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Stoichiometry of Manganese and Calcium Ion Binding to Concanavalin A†

C. Fred Brewer,* Rodney D. Brown, III, and Seymour H. Koenig

ABSTRACT: Using measurements of solvent nuclear (proton) magnetic relaxation dispersion (NMRD), we have previously shown that concanavalin A (Con A) can exist in two conformational forms and that, in the absence of Ca^{2+}, Mn^{2+} can bind to both the S1 and S2 sites of each monomer of Con A of at least one conformer [Brown, R. D., III, Brewer, C. F., & Koenig, S. H. (1977) *Biochemistry 16*, 3883–3896]. Recently other investigators have claimed that the stoichiometry of Mn^{2+} binding to Con A is only 1:1 for this conformational state, both in the absence and presence of saccharide; the same was claimed for Ca^{2+} under similar conditions. We now present titration and equilibrium dialysis experiments, both in the absence and presence of saccharide, using NMRD and atomic absorption spectroscopy, to investigate the stoichiometry of Mn^{2+} and Ca^{2+} binding to Con A. We have extended the NMRD method to include the determination of the total concentration of Mn^{2+} in samples of Con A. This, coupled with our previous use of NMRD to measure the concentration of free Mn^{2+} in protein solutions as well as the distribution of bound Mn^{2+} among different sites, allows us to measure the stoichiometry of binding with precision. We reconfirm that, at equilibrium in the presence of excess Mn^{2+}, the binding stoichiometry of Mn^{2+} to Con A is 2:1, both in the absence and presence of saccharide. Addition of Ca^{2+} to a solution of Mn^{2+}–Con A results in stoichiometric displacement of Mn^{2+} from the S2 site under the conditions investigated. Under nonequilibrium conditions, Mn^{2+} forms a metastable binary complex with the protein that persists for days at 5 °C. We also report, for the first time, values for all of the dissociation constants of binary and ternary complexes of Mn^{2+} with both conformations of Con A in solution. Atomic absorption measurements also indicate that Ca^{2+}, in the absence of Mn^{2+}, binds to both S1 and S2 sites in the absence and presence of saccharides.

Concanavalin A (Con A),[1] a metalloprotein isolated from the jack bean (*Canavalia ensiformis*), has attracted considerable interest as a probe for investigating the properties of cell surfaces (cf. Bittiger & Schnebli, 1976). Its utility is related to its saccharide binding activity, which is known to be influenced by the binding of metal ions. Each monomeric unit of Con A can bind divalent metal ions at two sites: S1, the "transition-metal" site, and S2, the "calcium" site. S2 is formed once S1 is occupied (Kalb & Levitzki, 1968). Considerable attention has been focused on the details of metal ion interactions with these two sites, interactions between the two sites, and the manner in which saccharide binding activity is controlled by their occupancy.

Brown et al. (1977) and Koenig et al. (1978), using solvent proton nuclear magnetic relaxation dispersion measurements (NMRD) to study the interaction of Mn^{2+} and Ca^{2+} with apo-Con A, concluded that binding of Mn^{2+} to S1 and Ca^{2+} to S2 forms a metastable state with a predominantly inactive conformation, called "unlocked", which converts to an active conformation, called "locked". The ground state free energies of the two conformations differ by only a few kcal M^{-1}, with the sign of this difference determined by the occupancy of S1 and S2 by metal ions. A relatively high energy barrier of about 22 kcal M^{-1}, which has been associated with a cis–trans isomerization in the polypeptide backbone of the protein (Brown et al., 1977), separates the two conformations, making their interconversion relatively slow. A major distinction in the properties of the two conformations is that the locked form has a much greater affinity for metal ions and for saccharides. These conclusions are consistent with earlier stopped-flow NMR studies of Con A reported by Grimaldi & Sykes (1975) and have been confirmed by subsequent polarographic studies of the kinetics of binding of saccharide and a variety of metal ions to Con A by Sherry et al. (1978), fluorescence stopped-

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[1] Abbreviations: Con A, concanavalin A; NMRD, nuclear magnetic relaxation dispersion; α-MDG, methyl α-D-glucopyranoside; MUM, 4-methylumbelliferyl α-D-mannopyranoside.

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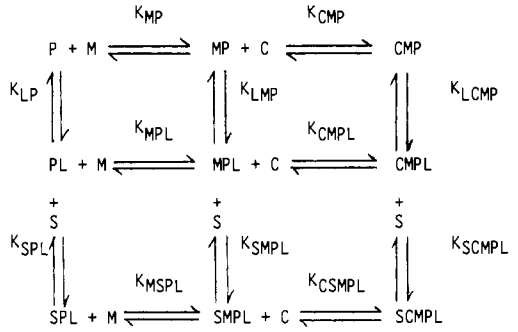
Scheme I



flow studies of metal ion and saccharide binding by Wilkins and co-workers (Harrington & Wilkins, 1978; Harrington et al., 1981), circular dichroism studies of metal ion substituted Con A (Cardin & Behnke, 1978), and ^{113}Cd NMR studies of the binding of Cd^{2+} to Con A by Ellis and co-workers (Palmer et al., 1980).

Koenig et al. (1978) and Harrington & Wilkins (1978) showed that substantial saccharide binding activity of Con A was associated with the locked conformation even in the absence of bound metal ions. Metal ion occupation of both the S1 and S2 sites of the locked conformation produced full activity, while locked apo-Con A was approximately an order of magnitude less active.

The findings of Brown et al. (1977) and Koenig et al. (1978) are described by Scheme I, which indicates the multiple equilibria of Mn^{2+}, Ca^{2+}, and saccharide with both the unlocked and locked conformations of Con A.

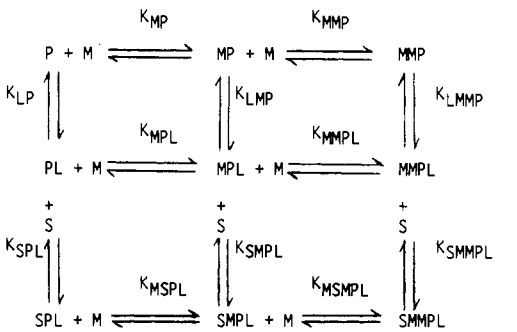
In Scheme I, P stands for apoprotein, M for Mn^{2+}, C for Ca^{2+}, and S for saccharide.[2] The suffix L indicates the locked conformation and its absence the unlocked. Binding of metal ions is sequential in that Mn^{2+} must occupy S1 before Ca^{2+} will bind at S2. The sequence of binding of metal ions in each complex is designated from right to left (i.e., CMPL indicates Mn^{2+} at S1 and Ca^{2+} at S2 in the locked conformation). Scheme I is a "first-order" description in that binding of Mn^{2+} at S2 and Ca^{2+} at S1 has been neglected, although conditions have been found where this does occur (Brown et al., 1977; Koenig et al., 1978), as described below.

At present, values are known for K_{MP}, K_{CMP} (Brown et al., 1978), K_{LP} (Brown et al., 1982), and K_{SCMPL} (Harrington & Wilkins, 1978) and estimated for K_{SPL} (Koenig et al., 1978). In some cases (e.g., K_{MP} and K_{CMP}) values are known at several values of pH and temperature (Brown et al., 1977). Moreover, the rate constants that relate to some of the equilibrium constants (e.g., K_{LCMP} and K_{LP}) are also known (Brown et al., 1977; Harrington & Wilkins, 1978; Harrington et al., 1981; Sherry et al., 1978; Grimaldi & Sykes, 1975).

Brown et al. (1977) showed that Mn^{2+} alone could bind sequentially to S1 and S2 to form a locked ternary complex. Studies by Harrington & Wilkins (1978) indicated that the final Mn^{2+}-locked complex possesses full saccharide binding activity. In a subsequent study, Koenig et al. (1978) reported that, in the absence of Mn^{2+}, Ca^{2+} could bind and activate the saccharide binding properties of the protein, presumably binding to both the S1 and S2 sites analogous to Mn^{2+}. These findings require that Scheme I be generalized to incorporate Scheme II, in which occupation of both S1 and S2 by Mn^{2+} (or by Ca^{2+}) can produce a change in the conformational equilibrium of the protein leading to a fully active ternary

[2] In Brown et al. (1977) the suffix *S* was used to denote the sum of the locked and unlocked species at equilibrium.

Scheme II



locked Mn^{2+} or Ca^{2+} complex. (Scheme II is written in terms of Mn^{2+} binding to Con A; a similar scheme can also be written for Ca^{2+} binding to the protein.)

An estimated value for K_{MMP} has been reported (Brown et al., 1977), while evidence suggests that K_{SMMPL} and K_{SCCPL} are essentially equal to K_{SCMPL} in Scheme I (Harrington & Wilkins, 1978). K_{SMPL} and K_{SCPL} are not presently known, although they might be expected to have a value between K_{SPL} and K_{SMMPL}. The rate for the MMP to MMPL transition has been estimated to be 100-fold slower than that of the analogous transition for CMP to CMPL at 25 °C (Brown et al., 1977).

Despite the success of Schemes I and II in describing a wide range of equilibrium and kinetic phenomena, several recent papers have questioned some of the principal findings of Brown et al. (1977) and Koenig et al. (1978). Magnuson and co-workers (Christie et al., 1979) concluded that only one Mn^{2+} ion could bind to each monomer of Con A, inducing full saccharide binding activity in the protein. In another study, this group (Christie et al., 1978) concluded that in the absence of Mn^{2+} only one Ca^{2+} ion need bind to the protein for full saccharide binding activity. More recently, Sherry and co-workers (Sherry et al., 1981) reached similar conclusions regarding the role of Ca^{2+} binding.

In light of our continuing interest in the role of metal ions in regulating the conformational and saccharide binding properties of Con A, we have reexamined the stoichiometry of binding of Mn^{2+} and Ca^{2+} to Con A and the relationship between this stoichiometry and the saccharide binding activity of the protein. In this paper we primarily address the issue of the stoichiometry of Mn^{2+} binding; more limited data on the stoichiometry of Ca^{2+} binding are also presented. The saccharide binding activity of these various metal ion complexes will be addressed separately.

