).

The result of plotting \(\log_{10} [\epsilon_1^* (t) - \epsilon_1^* (\infty)]\) versus time for

| pH     | \(\tau = 0\) | + Ca\(^{2+}\) | + Ca\(^{2+}\), + GlcMe* |
|--------|-------------|------------|------------------|
| 5.90   | 2.9         | 1.7        | 1.4              |
| 5.67   | 1.7         | 1.6        | 1.3              |
| 5.30   | 1.7         | 1.7        | 1.4              |
| 5.15   | 1.8         | 1.9        | 1.4              |
| 4.50   | 1.7         |            | 1.6              |

* All \(\tau = 0\) values are for times greater than 24 hours after Mn\(^{2+}\) addition when no further change in \(\epsilon_1^*\) was observed.

* Ca\(^{2+}\) concentration of 2.3 mm added as CaCl\(_2\).

* GlcMe concentration 1.2 mm, Ca\(^{2+}\) concentration 2.2 mm.
the $\epsilon_i^*$ values obtained in the presence of Ca$^{2+}$ and $\alpha$-methyl-$\beta$-glucoside appears in Table I. The addition of saturating Ca$^{2+}$ at any time after the Mn$^{2+}$ addition interrupts the kinetic process occurring with the Con A-Mn$^{2+}$ complex alone, and reduces $\epsilon_i^*$ rapidly (within 5 min) to a value which is time-independent. ESR results indicate that upon the addition of Ca$^{2+}$ in this situation there is no significant change in the fraction of Mn$^{2+}$ bound to Con A. At each pH studied the addition of $\alpha$-methyl-$\beta$-glucoside to the Con A in the presence of Mn$^{2+}$ and Ca$^{2+}$ further reduced the observed $\epsilon_i^*$ value (Table I), and the resultant value was also time-independent.

**Discussion**

The consistent decrease in the $\epsilon_i$ value observed upon addition of $\alpha$-methyl-$\beta$-glucoside to the Con A-Mn$^{2+}$-Ca$^{2+}$ complex suggests that the relaxation enhancement parameter may be useful in probing the interaction of Con A with cell surface components. Recent x-ray diffraction data (15, 16) indicate that the saccharide binding site is at least 20 A from the bound Mn$^{2+}$. This suggests that the reduction of $\epsilon_i$ caused by the binding of $\alpha$-methyl-$\beta$-glucoside is not the result of a direct interference with the bound Mn$^{2+}$ hydration sphere, but rather a more distant structural perturbation. The detection and characterization of such rearrangements with ligands analogous to the cell surface receptors should be important in understanding the structural basis of the lectin-cell surface interaction.

Equation 1 requires that the time dependence of $\epsilon_i^*$ derive from variations in either $\tau_M$, $\epsilon_i$, or both. The absence of a free Mn$^{2+}$ ESR signal under conditions where $\epsilon_i$ is decreasing indicates that the time dependence is not due to variation in $\tau_M$, but rather must arise from a change in $\epsilon_i$. Of the parameters which determine $\epsilon_i$ (12), $Q^*$, $\tau_M$, and $\tau_B$ are the only ones likely to be changing with time. $Q^*$, $\tau_M$, and $\tau_B$ are the number, lifetime, and $T_1$, respectively, of the water molecules in the first hydration sphere of the bound Mn$^{2+}$. Regardless of which is changing, the protein must be undergoing a conformational change since all three parameters are determined by the precise nature of the water-Mn$^{2+}$-protein interaction. At this time, without the knowledge of the radio frequency dependence of the time-dependent $\epsilon_i^*$, one cannot assess the possible contribution of a structural change influencing the magnitude of $\tau_M$. However, the alternative of a change in $Q^*$ contributing significantly to the decrease in $\epsilon_i^*$ is particularly attractive in the light of the 2 A crystal structure of Con A recently described by Edelman et al. (15).

Examination of the stereo diagrams for Con A (15) suggests that the NH$_2$-terminal 10 or 12 residues of the Con A backbone are buried within the structure, whereas residues 12 to 22 form a loop on the surface which almost closes the Mn$^{2+}$ site off from the bulk solvent. It seems most likely, therefore, in view of our results, that Mn$^{2+}$ initially binds at a site on the surface of the protein involving only the side chains of residues 8, 10, and 24 and perhaps the water which is hydrogen-bonded to the carbonyl of residue 32, while the loop of residues 12 to 22 remains out in solution. Thus, the bound Mn$^{2+}$ ion would have at least two coordinated waters in rapid exchange with bulk solvent water. Such a situation is consistent with the initial enhancement values of $\epsilon_i$ equal to 11 which we observed. The slow reduction in $\epsilon_i$ with time could then correspond to the reduction of $Q^*$ in an increasing proportion of molecules as the loop of residues 12 to 22 folds over the Mn$^{2+}$. In the folded structure the side chain of residue 19 would have displaced one of the coordinated water molecules, and the water hydrogen bonded to the carbonyl of residue 32 would be trapped inside the protein. The sixth coordination position would be occupied by the water molecule at the bottom of the channel described by Edelman et al. (15), and under this circumstance there will well have a $\tau_B$ significantly lengthened from the initial bound Mn$^{2+}$ state. Under such conditions one would predict that the enhancement would be very small as observed. The pH dependence of the rate of change in $\epsilon_i^*$ (Fig. 2) (i.e., the rate of loop folding over the Mn$^{2+}$) suggests the involvement of a residue with a $\mathrm{pK}$ in the range of 5.8. The protonation of this residue (possibly the Mn$^{2+}$ ligand His 24) accelerates the rate of folding as does the addition of Ca$^{2+}$. Presumably the presence of Ca$^{2+}$ further favors the completely folded structure in that it derives ligands from both the bulk of the structure (residue 10) and the mobile loop region of residues 12 to 22 (nearly residues 12, 14, and 19) (15). A study of the radio frequency dependence of the time-dependent relaxation times should yield the changes, with time, in the number of coordination positions open to water, and also shed some light on the possible contribution of a change in $\tau_M$ resulting from the structural transition. Such a study is in progress.

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