Part of the present study reproduces and extends the experimental techniques and conditions used by Magnuson and co-workers (Christie et al., 1979) in their study of Mn^{2+} binding to Con A: equilibrium dialysis and solvent proton relaxation measurements at one magnetic field (20.5 MHz) of Mn^{2+} titrated into solutions of apo-Con A. Interestingly, much of their relaxation data reproduced earlier results of Brown et al. (1977), data regarded by the latter as indicating two Mn^{2+} binding sites per monomer. However, the main conclusions reached by Magnuson and co-workers were based primarily on their equilibrium dialysis measurements, monitored using radioactive isotopes. Most of their NMR titration data were interpreted qualitatively and in terms of the conclusions drawn from the equilibrium dialysis data. Furthermore, the NMRD techniques used by Brown et al. (1977) were characterized by Magnuson and workers as an "indirect" method of determining the stoichiometry of Mn^{2+} binding, in contrast to equilibrium dialysis techniques which they regarded as "direct". This characterization of the NMRD method is

incorrect; to reaffirm this point, we provide further NMRD data, checked by atomic absorption spectroscopy, that demonstrates the utility of the technique for directly determining the concentrations of free and bound Mn^{2+} in solutions of Con A under a wide range of sample conditions. In addition, we have extended the NMRD technique to allow direct determination of total Mn^{2+} concentrations present in samples of Con A. We have also carried out extensive equilibrium dialysis experiments with Mn^{2+} and Ca^{2+}, monitored by both NMRD and atomic absorption spectroscopy, in order to compare these results with titration experiments that were monitored primarily by NMRD.

The present study demonstrates agreement between equilibrium dialysis and NMRD titration results and confirms the initial observations of Brown et al. (1977) that Mn^{2+} can indeed bind to apo-Con A and induce a conformational change in the protein leading to the locked ternary complex, MMPL (Scheme II). Equilibrium dialysis results show that two Ca^{2+} ions can also bind, presumably at S1 and S2. We also present, for the first time, a complete analysis of Mn^{2+} binding to apo-Con A taking into account the recently determined equilibrium ratio of [P] and [PL] ([PL]/[P] = 0.14 at 25 °C, pH 6.4) (Brown et al., 1982). The analysis includes the determination of all equilibrium constants in the upper two levels of Scheme II, the relaxation rate contribution of each species at various magnetic fields, and assignment of the respective relaxivity values,[3] R_M. The effects of saccharide binding on equilibrium Mn^{2+} complexes of Con A is also described, which again independently demonstrates that two equivalents of Mn^{2+} can bind to Con A. Similar results are found for Ca^{2+}. Finally, we resolve the apparent discrepancies in the literature regarding the stoichiometry of Mn^{2+} and Ca^{2+} binding to Con A under different conditions in terms of the multiple equilibria of Schemes I and II.

Materials and Methods

Native Con A was obtained from Miles-Yeda (lot 172), from which apo-Con A was prepared as described by Brown et al. (1977). Glass equilibrium dialysis cells were obtained from Bellco Glass, Inc. (Vineland, NJ), and dialysis membranes from Spectrum Medical Industries, Inc. (Los Angeles, CA). All reagents were from commercial sources. Microliter syringes were obtained from the Hamilton Co.

NMRD Measurements. The concentrations of free, bound, and total Mn^{2+} in equilibrium dialysis and titration experiments were monitored by the technique of NMRD, in which the spin–lattice relaxation rate (T_1^{-1}) of solvent protons is measured as a function of magnetic field between 0.01 and 20 MHz by using a field cycling technique.[4] The resultant variation (called a dispersion curve or NMRD spectrum) has an identifiable contribution from free Mn^{2+} ions below 0.5 MHz; their concentration can be determined quantitatively from ΔR, the difference in relaxation rates at 0.02 and 0.5 MHz, since field-dependent contributions to the dispersions curve by bound Mn^{2+} generally occur above 0.5 MHz. Sample volume was routinely 0.6 mL. Reproducibility of the rate measurements was generally better than 2%. The NMRD procedures are identical with those used previously (Brown et al., 1977); limited details can be obtained elsewhere (Koenig & Schillinger, 1969; Hallenga & Koenig, 1976).

Atomic Absorption Measurements. A Perkin-Elmer Model 603 atomic absorption spectrophotometer was used for analysis of Mn^{2+} and Ca^{2+} concentrations. Standard samples of Mn^{2+} and Ca^{2+} were run in the same buffer used in the protein studies. No effects on metal ion analysis were produced by the presence of saccharide or protein.

Equilibrium Dialysis Measurements. The glass portion of each cell was soaked in aqueous 1 N HCl at 25 °C for 6 h; the plastic screw caps were soaked in a pH 7.0, 10 mM EDTA solution at 60 °C for 6 h. Both caps and glass vessels were further soaked in 10 mM EDTA solution overnight and then rinsed with deionized, distilled water. Dialysis membranes were also soaked in pH 7.0, 10 mM EDTA solution overnight.

A 1.5-mL sample of 0.40 mM apo-Con A[5] in pH 6.4 buffer (0.1 M potassium acetate and 0.9 M potassium chloride) was placed in one side of the equilibrium dialysis cell and 1.5 mL of buffer in the other. To the buffer side was added 4 μL of a stock solution of 0.60 M $MnCl_2$ (or $CaCl_2$) to give approximately 1.6 mM metal ion initially free on this side of the cell. This sample composition was chosen, on the basis of previous experience (Brown et al., 1977), to optimize the sensitivity of the experiment to the stoichiometry of metal ion binding. Cells were rotated on a multipurpose rotator (Scientific Industries, Inc.) for 4 days at 21 °C. The buffer and protein side of the cells were emptied and characterized by atomic absorption analysis for Mn^{2+} and Ca^{2+} content and by NMRD measurements for free and total Mn^{2+}. A final determination of protein concentration was made by ultraviolet absorbance measurements by using $A_{280nm}^{1\%,1cm} = 12.4$ (Yariv et al., 1968), at pH 5.6. All protein solutions were centrifuged prior to determining their UV absorbance to minimize light scattering effects that otherwise were found to introduce inconsistencies in the absorption readings of the order of 10%.

NMRD Measurements of Free Mn^{2+}. A stock solution of 0.34 mM Mn^{2+} in pH 6.4 buffer (0.1 M potassium acetate and 0.9 M potassium chloride) was prepared and its Mn^{2+} concentration checked by atomic absorption analysis. The NMRD spectra of an aliquot (0.6 mL) of the stock solution was recorded at 5 and 25 °C in order to obtain R_M,[3] the relaxivity contribution of free Mn^{2+} at various fields, and ΔR_M, the difference in the relaxivities of free Mn^{2+} at 0.02 and 0.5 MHz. To the sample was added 10 μL of concentrated hydrochloric acid to give a pH 1.2 solution, and relaxation measurements were made at 5 and 25 °C to obtain R_M and ΔR_M at this pH. These latter values are used to determine total Mn^{2+} concentration.

NMRD Measurements of Total, Free, and Bound Mn^{2+} in Solutions of Apo-Con A, Mn^{2+}, and Ca^{2+}. Conditions were chosen such that three samples of apo-Con A containing the same total concentration of Mn^{2+}, in the absence and presence of Ca^{2+}, would form predominantly MP, MMPL, and CMPL, respectively, according to Schemes I and II. First a solution of 0.40 mM apo-Con A in pH 6.4 buffer was prepared and allowed to stand at 25 °C for several days to reach conformational equilibrium. The solution was chilled in an ice bath to freeze the conformational equilibrium, and then 0.83 mM Mn^{2+} was added. The NMRD spectrum of an aliquot (0.6 mL) of this solution, containing mostly MP (and some MPL) according to Scheme II and previous expectations (Brown et al., 1977), was immediately recorded at 5 °C. The sample was then acidified as above, and its NMRD spectrum recorded at 5 °C.

[3] R and ΔR are relaxation rates, expressed in units of s^{-1}, whereas R_M and ΔR_M are "relaxivities", or relaxation rates per unit concentration, expressed in units of $(mM\ s)^{-1}$.

[4] We measure magnetic field intensity in units of the Larmor precession frequency of protons in that field. The conversion is 4.26 kHz = 1 Oe = 1 G.

[5] Protein concentration is expressed throughout a monomer concentration.

Table I: ΔR_M and R_M of Free Mn^{2+} Aquo Ions at pH 6.4[a] and 1.2[b] at 5 and 25 °C

sample composition	pH	temp (°C)	R_M (0.02 MHz) [(mM s)$^{-1}$]	R_M (0.5 MHz) [(mM s)$^{-1}$]	R_M (20 MHz) [(mM s)$^{-1}$]	ΔR_M (0.02 − 0.5 MHz) [(mM s)$^{-1}$]
0.34 mM Mn^{2+}	6.4	5	51	34	13	17
0.34 mM Mn^{2+}	6.4	25	47	20	8.5	27
0.33 mM Mn^{2+}	1.2	5	52	32	12	20
0.33 mM Mn^{2+}	1.2	25	52	20	8.4	32
0.34 mM Mn^{2+} + 0.1 M α-MDM	6.4	5	49	33	13	16

[a] Buffer 0.1 M potassium acetate and 0.9 M potassium chloride. [b] pH 6.4 solutions (0.6 mL) were acidified with 10 μL of concentrated hydrochloric acid; all data are corrected for dilutions.

A second aliquot of the stock Mn^{2+}–Con A solution was prepared and 6 μL of 1.0 M $CaCl_2$ added to give 10 mM Ca^{2+}. After standing at 25 °C for 45 min to form CMPL, the NMRD spectrum of the sample was recorded at 5 °C, the sample acidified to pH 1.2, and the NMRD spectrum recorded again at 5 °C.

A third aliquot of the stock solution was allowed to equilibrate at 25 °C for several days to form predominately MMPL. Its NMRD spectrum was recorded at 5 °C and then acidified as above and its spectrum again recorded at 5 °C. The NMRD spectrum of a fourth aliquot of the apo-Con A stock solution, without metal ions, was determined at 5 °C, then 10 μL of concentrated hydrochloric acid added, and the NMRD spectrum again recorded at 5 °C to determine R_M of the apoprotein at pH 1.2 and 6.4.

On the basis of our previous results, the first three samples should have very different dispersion spectra before acidification; after acidification, if all Mn^{2+} is released from the protein, the spectra should become identical.

Titration of Apo-Con A with Mn^{2+}: NMRD Measurements. A stock solution of 0.39 mM apo-Con A in pH 6.4 buffer was allowed to stand at 25 °C for 1 weeks to ensure conformational equilibrium. Twelve samples, each containing 0.6 mL of the stock solution, were then chilled in an ice bath and maintained cold as increasing amounts of a 0.06 M $MnCl_2$ stock solution were added to each to give a range of total Mn^{2+} concentrations of 0.1–1.4 mM. NMRD measurements at 0.02, 0.5, and 20 MHz at 5 °C were made immediately upon addition of metal ions and repeated after 1 h to check the stability of the conformational distribution of the samples. All samples were then allowed to come to equilibrium at 25 °C for 6 days; NMRD measurements were again recorded at 5 °C.

Sufficient crystalline α-MDM was then added to each sample at 5 °C to give a final saccharide concentration of 0.1 M, after which NMRD measurements were recorded; the samples were then equilibrated a second time at 25 °C for several days and their relaxation rates measured at 5 °C. Total Mn^{2+} in the samples was verified in two ways: by acidification and NMRD measurements at 5 °C, as described above, and by atomic absorption analysis.

An analogous experiment was performed except that the stock apo-Con A solution was first equilibrated with 0.1 M α-MDM for 12 days at 25 °C, and then Mn^{2+} was added, after which the samples were allowed to stand at 25 °C for 5 additional days before NMRD measurements were made at 5 °C. Total Mn^{2+} in the samples was checked by acidification, as above. This procedure was used to determine if the order of addition of Mn^{2+} and α-MDM affected the final titration results.

Results

Calibration of Free Mn^{2+} Concentration by NMRD. Figure 1 shows dispersion curves of Mn^{2+} aquo ion solutions at 5 °C

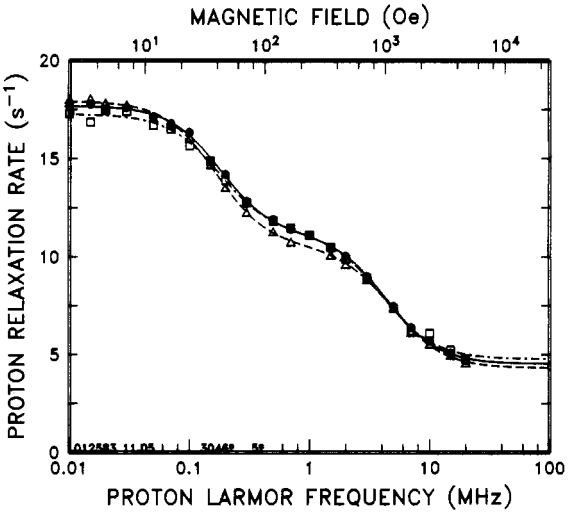


FIGURE 1: Solvent proton relaxation dispersion spectra of solutions of Mn^{2+} aquo ions at 5 °C at pH 1.2 (△), pH 6.4 (●), and pH 6.4 with 0.1 M α-MDM (□). The pH 6.4 solutions contained 0.34 mM $MnSO_4$ in 0.1 M potassium acetate and 0.9 M potassium chloride buffer. The pH 1.2 sample contained 0.33 mM $MnSO_4$ in the same buffer plus a small amount of concentrated hydrochloric acid. The lines through the data points result from a least-squares comparison of the data with the theory of relaxation for these aquo ions.

at pH 1.2, 6.4, and 6.4 in the presence of 0.1 M α-MDM. [Data for other conditions can be found in Brown et al. (1977).] The curve through the data points results from a five-parameter dispersion relation for solvent proton relaxation by Mn^{2+} aquo ions (cf. Koenig et al., 1973). Table I lists the relaxivity values, R_M, for Mn^{2+} aquo ions at 0.02, 0.5, and 20 MHz for these samples and others relevant to the present study. The standard relaxivity differences, ΔR_M, are also listed. These values are independent of free Mn^{2+} concentrations in the range used in the present studies. It is evident that varying the pH or adding saccharide at this concentration has a small but significant effect on the shape of the overall spectra: ΔR_M values in Table I for the pH 1.2 sample at 5 and 25 °C are somewhat larger than the corresponding values at pH 6.4, while ΔR_M for the saccharide containing sample is somewhat lower. All samples were found to be stable for at least 5 weeks.

NMRD Determination of Free, Bound, and Total Mn^{2+} in Solutions of Apo-Con A, Mn^{2+}, and Ca^{2+}. Since acidifying the Con A samples to pH 1.2 frees all bound Mn^{2+}, measurement of ΔR, together with the data in Table I, provides a direct determination of the total Mn^{2+} concentration. This is a new and quantitative procedure for determining total Mn^{2+} in solutions of Con A. The uncertainty in total Mn^{2+} concentration determined in this manner is the greater of ±3% or 20 μM. Moreover, unlike other methods, repeated recalibration of the apparatus is unnecessary; only the values in Table I need be known. (There are limitations in the use of

this method with very high concentrations of saccharide, >0.1 M, since not all of the Mn^{2+} is freed from the protein under these conditions.)

Parts A–C of Figure 2 illustrate the use of NMRD measurements for determining the free, total, and hence bound Mn^{2+} concentrations in solutions of Con A under three conditions. The same total Mn^{2+} and protein concentrations were used for each sample. The data in each figure show the NMRD of the sample at pH 6.4 with contributions from the apoprotein and from the free and bound Mn^{2+}, and also the NMRD of the acidified (pH 1.2) sample with all Mn^{2+} free. In each case the analysis of the pH 6.4 data is as follows: the apo-Con A and solvent contributions, determined independently from appropriate samples, were subtracted from the total NMRD. The free Mn^{2+} concentration was then determined by comparing the resulting dispersion between 0.02 and 0.5 MHz with that for a known concentration of Mn^{2+} under similar conditions, that is, from the measured difference in rates, ΔR, and the known difference in relaxivities, ΔR_M, from Table I. From the known functional form of the NMRD of the Mn^{2+} aquo ion (Koenig et al., 1973), the contribution of the free Mn^{2+} plus the apoprotein was subtracted from the total dispersion, leaving only the contribution of bound Mn^{2+}, which was also fit by a five-parameter dispersion function. The solid line through the data points is the sum of the apoprotein and free and bound Mn^{2+} contributions. The total Mn^{2+} was then found by a similar procedure for the pH 1.2 samples by using appropriate free Mn^{2+} and apo-Con A calibration samples. In this case, the total Mn^{2+} equals the free Mn^{2+}, and the dashed line through the data points is the sum of the apo-Con A and free Mn^{2+} contributions at pH 1.2.

Figure 2A shows the NMRD spectrum of a solution of 0.39 mM apo-Con A in pH 6.4 buffer at 5 °C immediately after addition of 0.83 mM Mn^{2+}. The spectrum of the acidified solution (pH 1.2) is also shown. The above analysis gives 0.47 mM free Mn^{2+} at pH 6.4 and a total of 0.83 mM Mn^{2+} from the pH 1.2 data. The total Mn^{2+} as determined by NMRD agrees with 0.82 mM found by atomic absorption analysis. The concentration of bound Mn^{2+}, 0.36 mM, was calculated from the difference between free and total Mn^{2+}. Thus, for these conditions, 0.9 equiv of Mn^{2+} is bound per monomer of Con A, with a relaxivity in the low-field limit of ~38 (mM s)$^{-1}$, in good agreement with the value of 35 (mM s)$^{-1}$ found previously for single Mn^{2+}–Con A complexes (Brown et al., 1977). In terms of Scheme II, MP and a small amount of MPL are predominantly formed, the latter due to the PL present at equilibrium in apo-Con A (Brown et al., 1982).

Figure 2B shows the NMRD spectrum of an aliquot of the same stock Mn^{2+}–protein solution shown in Figure 2A to which Ca^{2+} was added (in excess of the S2 site concentration), and the sample allowed to equilibrate at 25 °C. The observed rates are considerably less than those in Figure 2A. The above analysis of the dispersion data for the pH 6.4 solution in Figure 2B gives the solid line through the data points. The concentration of free Mn^{2+} thus calculated was 0.41 mM. The dispersion spectrum of the acidified solution is also shown, with the dashed line through the data points derived as above. The total Mn^{2+} in the acidified solution is calculated to be 0.81 mM, in agreement with the nominal value of 0.83 mM. The difference between total and free Mn^{2+} gives a value of 0.40 mM Mn^{2+} bound to the protein which corresponds to 1 equiv of Mn^{2+} bound per monomer of Con A. The contribution of both free and bound Mn^{2+} at pH 6.4 is also shown in Figure 2B. From Scheme I and our previous studies (Brown et al., 1977), only CMPL is formed under these conditions. The low-field relaxivity of the bound species, 11 (mM s)$^{-1}$, agrees

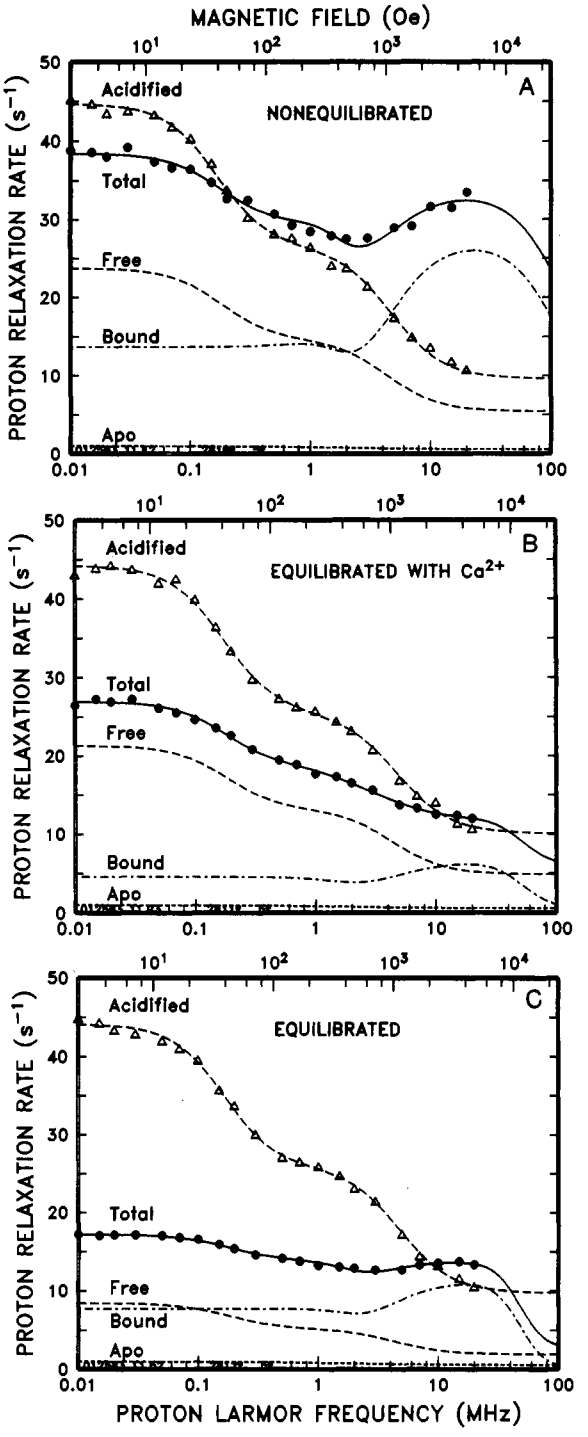


FIGURE 2: Solvent proton relaxation dispersion spectra for three samples of apo-Con A (0.39 mM) in pH 6.4 buffer containing the same total concentration of Mn^{2+} (0.83 mM) under different conditions. In (A), the sample was prepared in the cold (1 °C), and data (●) were immediately recorded at 5 °C. In (B), a sample identical with the one in (A) was allowed to equilibrate for 45 min at 25 °C in the presence of 10 mM $CaCl_2$, and then data (●) were recorded at 5 °C. In (C), a sample identical with the one in (A) was equilibrated at 25 °C for several days, and its spectrum (●) was recorded at 5 °C. All three samples were acidified to pH 1.2 and the dispersion spectra (△) obtained again at 5 °C. A comparison of these data (corrected for a small apo-Con A contribution) with the theory of relaxation by Mn^{2+} aquo ions is indicated in each case by a dashed line through the data points. (The results are essentially identical for each sample.) For all three samples, the solid line through the data of pH 6.4 is a reconstruction from three contributions, apo-Con A, indicated at the bottom of the figures, Mn^{2+} aquo ions, with contribution determined by the curvature of the data below 0.5 MHz, and bound Mn^{2+}–protein complexes, obtained from the data corrected for the other two contributions, and then smoothed by comparison with the theory of relaxation by bound Mn^{2+} ions.

reasonably well with the previous value of 9 $(mM\ s)^{-1}$ reported for CMPL at 5 °C, as does the form of the spectrum (Brown et al., 1977).

Figure 2C shows the dispersion data for a sample aliquot identical with that in Figure 2A, but which had been allowed to stand at 25 °C for several days before measurement at 5 °C. The spectrum is different from that in either part A or part B of Figure 2. A fit of the pH 6.4 data gives a value for free Mn^{2+} of 0.17 mM. Acidification of the sample yields a total Mn^{2+} concentration of 0.84 mM. This indicates that 0.67 mM Mn^{2+} is bound or that 1.72 equiv of Mn^{2+} is bound per monomer of Con A. In terms of Scheme II, MMP and MMPL predominate under these conditions. (An exact analysis of the proportion of the various species formed in Scheme II as a function of total Mn^{2+} is given below.) Under these same conditions, the presence of excess Ca^{2+} resulted in only 1 equiv of Mn^{2+} bound, to form CMPL (as shown in Figure 2B), implying that the additional Mn^{2+} in Figure 2C is bound to S2.

The above results, Figure 2A–C, indicate that by use of the acidification procedure to liberate all bound Mn^{2+} from various complexes of Con A, NMRD measurements can be used to determine quantitatively the total Mn^{2+} present, the concentration of free Mn^{2+} in solution in the original sample, and hence the concentration of bound Mn^{2+}. The above three examples also clearly show that under various conditions, both binary and ternary complexes can be formed between Con A and Mn^{2+}.

Additional studies indicated that the accuracy of the acidification procedure is limited at relatively high concentrations of α-MDM. At a concentration of α-MDM of 0.1 M, approximately 10–15% of the Mn^{2+} remains bound to the protein at pH 1.2, the percentage becoming greater at higher concentrations of α-MDM. Thus, the procedure is best used when the concentration of saccharide is less than 10 mM.

NMRD Measurements of Apo-Con A Titrated with Mn^{2+}. Figure 3A shows the results of titrating Mn^{2+} into 12 identical samples of 0.39 mM apo-Con A. The apo-Con A had been allowed to reach equilibrium at 25 °C and was cooled to 5 °C prior to addition of metal ions. Cooling the samples fixes the total concentration of the locked conformation in solution for a time long compared to these experiments, regardless of metal ion content, at the value determined by the 25 °C equilibrium ratio of [PL]/[P] of 0.14, recently determined by Brown et al. (1982). Thus, increasing amounts of Mn^{2+} result in changes in the relative concentrations of the species along the two upper horizontal pathways in Scheme II, but the total concentrations of locked and unlocked species, P + MP + MMP and PL + MPL + MMPL, are the same for all the samples. Data at 0.02, 0.5, and 20 MHz were recorded for each sample at 5 °C within 15 min of the addition of Mn^{2+}, to preclude drifts due to slow conformational changes between the locked and unlocked forms of the protein.

The lines in Figure 3A result from a fit of Scheme II to the data, as discussed below, after Figure 4A. For the first time, the presence of PL as well as P has been considered in the analysis of the stoichiometry of Mn^{2+} binding to apo-Con A, since it has not been known until recently that PL exists in appreciable concentration in solutions of apo-Con A (Brown et al., 1982). Qualitatively, as Mn^{2+} is added to apo-Con A, the S1 sites of P and PL fill to form MP and MPL (Figure 3B). With increasing Mn^{2+}, additional MP and MMPL form, and only after S1 of the unlocked protein and S1 and S2 of the locked protein are saturated do small amounts of MMP form (Figure 3B). The right-hand portion of Figure 3A is

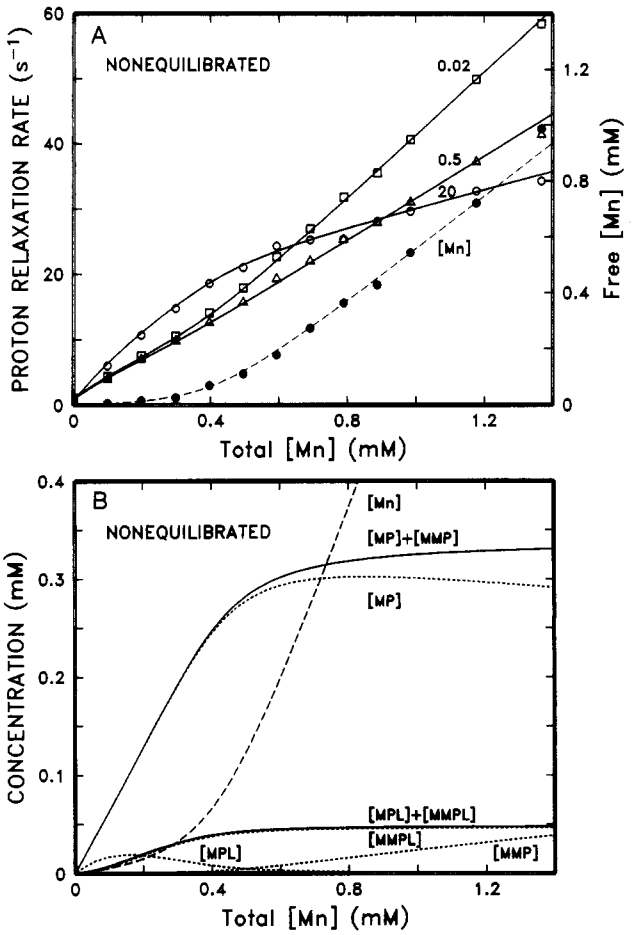


FIGURE 3: (A) Solvent proton relaxation rates at 0.02, 0.5, and 20 MHz[4] of 12 samples of 0.39 mM apo-Con A at pH 6.4 containing increasing concentrations of Mn^{2+}. Samples were prepared in the cold (1 °C) and the rates immediately recorded at 5 °C. The points are experimental data, and the lines are fits to the data according to Scheme II. In essence, the difference in the rates at 0.02 and 0.5 MHz was used to compute the actual concentration of free Mn^{2+} (●) of each sample. These were compared with the predictions of Scheme II by varying the values of the many equilibrium constants to optimize the comparison (dashed curve). Details are in the text. The other curves were constructed from Scheme II by assigning values to the relaxivity of the various components of the system that best fit the data. (B) The concentrations of MP, MMP, MPL, MMPL, and free Mn^{2+} as a function of total Mn^{2+}, computed from Scheme II by using values for the equilibrium constants obtained from the fit shown in (A).

dominated by K_{MMP} and the formation of MMP, because of its relatively high relaxivity, and the midrange by formation of MP and MMPL, and their respective relaxivities; the left-hand section of the data has comparable contributions from both MP and MPL.

Figure 4A shows the relaxation rates at 0.02, 0.5, and 20 MHz of the samples of Figure 3A after being equilibrated at 25 °C for 5 days and remeasured at 5 °C. Under these conditions, horizontal and vertical equilibria along the two upper pathways are established throughout the titration profile, in contrast to the restriction to the horizontal pathways in Figure 3A. The effect of increasing total Mn^{2+} concentrations in the samples in Figure 4A is to shift the overall equilibrium diagonally from the upper left portion of Scheme II, from P and MP, to the middle right-hand portion of the scheme to give predominantely MMPL. The lines in Figure 4A result from a fit of Scheme II to the data for these new equilibrium conditions, as discussed below. Since the vertical equilibria are reached between species in Scheme II for each sample,

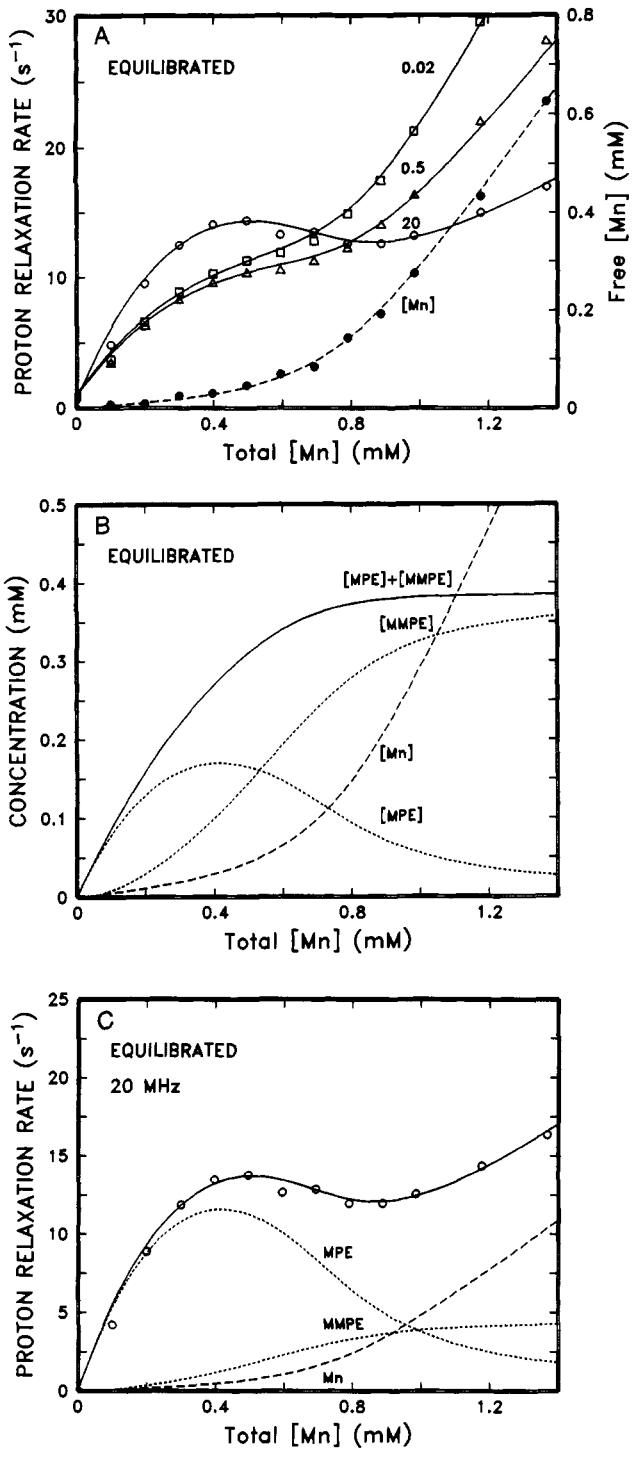


FIGURE 4: (A) Solvent proton relaxation rates at 0.02, 0.5, and 20 MHz of the samples in Figure 3A after equilibration at 25 °C for several days; measurements were recorded at 5 °C. The points are experimental, and the lines are fits to the data according to Scheme II. The fit, as for Figure 3A, involved adjusting the values of the equilibrium constants so as to optimize the comparison of the observed concentration of free Mn^{2+} (●) with the predictions of Scheme II (dashed curve). The values obtained here and from Figure 3A were constrained to be mutually consistent. The remaining curves were constructed from Scheme II by assigning appropriate relaxivity values to the various components of the system that best fit the data and were consistent with Figure 3A. (B) The concentrations of MPE (MP + MPL), MMPE (MMP + MMPL), MPE + MMPE, and Mn^{2+} as a function of total Mn^{2+} in (A), computed from Scheme II by using the equilibrium constants obtained from the fit shown in (A). (C) Contributions to the observed proton relaxation rates by MPE, MMPE, and Mn^{2+} at 20 MHz as a function of total Mn^{2+} in (A). Results of (B) were used, and relaxivity values were assigned to each species to obtain a best fit of the data.

the effect of added Mn^{2+} can be described as first forming binary equilibrium Mn^{2+} complexes with the two conformational forms of the protein at equilibrium, MPE (where [MPE] = [MP] + [MPL]), and then, at higher concentrations of Mn^{2+}, forming ternary Mn^{2+} complexes, MMPE (where [MMPE] = [MMP] + [MMPL]), as shown in Figure 4B. The primary difference between the results shown in Figures 3 and 4 is the predominant formation of binary Mn^{2+}–Con A complexes under the nonequilibrium conditions of Figure 3, since the total concentration of locked species is limited to the initial concentration of PL. By contrast, in Figure 4, with sufficient Mn^{2+} present (>1 mM total) under equilibrium conditions, locked ternary Mn^{2+}–Con A complexes form. These data are in complete accord with previous results of Brown et al. (1977), regarding the formation of MMPL under similar equilibrium conditions. The difference here is that the more recent data are taken at 5 °C rather than 25 °C, to compare with Christie et al. (1979).

The procedures for obtaining the fits to the data in Figures 3A and 4A are conceptually simple, but computationally complex. The fit to Figure 4A follows that of Brown et al. (1977) (cf. eq 10–16). For the fit in Figure 3A, for given concentrations of total Mn^{2+} and total protein, there are seven unknown concentrations in Scheme II (in the absence of saccharide) to be computed (M, P, MP, ..., MMPL) and seven equations that connect them. The latter include three summations, deriving from conservation conditions, that sum to the total Mn^{2+}, M + MP + MPL + 2(MMP + MMPL), and to the total unlocked and locked protein, P + MP + MMP and PL + MPL + MMPL, respectively. The latter two totals are known from K_{LP} and the initial conditions that freeze in the 25 °C equilibrium [P]/[PL] ratio. In addition, there are four equations that involve the four equilibrium dissociation constants K_{MP}, K_{MPL}, K_{MMP}, and K_{MMPL} [cf. eq 1–7 of Brown et al. (1977)]. With seven equations and seven unknowns, one can solve for all the concentrations for a given set of values of the dissociation constants. In fact, once the value of any one concentration is known the value of the six others can be derived from this one. We measure one concentration, namely, [M], for 12 values of the total Mn^{2+}. Then we search for a single set of values for the four equilibrium constants that, for all 12 values of the total Mn^{2+}, will give values for [M] that agree with the 12 observed values. The problem is clearly determined, so long as the range of total Mn^{2+} is significantly great so that all of the equilibrium constants are reflected in the titration plots. Therefore, a unique, best-fitting set of values for the equilibrium constants can be found. The solution of seven simultaneous equations is complicated by the fact that four of the equations are nonlinear in the unknowns, and therefore the equations were solved numerically. It was possible to compute 100 points on a curve of the expected values of [M] as a function of total Mn^{2+} for any set of values for the four dissociation constants and then search for the best set. The actual procedure was as follows.

First, values for K_{MP}, K_{MPL}, K_{MMP}, and K_{MMPL} were chosen, guided by our previous results (Brown et al., 1977, 1982). By use of these and the recently determined value of K_{LP} (7.1), the concentration of Mn^{2+} aquo ions, [M], was calculated for both sets of data in Figures 3A and 4A simultaneously, as a function of total Mn^{2+}. The values of the four dissociation constants were adjusted to give an optimum fit of the calculated [M] to the experimental values for [M] at each point along the titration curve(s), as determined from ΔR and the known ΔR_M. The comparison between fit and experiment is indicated by the dashed curves and solid circles in Figure 3A

and 4A. The vertical size of the data points for free Mn^{2+} is an indication of the uncertainty in the measurement.

This fitting procedure does not require knowledge of the relaxivities of the various species present but only the experimentally determined free Mn^{2+} concentration determined as a function of total Mn^{2+}. Values for the equilibrium constants that gave the fits shown in the figures are K_{MP} = 25 μM, K_{MPL} = 8 μM, K_{MMP} = 7 mM, and K_{MMPL} = 15 μM. The uncertainty associated with the values is estimated to be ±30% except for K_{MMP}, which has a greater uncertainity due to its larger value, which, in turn, can be influenced by weak binding elsewhere (Brown et al., 1977). Knowing the values of these four independent variables in Scheme II as well as K_{LP} permits calculation of K_{LMP} and K_{LMMP} [cf. Brown et al. (1977), eq 6 and 7]; these are 2.3 and 0.005, respectively. This represents the first complete determination of all of the equilibrium constants associated with Scheme II, in the absence of saccharide. [It must be noted that these values reflect measurements made at 5 °C on a system equilibrated at 25 °C, so that some uncertainty is associated with the temperature dependence of these values. However, previous results suggest that this dependence is relatively small (Brown et al., 1977), and to this extent the fitting procedure is rigorous.]

From a knowledge of the equilibrium constants in Scheme II, the amounts of free and total Mn^{2+} present in each sample, and total protein, it is relatively straightforward to compute the concentrations of each species present; these results are shown in Figures 3B and 4B. Finally, when these results were compared with the data, the relaxation rate contribution of each species could be computed in terms of an assigned relaxivity value, each of which was varied to give fits to the observed rates in both Figures 3A and 4A. An example is shown in Figure 4C for the 20-MHz data in terms of the contribution of MPE, MMPE, and M. At low concentrations of Mn^{2+}, MPE, which from K_{LMP} = 2.3 is about two-thirds unlocked and one-third locked, dominates the observed relaxation rates. With increasing Mn^{2+}, the rates decrease between 0.6 and 0.8 mM total Mn^{2+}, since [MPE] decreases as [MMPE] (which is almost 100% MMPL) increases, and the relaxivity of MMPL is lower than that of MP and MPL, which comprises MPE. At concentrations of total Mn^{2+} above 1 mM, the observed rates again increase as MMPE saturates, and the contribution of free Mn^{2+} becomes substantial. The individual relaxivity values (per Mn^{2+} ion) found for the various Mn^{2+} complexes in Scheme II at 0.02, 0.5 and 20 MHz, respectively, are the following: MP, 35, 35, and 55 (mM s)$^{-1}$; MPL, 43, 43, and 75 (mM s)$^{-1}$; MMP, 40, 40, and 100 (mM s)$^{-1}$; MMPL, 12, 12, and 12 (mM s)$^{-1}$.

Figure 5 shows the relaxation rates at 0.02, 0.5, and 20 MHz of the samples in Figure 4 after the addition of 0.1 M α-MDM followed by equilibration at 25 °C for several days and then cooled to 5 °C for the measurements. Measurements of the relaxation rates immediately after addition of saccharide showed little change. As can be seen, the observed rates are reduced compared to those in Figures 3 and 4, and the binding of Mn^{2+} is extremely tight with a sharp break at 0.78 mM total Mn^{2+} that corresponds to precisely 2.0 equiv of Mn^{2+} bound per monomer of Con A. The solid lines through the data points above 0.8 mM total Mn^{2+} were fits derived from the relaxivity values of free Mn^{2+} aquo ions in the presence of 0.1 M α-MDM at 0.02, 0.5, and 20 MHz (Table I). The linearity of the data to the left of the break and above 0.1 mM total Mn^{2+} shows that 0.1 M α-MDM exhibits a strong preference for forming a ternary Mn^{2+}–Con A complex (SMMPL, Scheme II) once a small fraction of S1 is filled (since it is unlikely that the single and double metal–Con–saccharide species have the same relaxivities).

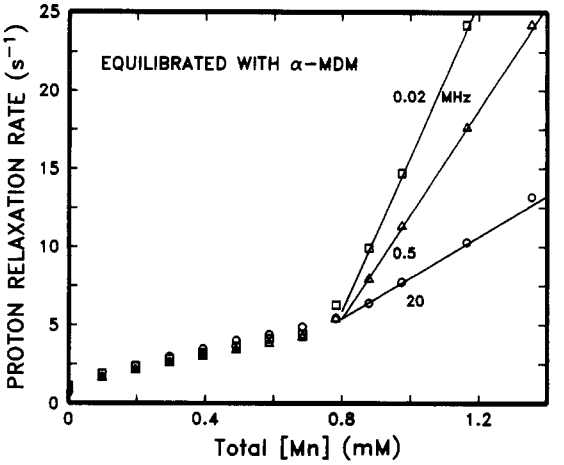


FIGURE 5: Solvent proton relaxation rates at 0.02, 0.5, and 20 MHz of the samples in Figure 4A after addition of 0.1 M α-MDM and equilibration at 25 °C for several days; measurements were recorded at 5 °C. (Only minor changes in relaxation rates were observed immediately after saccharide was added and before warming to 25 °C.) The points are experimental, and the lines to the right of the break are the results expected when the free Mn^{2+} relaxivity values in Table I are used, assuming that all Mn^{2+} added to the right of the break is unbound.

Table II: Equilibrium Dialysis Experiments with Mn^{2+} and Apo-Con A in pH 6.4 Buffer at 23 °C[a]

	experiment I		experiment II	
	protein side[b]	buffer side	protein side[b]	buffer side
total Mn^{2+} (mM)				
AA	1.12	0.35	1.05	0.38
NMRD[c]	1.23	0.41	1.21	0.41
total Ca^{2+} (mM)				
AA	0.085	0.005	0.051	0.005
free Mn^{2+} (mM)				
AA		0.36		0.38
NMRD	0.41	0.41	0.39	0.41
bound Mn^{2+} (mM)[d]				
AA	0.76 ± 0.03		0.67 ± 0.03	
NMRD	0.82 ± 0.03		0.80 ± 0.03	

[a] [Mn^{2+}] was determined by atomic absorption spectroscopy and NMRD and [Ca^{2+}] (adventitious) by atomic absorption spectroscopy. [b] Monomer protein concentration was 0.43 mM. [c] Using the acidification procedure discussed in the text. [d] The difference between the concentrations of total and free Mn^{2+}.

Equilibrium Dialysis Measurements of Mn^{2+} Binding to Apo-Con A. Results for two independent but similar equilibrium dialysis experiments are shown in Table II. The concentration of total Mn^{2+} and protein was chosen such that if only 1 equiv of Mn^{2+} bound per protein monomer, then the concentration of free Mn^{2+} would be greater than 0.6 mM, whereas if 2 equiv of Mn^{2+} bound per monomer, the concentration of free Mn^{2+} would be substantially less. As can be seen, the results from atomic absorption analysis and NMRD measurements agree very well with each other and indicate that 0.38 ± 0.03 mM Mn^{2+} is free on the buffer side of the cells. NMRD results for the contents of each cell also provide a direct determination of their free Mn^{2+} concentration; these agree with the concentration found on the buffer side by both techniques. Both the atomic absorption and NMRD measurements provided values for the total concentration of Mn^{2+} in the protein side of each cell, 1.13 ± 0.08 mM. The concentration of adventitious Ca^{2+} in both sides of the cells, de-

Table III: Equilibrium Dialysis Experiments with Ca^{2+} and Apo-Con A in pH 6.4 Buffer at 23 °C[a]

	experiment I		experiment II	
	protein side[b]	buffer side	protein side[c]	buffer side
total Ca^{2+} (mM)	1.25	0.29	1.25	0.31
free Ca^{2+}		0.29		0.31
bound Ca^{2+} (mM)[d]	0.96		0.94	
equivalents of bound Ca^{2+}	2.0		2.0	

[a] $[Ca^{2+}]$ was determined by atomic absorption spectroscopy. [b] Monomer protein concentration was 0.49 mM. [c] Monomer protein concentration was 0.47 mM. [d] The difference between the concentration of total and free Ca^{2+}.

termined by atomic absorption, was found to be less than 10% of the total Mn^{2+} concentrations. The amount of bound Mn^{2+} per monomer of Con A was calculated to be 1.8 and 1.7 ± 0.1 equiv/mol by both methods. This is in excellent agreement with the anticipated 1.8 equiv predicted from Schemes I and II taking into account the amount of Ca^{2+} present by using the known equilibrium constants [Brown et al. (1977) and redetermined above]. Further experiments (not shown) demonstrated that upon addition of excess Ca^{2+} (3 equiv per monomer of Con A), only 1 equiv of Mn^{2+} remained bound to the protein, with Ca^{2+} having displaced the Mn^{2+} from S2, judging from the observed relaxation rates.

In separate experiments (not shown), the presence of 10 mM α-MDM was found not to alter the observed stoichiometry of Mn^{2+} binding to Con A (i.e., ~2 equiv per monomer at equilibrium).

In initial experiments, in which the dialysis cells were not exhaustively treated with EDTA and acid prior to usage, the amount of Mn^{2+} found bound was 1 equiv. NMRD analysis of the sample indicated the presence of CMPL. Atomic absorption analysis revealed that the cells used for the dialysis experiments contained a relatively high content of endogenous Ca^{2+} that leaked into the buffer solutions, when not removed by prior treatment with EDTA and acid.

The above experiments clearly show that 2 equiv of Mn^{2+} can bind to apo-Con A under equilibrium conditions, 23 °C, pH 6.4, in the absence of added Ca^{2+}, in complete agreement with our titration results.

Equilibrium Dialysis Measurements of Ca^{2+} Binding to Apo-Con A. The results from two independent but similar equilibrium dialysis experiments with Ca^{2+} and apo-Con A are shown in Table III. The concentration of total Ca^{2+} was chosen to be similar to that used for Mn^{2+} in Table II. The results, monitored by atomic absorption analysis of both sides of each dialysis cell, show that 2.0 equiv of Ca^{2+} is bound per monomer of Con A. (Note that monitoring calcium concentrations by atomic absorption rather than by radioactive techniques obviates problems relating to adventitious Ca^{2+}.) When excess Mn^{2+} is added (>2 equiv per monomer), only 1 equiv of Ca^{2+} remains bound and 1 equiv of Mn^{2+} binds (not shown), as determined from the NMRD spectra and the known relaxivity of CMPL (Brown et al., 1977), and atomic absorption analysis. Addition of 10 mM α-MDM to a similar solution of Ca^{2+} and apo-Con A, as above, results in at least 2 equiv of bound Ca^{2+} and up to 4 equiv of bound Ca^{2+} at higher Ca^{2+} concentrations (not shown). Addition of excess Mn^{2+} to such a solution results in replacement of only 1 equiv of bound Ca^{2+} (ostensibly from S1).

The above results show that in the absence of saccharide and Mn^{2+}, 2 equiv of Ca^{2+} binds under equilibrium conditions (23 °C, pH 6.4) to both the S1 and S2 sites of Con A and forms a locked ternary complex. Surprisingly, the presence of saccharide (α-MDM) increases the amount of Ca^{2+} binding to the protein under similar conditions.

Discussion

Stoichiometry of Mn^{2+} Binding to Con A. The findings in the present paper reaffirm and extend the observations of Brown et al. (1977) that, in the absence of Ca^{2+}, Mn^{2+} can bind to both the S1 and S2 sites of Con A to form a ternary locked complex, MMPL (Scheme II). The present investigation used a combination of equilibrium dialysis and titration experiments, monitored by both atomic absorption spectroscopy and NMRD measurements, to give additional and conclusive evidence for the formation of ternary Mn^{2+}–Con A complexes, both in the absence and presence of saccharide. Indeed, each of several experiments in the present study independently demonstrates this conclusion. For example, NMRD data in Figure 2C clearly show that when approximately 2 equiv of Mn^{2+} (0.83 mM) is equilibrated with apo-Con A (0.39 mM) at 25 °C for several days, 1.72 equiv of Mn^{2+} binds per monomer of protein. (A 2:1 stoichiometry requires more total Mn^{2+} because K_{MMPL} is rather large.) Adding increasing amounts of Mn^{2+} to solutions of apo-Con A (0.39 mM) and allowing conformational equilibrium to be reached also show that above 2.5 equiv of total Mn^{2+} essentially all of the protein is present as MMPL (Figures 4A,B). When Mn^{2+} is present in lesser concentrations (the left of Figure 4B) or if the locked forms are not allowed to reach equilibrium (Figure 3B), or if Ca^{2+} is present (Figure 2B), a maximum of 1 equiv of Mn^{2+} binds per monomer, in agreement with the findings of Brown et al. (1977), as summarized in Schemes I and II. In addition, saccharide at high concentrations (0.1 M α-MDM) also selectively binds to a ternary Mn complex, as shown by the results in Figure 5. The linearity of the data over most of the range indicates that mass-action effects of the saccharide have pulled all of the Mn^{2+} on as doubles at equilibrium. And last, the results of equilibrium dialysis studies, using both NMRD and atomic absorption spectroscopy to monitor Mn^{2+} binding, clearly and independently show that 2 equiv of Mn^{2+} binds to the protein in the absence and presence of saccharide (Table II and Results).

In contrast, Magnuson and co-workers (Christie et al., 1979) have argued that in the absence of Ca^{2+}, only one Mn^{2+} binds per monomer of Con A and activates the protein and further that Mn^{2+} does not form a ternary Mn^{2+}–Con A complex but instead binds only at or near the S1 site (Pandolfino et al., 1980). Several experiments in the present studies were deliberately made similar to those performed by Magnuson and co-workers in order to compare the results where possible. Furthermore, in response to the implication (Christie et al., 1979) that the use of NMRD techniques, as in our previous studies, is an indirect method of monitoring Mn^{2+} binding to Con A (Brown et al., 1977), we have provided new and independent data showing unequivocally that NMRD measurements can provide a direct and precise determination of the concentrations of free, total, and therefore bound Mn^{2+} in solutions of Con A under a variety of conditions (Figure 2A–C). In fact, few other methods are as direct and versatile, since most (e.g., atomic absorption, EPR, and radioactive isotopes) require standardization each time they are used, whereas once ΔR_M is established, the NMRD method is absolute. Furthermore, the NMRD results have been shown to agree very well with independently obtained atomic absorption analysis for a variety of conditions (cf. Table II).

The two techniques used by Christie et al. (1979) in their study of Mn^{2+} binding to Con A were equilibrium dialysis

using radioactive Ca^{2+} and Mn^{2+} and solvent proton relaxation measurements at 20.5 MHz. However, their conclusions regarding the stoichiometry of Mn^{2+} binding were based exclusively on equilibrium dialysis data carried out at 5 °C. The Scatchard plot of their data (their Figure 1) clearly shows ~0.9 equiv of Mn^{2+} bound per monomer of Con A. In relation to the findings in the present study, it appears that under the conditions of their experiment, namely, low temperature, they monitor the formation of primarily MP and MPL (Scheme II), in samples which have not come to conformational equilibrium; there is little MMPL present due to the slow kinetics of the vertical pathways at 5 °C in Scheme II. That is, the locked form of the protein has not formed under their conditions. Their results in this instance are equivalent to the data in the present paper shown in Figures 2A and 3A and therefore are not a test of the stoichiometry of binding of Mn^{2+} to PL.

On the other hand, their data for solvent proton relaxation measurements at 20.5 MHz of 0.37 mM apo-Con A titrated with up to 4.0 equiv of Mn^{2+} clearly show that two types of Mn^{2+}–protein complexes are formed depending on whether the samples were kept at 5 or 23 °C for an extended period (their Figure 2). Samples kept at 5 °C and measured immediately after warming to 23 °C gave a titration profile that resembles the 20-MHz data in Figure 2A of the present study, while the same samples after being kept at 23 °C for several days gave a relaxation rate profile at 23 °C very similar to that previously reported by Brown et al. (1977) for similar measurements made at 25 °C and to that in Figure 4A of the present study. Christie et al. (1979) offer no quantitative explanation for the difference in rates and shapes of the two profiles other than to suggest that Mn^{2+} seemed to be in "two different temperature-dependent states..." when bound to apo-Con A. The authors rely primarily on the results of their equilibrium dialysis measurements (at 5 °C) to conclude that only 1 equiv of Mn^{2+} binds to Con A at the two temperatures used in their Mn^{2+} titration study. They state that ESR measurements were used to check the stoichiometry of free and bound Mn^{2+} in some of the samples, though no data are presented, and there is difficulty in using ESR to distinguish between free and bound Mn^{2+} since the bound signal is intense and overlaps with the free Mn^{2+} signal (Meirovitch et al., 1974a,b).

The results of our present study show conclusively that the two titration profiles observed in our Figures 3A and 4A, which closely correspond to the two curves in Figure 2 of Christie et al. (1979), are due to Mn^{2+} binding to form predominately MP in Figure 3A and, with total concentrations of Mn^{2+} above 0.6 mM, the formation of predominantly MMPL in Figure 4A. The decrease in rates in Figure 4A between 1 and 2 equiv of total Mn^{2+} in the samples equilibrated at 25 °C is diagnostic of the formation of MMPL from MP and MPL since the former at 5 °C has a much smaller relaxivity (12 mM^{-1} s^{-1}) than the latter two (55 and 75 mM^{-1} s^{-1}, respectively) at 20 MHz (see also Figure 4C). [Furthermore, no evidence of large allosteric effects during Mn^{2+} binding to Con A has been reported which might produce the decrease in rates in the middle of the curve in our Figure 4A and the lower curve in Figure 2 of Christie et al. (1979).] The observed decrease in rates therefore absolutely requires the interaction of a pair of Mn^{2+} ions on each monomer of Con A. Thus, the relaxation measurements reported by Magnuson and co-workers on samples of apo-Con A titrated with Mn^{2+} are entirely consistent with the observation in the present paper which shows that Mn^{2+} forms predominantly binary complexes (MP and MPL, in Figure 3B) at low temperature (5 °C) and nonequilibrium conditions, while after conformational equilibrium is obtained at higher temperatures (25 °C) in the present of sufficient amounts of Mn^{2+}, MMPL, the most stable ternary complex, predominates (Figure 4B).

In the same report, Magnuson and co-workers also argued that only 1 equiv of Mn^{2+} bound to Con A was sufficient to induce the full saccharide binding activity of the protein. We will present data on the relation of the stoichiometry of Mn^{2+} binding to Con A to its saccharide binding activity in a subsequent paper. However, the results in Figure 5 show a strong preference for binding of 0.1 M α-MDM to a ternary Mn–Con A complex. It is important to note that K_{MPLS} for saccharide binding to MPL (Scheme I and II) is not known, though it is expected to fall between K_{LPS} for PL and K_{MMPLS} for MMPL.

Stoichiometry of Ca^{2+} Binding to Con A. The limited data in Table III were obtained under conditions chosen to be similar to that for Mn^{2+} binding to apo-Con A to form MMPL; i.e., excess Ca^{2+} was equilibrated with the demetalized protein at room temperature for several days. As predicted from the earlier results of Koenig et al. (1978) and the present findings for Mn^{2+}, 2 equiv of Ca^{2+} is observed to bind under these conditions. That one of the Ca^{2+} ions can be displaced with added Mn^{2+} to form CMPL supports binding of Ca^{2+} at S1 and S2. These results, though not intended to prove that Ca^{2+} binds in the same detailed manner as Mn^{2+}, demonstrate that Ca^{2+} also forms a ternary complex, CCPL.

Although a detailed study of Ca^{2+} binding to Con A will be presented elsewhere, a few comments are in order regarding published reports in the literature. Magnuson and co-workers (Alter et al., 1977) report equilibrium dialysis data of Ca^{2+} binding to apo-Con A at pH 7 at 2 °C in which the resulting Scatchard plot is highly convex toward the origin, with more than 1 equiv of Ca^{2+} bound per monomer of Con A. The authors concluded that Ca^{2+} binding was "obviously complex and difficult to interpret...". This report is consistent with our findings for Mn^{2+} binding in which binary and ternary complexes of P and PL are formed.

On the other hand, Sherry and co-workers (Sherry et al., 1981) report kinetic and thermodynamic studies of Ca^{2+} binding to Con A which they present as evidence for only one Ca^{2+} bound per monomer of protein. Specifically, they have observed the kinetics of quenching of the fluorescence of 4-methylumbelliferyl α-D-mannopyranoside (MUM) upon binding to Con A as Ca^{2+}, added to apo-Con A in solution, binds and induces saccharide binding activity. The data for MUM quenching (their Figure 2) show an apparent first-order dependence on Ca^{2+} concentration over a range of 1–30 mM at pH 6.4, 25 °C (also at pH 7.2). The authors conclude from these data that only one Ca^{2+} is bound and required to induce the saccharide binding activity of the protein. However, their scheme (their eq 1–3) which describes the kinetics of MUM quenching due to Ca^{2+} binding to apo-Con A

$$C + P \overset{K_a^M}{\rightleftharpoons} CP \xrightarrow{k_2} CPL + S \overset{K_a^S}{\rightleftharpoons} SCPL$$

where S is MUM, is kinetically equivalent, under their conditions, to the model for protein locking by both Mn^{2+} and Ca^{2+} proposed by Brown et al. (1977) and used by Harrington & Wilkins (1978), Schemes I and II, and for locking by two Ca^{2+} ions proposed by Koenig et al. (1978). Sherry and co-worker's scheme is equivalent to

$$CP + C \overset{K_a^M}{\rightleftharpoons} CCP \xrightarrow{k_2} CCPL + S \overset{K_a^S}{\rightleftharpoons} SCCPL$$

where CP has a greater affinity for Ca^{2+} than CCP such that

the observed first-order dependence of MUM quenching on Ca^{2+} is due to Ca^{2+} saturating S1 and then binding to CP to form CCP which, in turn, locks to form CCPL, the active complex. Thus, the kinetic experiments described by Sherry et al. (1981), which use excess concentrations of Ca^{2+} over protein to observe locking of Con A, do not provide evidence for only 1 equiv of Ca^{2+} binding to the protein.

Data were also reported by this group for a much lower concentration of Ca^{2+} (25 μM) (their Figure 1B). Here, the rate of quenching of MUM is nearly 100-fold faster than that predicted by extrapolating their data at higher Ca^{2+} concentrations (their Figure 2), indicating a different mechanism of Ca^{2+} activation under these conditions. These latter results can be explained by Ca^{2+} binding to S1 and S2 of PL, the latter present in small but not negligible amounts ($\sim$12%) due to its equilibrium with P (Brown et al., 1982). This rapid activation of PL by Ca^{2+} is analogous to that observed for Mn^{2+} activation of the protein observed by Harrington & Wilkins (1978) (their Figure 2), which have rates also nearly 100 times faster than the locking of CMP to CMPL (Scheme I).

The same workers (Sherry et al., 1981) also present equilibrium dialysis data for binding of $^{45}Ca^{2+}$ to locked apo-Con A (PL) which show a relatively straight Scatchard plot with one Ca^{2+} bound per monomer at pH 6.4 and 7.2 at 25 °C (their Figure 4). They concluded from this that PL binds only one Ca^{2+}, and most likely at S2. However, these experiments were carried out with locked apo-Con A (concentration not given), prepared at 5 °C, which was dialyzed at 25 °C against low concentrations of $^{45}Ca^{2+}$ (total concentrations not given) at pH 6.4 and 7.2 for "at least" 5 h. Since PL reverts to P at 25 °C with a half-life of 3.2 h (Brewer et al., 1983), it would appear that under the conditions of the study, a large proportion of this protein is unlocked P. Thus, it is difficult to interpret the results of this experiment.

Finally, the results of the present study show that in the presence of saccharide such as α-MDM, Con A binds two Ca^{2+} and up to 4 equiv of Ca^{2+} per monomer of Con A at excess Ca^{2+} concentrations of >5 mM. One of these equivalents is displacable by Mn^{2+} and is presumably bound at the S1 site. (Interestingly, Mn^{2+} under the same conditions, in the absence of Ca^{2+}, only shows 2 equiv of bound ion.) These results are consistent with the minimal requirement of two Ca^{2+} bound at S1 and S2 for saccharide binding activity and with additional weaker Ca^{2+} binding sites generated by the presence of bound saccharide.

Additional Observations. The complete analysis of all of the equilibrium constants in Scheme II for Mn^{2+} interaction with the protein is the first such to include interactions with both P and PL, for both equilibrium and nonequilibrium conditions. The equilibrium constant K_{LP} has only recently been reported to be 7.1 at 25 °C (Brown et al., 1982).[6] We now have the first reported values for K_{LMP} and K_{LMMP} (2.3 and 0.005, respectively, at 25 °C) as determined from measurements at 5 °C. The small value of K_{LMMP}, which corresponds to an energy difference of 3 kcal M^{-1} favoring the formation of MMPL, is not surprising in light of the relative stability of CMPL to CMP in Scheme I (Brown et al., 1977). Interestingly, binding of Mn^{2+} to PL to form MPL (K_{MPL} = 8 μM) is only somewhat tighter than Mn^{2+} binding to P to form MP (K_{MP} = 25 μM). Our previous estimate of K_{MMP} of 0.05 mM (Brown et al., 1977) has been revised to $\sim$7 mM, showing the weak affinity of Mn^{2+} for S2 of MP, at pH 6.4. This should be compared to the K_{CMP} value of 0.3 mM at 5 °C, pH 6.4 (Brown et al., 1982). Also of interest is the observation that MPL has somewhat higher relaxivity values than MP at the three fields used and that MMP also has much higher relaxivity values than MMPL (whereas CMP and CMPL have comparable relaxivities). The flat relaxivity profile for MMPL (12 mM^{-1} s^{-1}) indicates that the relaxation rates of MMPL are entirely limited by τ_M, the residence lifetime of the exchanging water ligands of the Mn^{2+} ions, which is $\sim$16 μs, assuming Mn^{2+} at S1 and S2 behave similarly.

Summary. We have confirmed and extended previous observations by Brown et al. (1977) that apo-Con A in the presence of sufficient concentrations of Mn^{2+} forms MMPL, a ternary complex with the locked form of the protein (Scheme II), with Mn^{2+} at both S1 and S2. If equilibrium is not reached so that the protein is predominately in the unlocked conformation, or sufficient Mn^{2+} is not present, or Ca^{2+} is present, only 1 equiv of Mn^{2+} binds (at S1). We also show that 0.1 M α-MDM (a large excess over the K_d of 10^{-4} M for sugar) preferentially binds to a ternary Mn^{2+}–Con A complex (SMMPL); details will be presented elsewhere. For the first time, a complete analysis is presented of the multiple equilibria of the upper two levels of Scheme II involving the interaction of Mn^{2+} with P and PL. We also demonstrate quantitative agreement of NMRD and atomic absorption spectroscopy when used to monitor the binding stoichiometry of Mn^{2+} to Con A. In addition, use of NMRD has been extended to include determination of the total Mn^{2+} concentration present in solutions of protein (by acidification), in addition to being able to determine directly the concentration of free Mn^{2+} present in the unacidified solution, which we have previously used (cf. Brown et al., 1977). This has permitted us to use NMRD to determine the concentrations of free, total, and hence bound Mn^{2+} in solutions of Con A.

We also present equilibrium dialysis data that show that apo-Con A in the presence of Ca^{2+} forms a ternary complex, similar to MMPL above, which when Mn^{2+} is added readily converts to CMPL (Scheme I). Finally, we argue that the majority of data in the literature regarding the interactions of Mn^{2+} and Ca^{2+} with Con A are consistent with Schemes I and II.

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Registry No. Mn, 7439-96-5; Ca, 7440-70-2; Con A, 11028-71-0; α-MDM, 617-04-9.

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[6] After the present paper was essentially complete, a note appeared by Strazza & Sherry (1982) stating that the equilibrium ratio of [PL]/[P] is zero when the protein is prepared in the presence of EDTA. However, the conclusions of this study are now recognized to be incorrect by Dr. Sherry's laboratory (personal communication). A detailed reanalysis of their study has been published elsewhere (Koenig et al., 1982) which demonstrates complete agreement with the original report by Brown et al. (1982) on the equilibrium ratio of [P] and [PL].

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Amino Acid Sequence of the Catalytic Subunit of Bovine Type II Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase†

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ABSTRACT: The 350-residue amino acid sequence of the catalytic subunit of bovine cardiac muscle adenosine cyclic 3′,5′-phosphate dependent protein kinase is described. The protein has a molecular weight of 40 862, which includes an *N*-tetradecanoyl (myristyl) group blocking the NH_2 terminus and phosphate groups at threonine-197 and serine-338. Seven methionyl bonds in the S-carboxymethylated protein were cleaved with cyanogen bromide to yield eight primary peptides. These fragments, and subpeptides generated by cleavage with trypsin, pepsin, chymotrypsin, thermolysin, and Myxobacter AL-1 protease II, were purified and analyzed to yield the majority of the sequence. The primary peptides were aligned by analyses of overlapping peptides, particularly of methione-containing tryptic peptides generated after in vitro [^{14}C]methyl exchange labeling of methionyl residues in the intact protein.

The varied physiological actions of adenosine cyclic 3′,5′-phosphate (cAMP) in eukaryotic organisms are mediated primarily by the specific phosphorylation of cellular proteins by cAMP-activated protein kinases (ATP:protein phosphotransferase, EC 2.7.1.37). They were widely distributed and well recognized to play a major general role in the regulation of energy metabolism and other physiological functions (Krebs, 1972; Rubin & Rosen, 1975; Rosen et al., 1977; Cohen, 1978, 1982; Krebs & Beavo, 1979). The enzyme is known to be composed of two regulatory (R) and two catalytic (C) subunits that together constitute an inactive holoenzyme (R_2C_2). The addition of cAMP activates protein kinase according to the following scheme:

$$R_2C_2(\text{inactive}) + 4\text{cAMP} \rightleftharpoons R_2(\text{cAMP})_4 + 2C(\text{active})$$

The enzyme is present in mammalian tissues in two forms, type I, the predominant form in skeletal muscle, and type II, the predominant form in bovine cardiac muscle (Reimann et al., 1971; Rubin et al., 1972). The differences between the two types are attributed to differences in the structure of the R subunit; the C subunits are thought to be identical (Hofmann et al., 1975). Recently, the roles, function, and location of critical sulfhydryl groups and of the ATP–Mg binding site of the C subunit of the enzyme were described (Zoller et al., 1981; Nelson & Taylor, 1981; Jimenez et al., 1982; Kupfer et al., 1982). In order to relate its molecular structure to the mechanism of action of the enzyme and the manner in which it is regulated, we have determined the amino acid sequence of the protein. The location of two sites that are phosphorylated and the occurrence of the unusual myristyl blocking group have already been described (Shoji et al., 1979; Carr

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